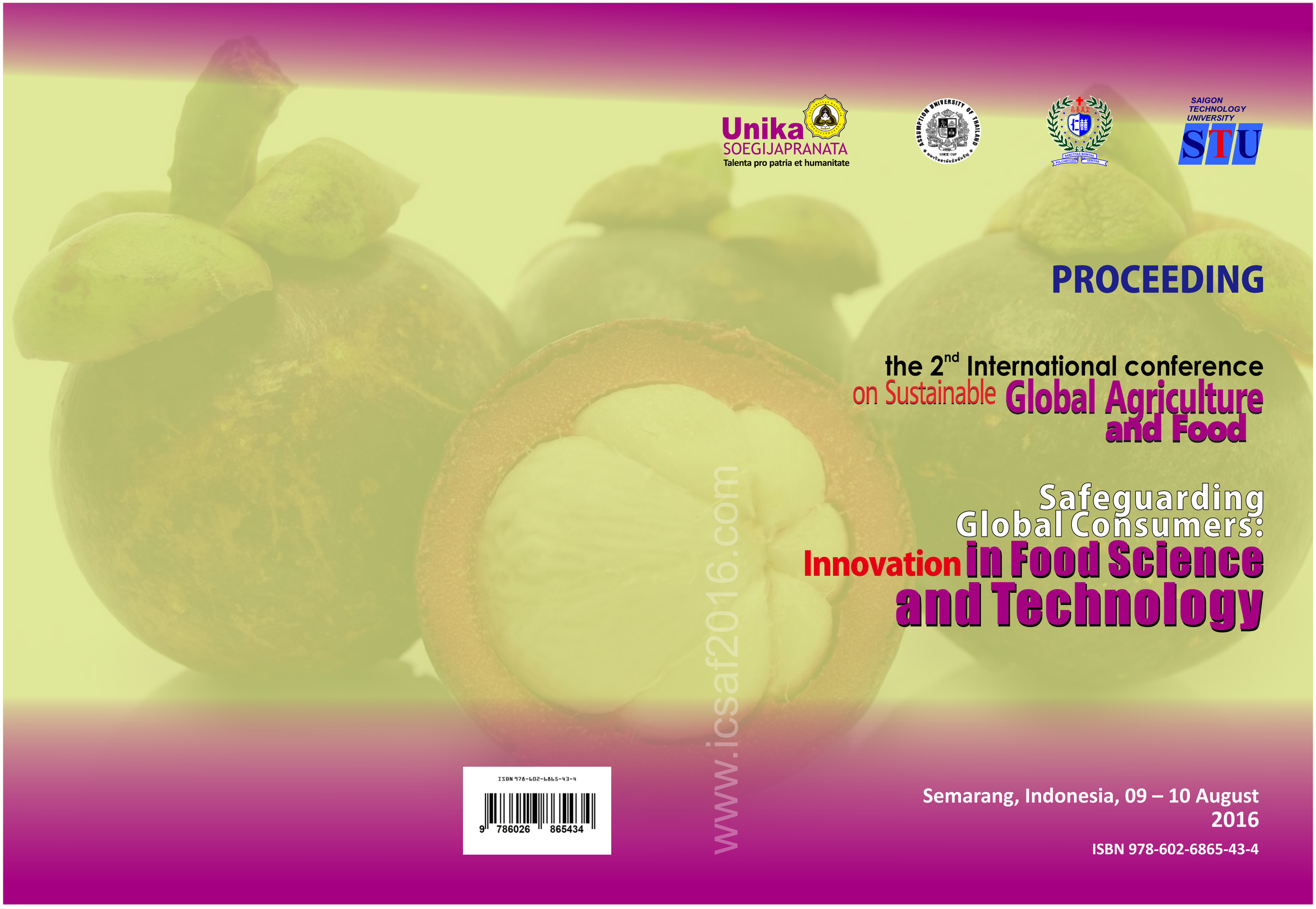


PROCEEDING

the 2nd International conference on Sustainable Global Agriculture and Food

Safeguarding Global Consumers: Innovation in Food Science and Technology

www.icsaf2016.com

Semarang, Indonesia, 09 – 10 August 2016

ISBN 978-602-6865-43-4

Unika SOEGIJAPRANATA
Talenta pro patria et humanitate

ASSUMPTION UNIVERSITY OF THAILAND
SINCE 1969

SAIGON TECHNOLOGY UNIVERSITY
STU

PROCEEDING

the 2nd International conference on Sustainable Global Agriculture and Food

Safeguarding Global Consumers: Innovation in Food Science and Technology

Editor :
Dr. R. Probo Nugrohedi, Msc.
Katharina Ardanareswari, STP., Msc.
Ivone E. Vernandez, SSi, Msc.

Reviewer :
Dr. V. Kristina Ananingsih, MSc.
Dr. Lindayani, MP.

TABLE OF CONTENTS

PREFACE

On behalf of Faculty of Agricultural Technology, Soegijapranata Catholic University (SCU), I would like to express my gratitute and warmest welcome to all of the participants of the 2nd International Conference on Sustainable Global Agriculture and Food Security (ICSAF). It is a great honour and pleasure for Soegijapranata Catholic University to host this ICSAF. This conference is conducted biannually to share the research findings, experiences and knowledge in recent trends of food science and technology.

The 1st ICSAF has been succesfully held in Bangkok on 16-18 July 2014. As all the advisory board agreed, the organizing committee of this biannual ICSAF is consist of 4 (four) Universities, i.e.: Assumption University Thailand, Fu Jen Catholic University Taiwan, Saigon Technology University Vietnam and Soegijapranata Catholic University Indonesia. These events are only made possible due to cooperation and generous supports of advisory board from those four universities. I would therefore like to express my sincere gratitude to advisory board for trusting Soegijapranata Catholic University to host this 2nd ICSAF.

The theme of this 2nd ICSAF is "Safeguarding Global Consumers: Innovation of Food Science and Technology". This conference is intended to bring together the perspectives and knowledge in safeguarding global consumers. The existing various disciplinary approaches and different research areas are accommodated in this conference to confer a good viewpoint and furnish a critical thinking about recent innovation in food science and technology.

In this special occasion, I would like to thank Rector of Soegijapranata Catholic University, Prof. Budi Widianarko for generous support to this conference. Please also allow me to express my gratitude to all the keynote speakers, presenters and all participants. I would like to thank all participants from 7 countries, i.e.: Netherlands, USA, Malaysia, Thailand, Vietnam, Taiwan as well as Indonesia. I would like to thank all the sponsors which provide the things that we need so that this 2nd ICSAF can be held. Also, I would like to convey my sincere thank to all the Committe members, who have contributed their time and effort to make this conference possible.

Finally, I hope we all have a fruitful discussion, meet new colleagues and experiencing in exchanging ideas and sharpening them so that we will be benefited by this conference. Also, I wish all of our guests to have a pleasant and meaningful stay here in Semarang. Thank you very much.

Sincerely yours,

Dr. Victoria Kristina Ananingsih
Dean of Faculty of Agricultural Technology
Soegijapranata Catholic University

KEYNOTE SPEAKER

The 7 Challenges of Indonesian Rice Farmers

Aart Schrevel
Alterra, Wageningen UR, Wageningen, the Netherlands
email: aart.schrevel@wur.nl

ABSTRACT

Indonesian rice farmers face many challenges producing rice on their fields and selling that to generate income for their families. This paper investigates the 7 major challenges: 1. My farm is too small, have to find other sources of income, 2. How do I maximize output from my rice field?, 3. How can I finance my crop?, 4 Where do I find laborers?, 5. How do I secure enough water at the right time?, 6. How do I apply seeds, fertilizers, pesticides?, 7. Who will succeed me when I retire? For a complete picture, these challenges must be understood within the broader context of the Indonesian rice production sector. The paper starts with an overview of the main macro-economic parameters that describe the rice production sector, and continues with picturing the 7 challenges.

Keywords: rice farming, land distribution, off-farm incomes, irrigation, succession

Introduction

Every year around 12 million ha of rice fields are being harvested in Indonesia. The work is done by over 20 million rice farmers: they are the food producers responsible for producing the rice that the Indonesian citizens consume. In this paper the focus is on the micro-economics of rice farming. We will discuss things as size of operations, investments, management decisions, market and other constraints, profitability, etc. The way that the rice sector is structured, a macro-economic topic, constitutes the framework within which the rice farmer is operating. We will start with picturing the macro-economics of rice farming.

The focus is on the micro-economics of rice farmers. That means we will not discuss other aspects that the rice farmer has to deal with, like for example his relations with agricultural laborers, interpersonal relations with the members of his household, his qualities as a farmer or as a trader. These aspects of farm management belong to the sphere of business management and psychology and are not a topic in this paper.

Part of the data presented come from a base line survey organised by Universitas Brawijaya, Malang, East Java, within the framework of the G4INDO project (Brawijaya Universitas, 2015), (www.G4INDO.org). The survey was held in three villages in East Java, 2015. East Java is Indonesia's main rice producing region. The total number of respondents was 150 (n=150). The conclusions of the survey can be generalised to describe conditions in East Java as a whole and other parts of Java. Other data presented in this paper mainly come from trustworthy website on the internet.

The title of the paper is "7 challenges for Indonesia's rice farmers: a micro-economic analysis". We prefer to speak of "challenges" instead of the more neutral term "characteristics", to emphasize that rice cultivation is a demanding task, even in the most fertile and productive provinces of Indonesia. In the sections below it will soon be evident why this is the case.

Rice farming in Indonesia: the statistics

China is the largest producer in the world, followed by India, and Indonesia is third on the list of the world's largest producers of rice (http://www.indonesia-investments.com/nl/business/grondstoffen/rijst/item183).

Compared to selected countries in Southeast Asia, Indonesia is producing average levels per ha (5.2 t/ha; source FAO's FAOSTAT database online and AQUASTAT database online, as of September 2012, quoted in Ricepedia.org). In 2010, Vietnam produced 5.3 tons/ha, Malaysia 3.6, China 6.5 (in the last 3-5 years), Thailand 2.9 tons/ha. Production levels in Thailand are relatively low. This is explained by the fact that rice fields in this country are mostly rain-fed . Thai farmers prefer to grow high-quality, low-yielding traditional varieties that fetch relatively high prices at world markets. Productivity per ha in China is comparatively high, 6.5 tons per ha; in China mostly hybrid seeds are used. Production levels in the USA reach 7.5 tons per ha; the country produces <2% of the global production. The

data are from Ricepedia, an online source of information on rice as a commodity (http://ricepedia.org/).

Indonesia has about 15 major rice producing provinces. Table 1 presents a list of these provinces. The table presents the total farm area per province and the total harvested area per province, as well as the total production. The data are given for three years, 2013, 2014, and 2015.

Table 1. Average production levels in Indonesia's main rice producing provinces, years 2013-15

Provinces	Padi						
	Farm Area (Hektar)	Production (Ton)			Harvest Area (Hektar)		
	2013	2015	2014	2013	2015	2014	2013
JAWA TIMUR	1,102,863	13,154,967	12,397,049	12,049,342	2,152,070	2,072,630	2,037,021
JAWA BARAT	925,042	11,373,234	11,644,899	12,083,162	1,857,626	1,979,799	2,029,891
JAWA TENGAH	952,525	11,301,422	9,648,104	10,344,816	1,875,793	1,800,908	1,845,447
SULAWESI SELATAN	602,728	5,471,806	5,426,097	5,035,830	1,044,030	1,040,024	983,107
SUMATERA SELATAN	612,424	4,247,922	3,670,435	3,676,723	872,737	810,900	800,036
SUMATERA UTARA	438,346	4,044,829	3,631,039	3,727,249	781,769	717,318	742,968
LAMPUNG	360,237	3,641,895	3,320,064	3,207,002	707,266	648,731	638,090
SUMATERA BARAT	224,182	2,550,609	2,519,020	2,430,384	507,545	503,198	487,820
NUSA TENGGARA BARAT	253,021	2,417,392	2,116,637	2,193,698	467,503	433,712	438,057
ACEH	300,808	2,331,046	1,820,062	1,956,940	461,060	376,137	419,183
BANTEN	194,716	2,188,996	2,045,883	2,083,608	386,676	386,398	393,704
KALIMANTAN SELATAN	440,429	2,140,279	2,094,590	2,031,029	511,213	498,133	479,721
KALIMANTAN BARAT	330,883	1,244,485	1,372,695	1,441,876	433,928	452,242	464,898
SULAWESI TENGAH	146,721	1,015,368	1,022,054	1,031,364	209,057	219,613	224,326
NUSA TENGGARA TIMUR	169,063	948,088	825,728	729,666	266,242	246,750	222,469
INDONESIA	7,053,988	68,072,338	63,554,356	64,022,689	12,534,515	12,186,493	12,206,738

source: National Bureau of Statistics (BPS) of Indonesia

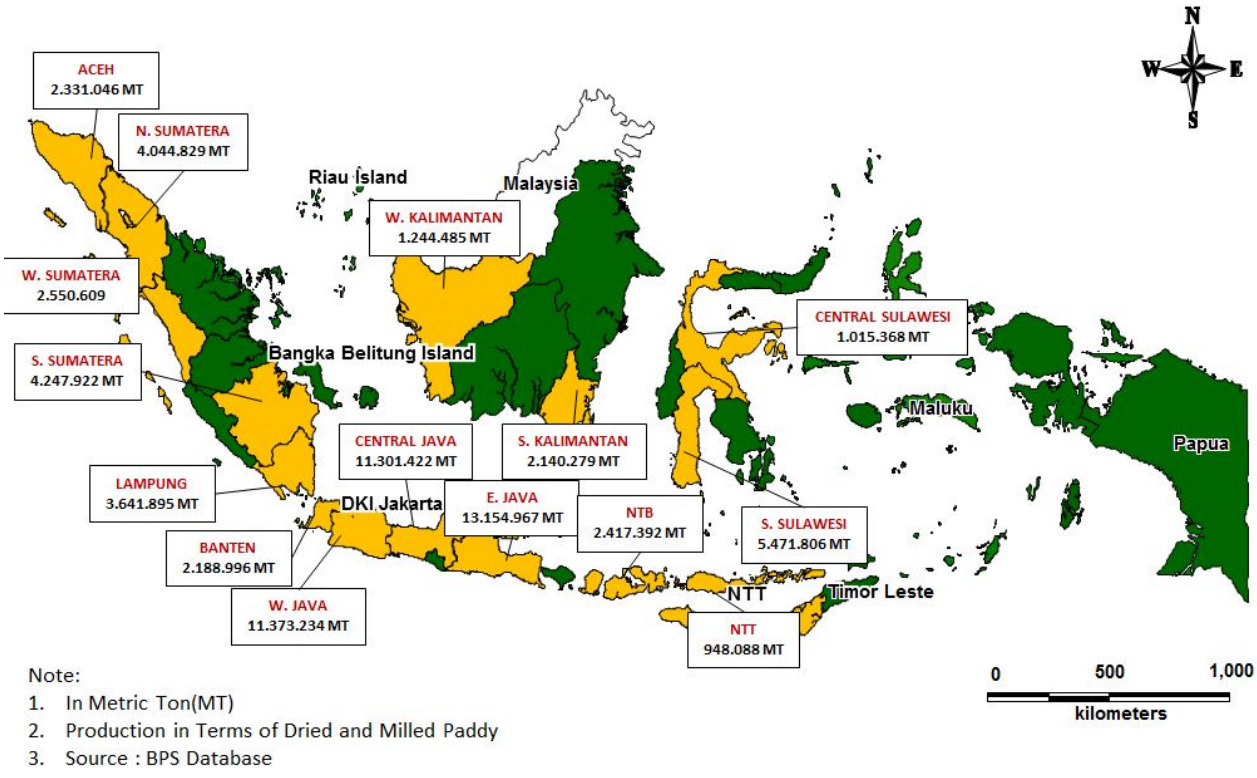
A number of conclusions can be drawn from the table:

- The total farm area is about 7 million ha. The total harvested area is in the order of 12 million ha. The difference is explained by the fact that in many area two rice crops, and sometimes even 3 rice crops are possible. But not in all areas. Out of the 7 million ha, about 2 million ha is planted only one time per annum with rice. Note: the Ministry of Agriculture uses a different total farm area, namely 8 million ha, as we will see below.
- The three producing areas on Java, East Java, Central Java, West Java, and Banten, cover almost half of the total farm area, are good for more than half of the total rice production in 2015 (38 million ton of rice out of 68 million ton), and cover about half of the total harvested area. Not only are ecological conditions in Java exceptionally suitable for cultivating rice, an attribute explained by the fact that Java has predominantly soils of volcanic origin, also there is a lot of land available that can be planted.

- East Java is the largest producer of rice in Indonesia.
- According to these statistics, the total harvested area in East Java is still increasing; an increase of about 100,000 ha in the period 2013-2015. That is equal to 2-3 average sized technical irrigation systems. The total harvested area in West Java is decreasing, however. The main reason is likely urban expansion. The other provinces all appear to see an increase in the harvested areas. Central Sulawesi is the exception, as in this province the harvested area decreased over the years. The reason for this is not known. It should be added that differences in harvested area from year to year can also be due to things like droughts, floods, pests, diseases, etc, and do not necessary reflect differences in areas planted.
- Production levels appear to follow the same trend: they increase in almost all provinces, but decrease in West Java, parallel to the decrease in harvested area. Production levels also decrease in West Kalimantan, a phenomenon possibly explained by the conversion of rice with oil palms.

The production data in the year 2015 are plotted on a map, figure 1.

Figure 1. Productions levels in 15 rice producing provinces of Indonesia in 2015, colored yellow (data BPS).



The map shows the relative location of the main rice producing provinces in Indonesia. The green areas on the map are not included in the data in the table available from the Central Bureau of Statistics. The reason why is not clear. Clearly rice is also produced on e.g. Bali and East Sulawesi. (This could be the explanation why the total area of farm land as stated by BPS deviates from the official numbers from the Ministry of Agriculture.)

As was said above, total production levels of the four rice producing regions of Java are higher than that of the 11 other regions together. Is that also true for the production per unit of land? Table 2 provides the answer. The unit in which BPS traditionally reports productivity is quintal. A quintal is equivalent of one/tenth of a metric ton, or 100 kg.

Indonesia has different climate zones, each with typical rainfall patterns, hours of sunlight characteristics of their own, leading to different conditions to grow rice. The soils on which rice is grown on Java are volcanic soils and are exceptionally fertile. Generally spoken physical conditions to grow rice are favourable in many parts on Indonesia, but are most favourable on Java (and on Bali, not presented in the table). This is reflected in the production levels per ha in each of the regions. In the table they are ordered from highest till lowest (2015 data).

Table 2. Production levels of rice per ha or land, 15 main rice producing regions of Indonesia, years 2013-2015

Provinces	Productivity (Kuintal/Hektar)		
	2015	2014	2013
JAWA TIMUR	61.13	59.81	59.15
JAWA BARAT	61.22	58.82	59.53
JAWA TENGAH	60.25	53.57	56.06
SULAWESI SELATAN	52.41	52.17	51.22
SUMATERA SELATAN	48.67	45.26	45.96
SUMATERA UTARA	51.74	50.62	50.17
LAMPUNG	51.49	51.18	50.26
SUMATERA BARAT	50.25	50.06	49.82
NUSA TENGGARA BARAT	51.71	48.80	50.08
ACEH	50.56	48.39	46.68
BANTEN	56.61	52.95	52.92
KALIMANTAN SELATAN	41.87	42.05	42.34
KALIMANTAN BARAT	28.68	30.35	31.01
SULAWESI TENGAH	48.57	46.54	45.98
NUSA TENGGARA TIMUR	35.61	33.46	32.80

Clearly visible in the table is that higher production levels per ha are realised on Java, Sulawesi and Sumatra. Kalimantan and the islands to the East are less productive.

The 7 Challenges of the Indonesian Rice Farmer

Challenge 1: my farm is too small, have to find other sources of income

It is easy to understand that a direct relation exists between size of farm and income from agriculture. The larger a farm, the higher the income from agricultural activities. But what is the size of rice farms in Indonesia, what is the range in farm sizes? How can the difference in farm sizes be explained? Why are so many rice farms in Indonesia's main production areas so small? And more importantly, how much income can be derived from farms of different sizes, and is that income sufficient for the needs of the rice farmer's household?

Table 3, below, presents data on land uses and the tenure status of land in 3 different villages in East Java. These are the village Kapi, Ngepeh, and Tanjung Gunung, located respectively in district Kediri, district Nganjuk, and district Jombang.

Table3. Average size of rice farm in 3 villages in East Java, year 2015 (ha) (n=150)

Type of Land	Tenure Status	Kapi (ha)	Ngepeh (ha)	Tanjung Gunung (ha)
Irrigated land	Privately owned	0,387	0,440	0,313
	Rented	0,112	0,162	0,113
	Share cropped	0,020	-	0,021
	Office land	0,170	0,085	0,058
Subtotal		0,689	0,687	0,505
Dry land (not irrigated)	Privately owned	-	0,101	0.012
	Rented	-	-	-
	Share cropped	-	0.004	-
	Office land	-	-	-
Subtotal		-	0,105	0,012
Garden	Privately owned	0,016	0,030	-
	Rented	-	-	-
	Share cropped	-	-	-
	Office land	-	-	-
Subtotal		0,016	0,030	-
Yard	Privately owned	0,005	0,020	0,001
	Rented	-	-	-
	Share cropped	-	-	-
	Office land	-	-	-
Subtotal		0,005	0,020	0,001
Fish Pond	Privately owned	0,0024	0,0002	-

	Rented	-	-	-
	Share cropped	-	-	-
	Office land	-	-	-
Subtotal		0,0024	0,0002	-
Total	Privately owned	0,4104	0,5912	0,326
	Rented	0,112	0,162	0,113
	Share cropped	0,020	0,004	0,021
	Office land	0,170	0,085	0,058
		0,7124	0,8422	0,518

Source: Sangor Kanto, 2015

As can be seen, a typical rice farm combines different types of land use: irrigated land, dry land, a yard on which the house is built, perhaps a garden with trees, and maybe a fish pond. The purpose of a piece of land is in first instance dictated by physical conditions, and in second instance by market conditions. That is easily explained. If a farmer expects the market to be very good for, say, irrigated rice, but he has no way to bring irrigation water to his plot, he has to accept that irrigated rice is not an option for him. Clearly the physical conditions precede over market opportunities. On the one hand a farmer may change the use of his land from maize to fruit trees, if he thinks that is more profitable. It is usually possible to make that kind of choice within the boundary conditions set by the physical system in which he is operating. This is one explanation why the diversity of crops on dry fields is much higher than in the lowlands. Rice is the preferred crop and in the lowlands irrigated rice is possible.

Not shown in the table is that farmers often have more than one irrigated field or dry field, and that the fields belonging to a farm are most often not one continuous block, but are located in different parts of the village, and sometimes even in another village. Table 4 provides further details regarding the number of fields owned by farmers. One third to half of all farmers have more than one rice field, some even have more than 4 plots.

Table 4. Percentage of farms with one or more rice plots, 3 sample villages, East Java, 2015 (n=150)

number of plots	Kapi	Ngepeh	Tanjung Gunung
1	56	62	70
2	24	22	18
3	16	12	12
4	4	0	0
>4		4	0

Source: Sangor Kanto, 2015

Not all land is privately owned. Part of the land cultivated by the farmers in the sample is owned by persons who are not the cultivators. Land may be rented out, or is cultivated on a share cropping basis. In the sample villages, the average cost of land rent per hectare is around Rp.15-20 million per year, depending on the fertility of the soil; in Ngepeh village, the cost of land rent per *pekulen* (200 ru) is around 5-6 million per year. The profit-sharing system in the villages is mostly in the form of *maro*, where the share is 1:1 for the owner

and tennant. In addition to that some farmers have a formal position in the village administration, and are compensated for their efforts with granting them the use of office land. Office land is land owned by the village that is given as compensation to individuals that do work for the village. Some villages have more office land than others.

All the land in the table 3 is productive land. Irrigated fields are used to grow rice during the rainy season. And if it expected that there will be enough rain or irrigation water during the dry season, and if the farmer can organise sufficient labour to prepare his irrigated fields for a second crop, another rice crop is grown during the dry season. With the increasing availability of water pumps in recent years, farmers in East Java nowadays always have enough water for the second crop, and even grow a third crop. If nevertheless at the beginning of the dry season not enough water is expected to be available, or not enough labour can be organised, farmers refrain from growing rice again, and switch to another crop, usually a maize crop.

Dry fields are less productive as rule. The limiting factor is water. Dry fields can only be planted one time a year, which is during the rainy season. The situation described above is generally the case all along the north coast of Java (production regions East Java, Central Java, West Java, and Banten). Another conclusion that can be drawn from the table is that the average rice farm is small: 0.7 ha in Kapi village, 0.8 ha in Ngepeh village, and 0.5 ha in Tanjung Gunung village. The average size of rice farms in the 3 villages combined is 0.6 ha. Later we will see that a farmer should have 0.7 ha to generate enough income to feed his family. How is the land distributed over the farms, or in other words, how large are the largest farms, how small are the smallest farms, and how many are there of each? Again we look at the villages from which the samples were taken. Table 5 shows the differences in farm sizes in the sample villages. The data from the three sample villages are combined.

Table 5. Distribution of productive land over farms in the three sample villages

Strata (Levels) of Land Size Ownership (Ha)	Total Household	Total Size of Land Tenure(Ha)	% of Total Household
<0,25	61	6.637	41
0,25-<0,50	47	15.67	31
0,50-<0,75	24	14.013	16
0,75-<1,00	7	6.14	5
≥ 1,00	11	23.92	7
Total	150	66.38	100%

Source: copied from Sangor Kanto, 2015

A number of important conclusions are drawn from the table. The first is that the majority of the farms belong to the smallest of land tenure categories. In total 61 out of the 150 farms in the sample have a size of less than 0.25 ha; these are minute farms indeed! In the villages it is commonly accepted that 0.7 ha of good productive land (2 harvests per annum) is minimally required to feed an average sized family. And yet, 88% of all farms is smaller

than 0.75 ha. And all these farms are so small that they do not produce enough income for the farmer and his family. It seems to be a contradiction, but the bulk of the rice that is being produced in Indonesia is produced on farms that are so small that they do not generate enough income to make a decent living!

There is only one conclusion possible: the majority of farmers in the sample village needs other sources of income to make both ends meet.

<u>This is first challenge of the rice farmer: how to get access to sufficient sources of income.</u>

Typically rice farmers in the sample villages, and in all other rice producing regions in Java, find other employment in a range of activities: an official office of some sort (one respondent in the sample is a policy officer), agricultural labourer, trader, construction worker, driver, carpenter, computer maintenance man, teacher, shop keeper, etc. Roughly half of all able-bodied household member appeared to be engaged of such activity. In fact that is lower than what seems logical. The question is what the other half is doing. Probably they do not all follow some kind of education, and are also not sitting idle at home. Further, a certain part of the income of the households comes from remittances. These come from household members staying elsewhere. The combined income per household from farm proceeds and from off-farm activities is shown in Table 6.

Table 6. Absolute and relative importance of income from crops and from off-farm work and remittances, three sample villages, East Java, 2015, IDR (n=150)

		Kapi	Ngepeh	Tanjung Gunung	total sample	%
income from agriculture		6156791	10710792	11483410	28350993	32
off-farm income	odd jobs	15679540	15741100	22132840		
	remittances	3351000	2064000	1638000		
subtotal off farm income		19030540	17805100	23770840	60606480	68
total		25187331	28515892	35254250	88957473	

Source: copied from Sangor Kanto, 2015

What is striking is that the income from off-farm is 2 times higher than the income from crops. This conclusion is not different from what was found in a similar survey done close to the city of Malang, East Java, about 30 years ago. In this study it was found that 70% (area dominated by irrigated rice fields) and 62% (area dominated by dry fields) of the total household income came from off-farm activities (Schrevel, 1981, p.61). Indonesia's rice farm households largely depend on other activities to survive!

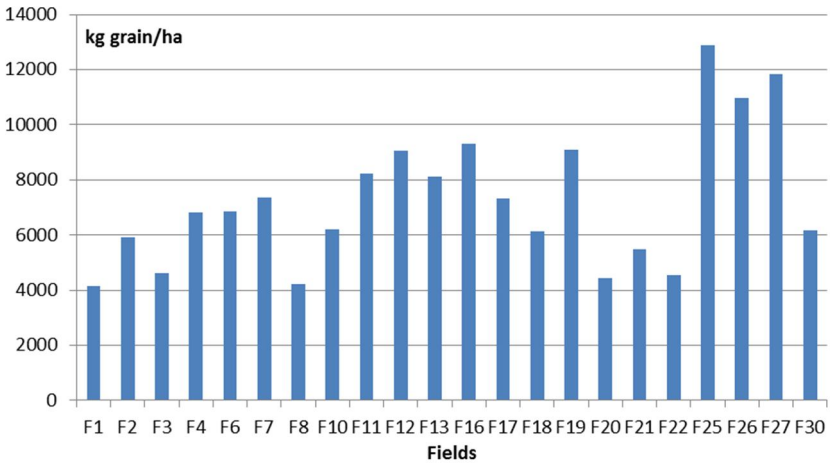
Challenge 2: How do I maximize output from my rice field?

This is the second challenge of the rice farmer: how to maximize output from my rice field. Having said that, it must immediately be added that maximizing output is not always the main strategy of the Indonesian rice producing farmer. It has been found in farmer surveys

that going for a good crop is often the preferred strategy of farmers, rather than trying to maximize output. Risk-avoidance is often taking precedence over maximizing output. For our purpose, however, the precise strategy of the rice farmer does not make much difference. What we discuss now are the constraints that the rice producing farmer faces in his attempts to produce a crop, regardless whether he aims at producing enough or as much as possible.

Average production levels do not say much about the production levels in the case of individual rice farmers. This is clearly illustrated in table 7, which shows the production per ha in kg in the case of 30 plots located in the three sample villages. Development of the rice crop in these plots was closely monitored over one season, the rain season of 2014-15; the results were presented in a report (Hengsdijk et al, 2015). The work was done within the framework of the G4INDO project, a cooperation project between Indonesia and the Netherlands.

Table 7. Production per ha in kg, 30 plots in three sample villages, October-March season, East Java, 2014-15



Source: Hengsdijk 2015

This is a quotation from the report (p. 8):
"Highest yield was observed in field F25 with almost 13 t dry matter grain/ha and the lowest yield in F1 with 4.1 t dry matter grain/ha. The average grain dry matter yield (unmilled) was 7.3 t/ha. Interviewed farmers at harvest mentioned frequently a grain yield of 7 t/ha, but this is probably fresh weight. Assuming a moisture content of 20-25%, dry matter yields in practice would be in the range of 5.3-5.6 t/ha, which is considerably lower than observed in our fields."

The difference in yield per ha is striking: 4.1 ton/ha compared to 13 ton/ha, a difference of 300%! Asking the main author of the report, who is also the one who directed the study, revealed that the difference must largely be attributed to the way in which the yields were measured. No conclusions should therefore be attached to the observed large difference in yields in the different plots. In this case the average yield is much more relevant. Yet farmers do have different yields from season to season, and from year to year.

Another point to be made is the difference between the estimated yield by farmers and the yield as measured by the research team, and the uncertainty that is caused by the difference in outcomes. Farmers are expert rice growers and seems logical to assume that they have a good idea of the productivity of their field. The research team were outsiders, however they applied the best possible methodology to monitor crop development and measure yields. And when comparing the two outcomes we seen a difference in the order of (7.3-5.6 =) 1.7 and (7.3-5.3 =) 2 t/ha tons per ha. Of course the production of each field is not always carefully measured, so one wonders how the national production statistics are calculated, and for that matter, how accurate they are. According to the information available the national production figures are based on sample cuttings taken in selected irrigated rice fields. The sample cuttings have a space of $1m^2$.

Clearly size of farm is not the only factor to take into account when assessing the wealth base of a rice farmer, although of course it remains an important factor. Other factors concern the actual productivity of his fields, which depends on many factors, including quality of the soil in which the rice plant grows, availability of irrigation water, applied fertilizer, availability and ability to purchase insecticides and pesticides, his skills as a farmer, and factors that apparently cannot be manipulated too easily, like exposure to pests and diseases.

Table 8. Average production costs of rice fields per farm and revenues, three sample villages, East Java, 2015 (n=150)

	1st crop	2nd crop	3rd crop	year
Production costs	18489677	14944063	14294146	47727886
Price obtained	26176417	23074316	18727129	76078879
Revenues	7686740	8130253	4432983	28350993

Source: copied from Sangor Kanto, 2015

Farmers are very active throughout the season to create the best possible conditions for their crops to flourish. They have to, as their income and the wellbeing of their families depends to an important degree on the success of their crops. They are careful when to start planting (when they feel certain that the rains start, and when they have enough labor at their availability to perform the tasks of land preparation, seed bed construction and transplanting), they plant the seedlings in the best optimal pattern (*legowo* pattern or some other pattern), they regularly check the health of the crop and act when the crop suffers from some kind of disease by spraying (provided they have access to cash to buy insecticides (and the success of their actions also depending on their expertise to correctly identify the pest), they manipulate water resources (irrigation water, groundwater) to the best of their ability, and they try to harvest at the optimal time or to sell their crop while still on the field against the best possible price.

Table 8 shows what these efforts bring them in terms of incomes from crops. The data show production costs, price obtained, and revenues after the crop is sold. The data differentiate between the rainy season crop (1^{st} crop), the dry season crop, and the third crop. Clearly revenues from the third crop are lower than from the other crops. More accurately, also the second crop givers a lower return than the first crop. Availability of water is the limiting

factor. Clearly how to secure a good harvest is a serious challenge for every rice growing farmer, every season again.

Challenge 3. How can I finance my crop?

There are many reasons why a rice farmer in Indonesia has problems financing his crop. Of course the situation for the farmer with only a small farm is more precarious than that of his fellow farmer who has a larger farm, even though, as we have seen, yields per unit of land can differ from season to season. The latter simply has a bigger financial buffer. The reasons why Indonesian rice farmers may be short of cash at the beginning of the planting season and thereafter, actually during the entire crop season until he is paid for his crop at harvest time, are one of the following, or a combination of:

- previous harvest has been disappointing, as a result of which he has not much cash to begin with,
- money earned with previous harvest was used for some urgent expenditure or was used to finance a luxury item,
- price obtained for his harvested rice was disappointing.

Moreover, we have seen the majority of farms is smaller than what is minimally required to provide sufficient income for an average household. In fact the situation is such that:

Challenge 3: the majority of the rice farmers faces the challenge how to finance their next crop.

The situation is such that he faces this challenge every new planting season again, thus in fact, throughout the year. Farmers themselves relate to this as the continuing cycle of debt. Undoubtedly the income from other sources than rice, generated by himself and other members of his household, provide the farmer with much needed cash at the time of starting the rice crop. Still there are occasions that he has shortage of cash and needs to find other ways to pay for the necessary expenses. Here is what the 150 farmers in the sample answered when asked how they financed their crop when they do not have enough money themselves (Table 9). The questions in the table may not always mutually excluding, but they give a clue of what the situation is.

Table 9. Farmer's perceptions on the impact of crop failure in three villages in East Java, 2015 (n-150)

Item	Kapi Village (%)	Ngepeh Village (%)	Tanjung Gunung Village (%)
1. Ever experienced difficulties paying loans due to crop failure?			
a) Yes	22	18	8
b) No	78	82	92
2. Options for next planting season if crop failure occurs			

a) Borrow money for production costs	10	9	-
b) Use previous savings	-	91	12
c) Change seeds	13	-	-
d) Change and decrease fertiliser	14	-	-
e) Plant like before	23	-	76
f) Change cropping pattern	10	-	-
g) No change	7	-	-
h) Follow recommendations from Department of Agriculture	-	-	12
i) Change plants	3	-	-
3) Other risks associated with crop failure			
a) Disturbance on the economy of household	45	67	25
b) Lack of capital for next PS	1	11	-
c) Embarrassed (ashamed) due to late repayments on loans	11	11	-
d) Taking additional loans	3	11	75
e) Loss of energy, time and cost	40	-	-

Source: copied from Sangor Kanto, 2015

During a group interview in the same villages at about the same time, and also according to the answers that female farmers gave when asked, the impression is that having to borrow money to pay for necessary expenses is far more common than would appear from the percentages in the table. Farmers speak of caught in a circle of debt.

When asked specifically about borrowing money from a trader or middleman, it became clear that this is indeed common practice; table 10. They often borrow money from the trader (tengkulak) that always buys their crop. The trader is willing to provide a loan and agrees with the farmer that he will sell his crop to him when it is ready for harvesting against a fixed price. The price is relatively low, usually an interest in calculated that is higher than the 19%/year interest that a bank loan costs. Alternatively farmers borrow money from a bank. But this requires the necessary paper work, and more importantly, collateral of some kind. In the absence of an ownership certificate of his rice field (only a small proportion of all fields is certified) farmers often do not have a collateral, which practically means that this way of financing their crop is not open to them.

Table 10. Percentage of farmers who borrow money from middlemen (traders) and average size of loan, data from three villages in East Java, 2015 (N=150)

	Kapi	Ngepeh	Tanjung Gunung
1) Borrow money from middleman (% of Household)			
a) Yes	72	2	2
b) No/Never	28	98	98

2) Average amount of loan (Rp)	1.894.000	2.000.000	4.000.000

Source: copied from Sangor Kanto, 2015

According to this table far more farmers from Kapi borrow than from the other two villages. The size of the loans are also interesting: Rp. 2-4 million. Generally the investment costs to grow a rice crop on 1 ha of land is Rp. 6 million. Unfortunately the figures in the table give no clue of the size of farms that the loans are required for. If it would be for farms of 0.5 ha, the loans would cover all the investment costs. Many farms are smaller.

The government of Indonesia has started a new initiative that potentially eases the life of farmers. Farmers throughout the country will be offered a crop loss insurance. If they have paid the premium and their crop gets lost because of certain reasons specified in the insurance contract they get compensated for their loss. According to the procedures the compensation money is paid out within days of issuing a claim. This will help them starting again with a new crop without it being necessary to borrow money at high interest rates. The insurance program is based on law 19:2013 on the "Protection and empowerment of farmers in Indonesia". The first insurances were issued in the October-March season 2014-15. The G4INDO project mentioned earlier is designed to assist the government of Indonesia in successfully implementing the law.

Challenge 4 Where do I find laborers?

The three most laborious tasks in the rice growing cycle are land preparation, transplanting, and harvesting. Land preparation is a task that requires physical strength and is traditionally a man's job. Fields are often small and often not suitable to be prepared mechanically. Most farmers also do not have a tractor. In 2015 the government handed out tractors for free in an effort to boast production, but the number of tractors distributed was roughly 6000. If one tractor would be used to plough 100 ha per season, which is practically impossible, the 6,000 tractors together still would not plough more than 600,000 ha. If it is realized that the total rice production area in Indonesia is 7 million ha, it is clear that the program helps, but the effect is limited.

These websites provide interesting reading on the subject:
http://www.setneg.go.id/index.php?option=com_content&task=view&lang=en&id=8680;
http://setkab.go.id/en/submit-thousands-of-free-tractors-president-jokowi-in-2-3-years-we-should-beve-rice-self-sufficiency/; and http://www.agronomers.com/2015/10/laporan-pendampingan-upsus-pajale-2015.html

The fact is that most farmers use animal power, or their own labour and hired labour, to plough their fields. This is an important reason why the planting season in one area can spread over many weeks. Especially when the second crop needs to be planted the challenge to find enough labour to do land preparation becomes real. The second crop needs to be planted immediately after the first crop is harvested, as the longer it takes before the land is prepared, the higher the risk is that the crop will suffer from drought at the end of the dry season. In fact not being able to secure enough labour is an important reason why in East

Java the second crop is also recorded to be maize. Traditionally transplanting is a task for women. Unfortunately information on the demand for transplanters and their availability at the beginning of the planting season is not available.

Harvesting is another labour-intensive task in the rice growing cycle. It includes threshing, which is often done in the field. Harvesting is a task that involves both men and women. Timing is critical. Waiting may result in more and heavier grains, but exposes the crop longer to rats and other plagues, as well as to the threat of absorbing more moisture when hit by rain. Thus a farmer must have organised sufficient labour at the most optimal day. Many farmers can do this. But also many farmers avoid the problem and sell their crop while still on the field, to a middlemen. The middlemen works with groups of agricultural labourers and offers his services in one village after the other. He is experienced in estimating the weight of the crop while still on the field. He makes the farmer an offer, and when both agree on the price, the middlemen sends his men into the field and they harvest the crop. Mechanically harvesting rice is possible, but to our knowledge it is not practiced anywhere on Java.

The conclusion is clear:

Challenge 4: Every season again, it is a challenge of rice farmers to secure enough labour to prepare his fields, to transplant his seedlings, and to harvest his crops.

Of course this challenge is not new to farmers. Also the situation at present does not seem to be much different from how it was 10 years ago, of for that matter, 30 years ago. There are no examples of major land consolidation programs in Indonesia. Land consolidation program are designed to create more uniform and larger fields, and straight access roads that are sufficiently wide to allow large agricultural machinery to pass. Such programs could help realizing higher yields. They can also help to solve the labour question, as more machine power is more brought in the equation. The downside is of course that it would force many agricultural labourers out of their jobs.

Challenge 5. How do I secure enough water at the right time?

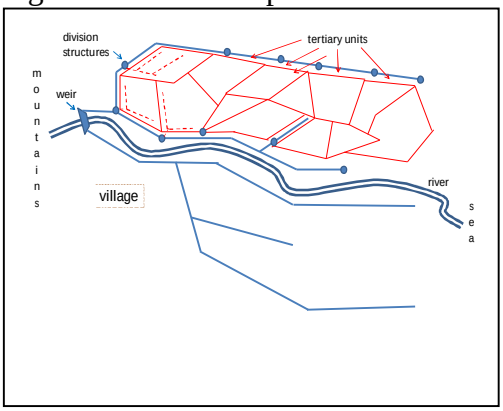
Irrigation is the art of bringing enough water of sufficient quality at the right time to where you want it to be. For irrigated rice the quality of water is not so much an issue. Much more important is the quantity of water and the timing of the irrigation. And of course the efficiency in transporting irrigation water. Losses because of canal leakages, unauthorised removal of water from canals, etc. cause lower efficiencies.

The irrigation systems in Indonesia are of the gravity irrigation type. Water flows from a source to an irrigation field. The source is a canal, usually a small canal that is fed by a bigger canal, that in turn receives its water from a river. Upstream in the river may be a man-made reservoir. This type of irrigation system is referred to as “technical irrigation” in Indonesia. Technical irrigation systems are constructed by the government. Many systems date from the first quarter of the 20th century. There are also “non-technical” systems, or more correctly phrased “village irrigation” systems. These are indigenous systems built by local people

using their own resources. Many of those systems are hundreds of years old. The village irrigation systems on Bali are famous. These systems are managed by the villagers themselves. The irrigation management system in Bali is called Subak. In technical irrigation systems only the smallest canals, called tertiary and quaternary canals, are the responsibility of the farmers. The main canals are property of the irrigation service, and are also managed by the irrigation service.

Figure 2 presents the typical layout of a technical irrigation system. As can be seen, it consists of a hierarchy of canals; the highest order canal is called primary canal, and it supplies water to the secondary canal, which supplies the tertiary canals, which in turn brings water to the quaternary canal. At the junction of canals distribution structures are placed; if smaller these are called boxes. They are made of concrete, and are furnished with gates, which are mostly made of iron. A closed gate blocks the water flow to the canal that lies behind it, if a gate is open water can flow through that gate into the canals lying downstream of it. The systems are built to allow water to be rotated over blocks of land within the irrigation scheme. Public Works operates the weir and the primary and secondary canals in the technical irrigation systems. The farmers are responsible for the tertiary and quaternary canals, as was mentioned earlier.

Figure 2. Schematic presentation of a technical irrigation system



Technical irrigation systems in Indonesia usually irrigate several thousand hectares with an estimated average of about 25000 ha. They are small in comparison to, for example, the systems in North India, that easily cover several hundred thousand hectares. This has to do with the topography of the areas to be irrigated. Irrigation systems on Java, for example, receive their water from the rivers that flow from the line of volcanoes that runs East-West. The distance from the mountains to the sea is relatively short. Indian irrigation systems receive their water from the mighty rivers that flow from the Himalayas and are many hundreds of kilometres long. These rivers, like the Bramaputra or the Ganges, carry much more water than those on Java, or for that matter, any river in Indonesia.

According to one source about 60% of all land under cultivation in Indonesia is under irrigation, 20% is under rainfed rice fields, and the rest are rice fields in tidal cultivation systems or swamps (https://en.wikipedia.org/wiki/Rice_production_in_Indonesia). The

figures are from 1983. Since then the area under technical irrigation has only increased. On Java practically all rice is cultivated under irrigated conditions.

It is not guaranteed that a farmer cultivating rice in a technical irrigation system will always have water on his fields when he needs it. He has to wait for the rains to start. There must be enough water in the river, and if there is one, in the reservoir upstream of the weir that feeds the primary and secondary canals. If he requires irrigation, he must wait for his turn in the rotation scheme. If his field is in the tertiary unit that is in the third secondary block on the left hand bank of the river, he must first wait till the left hand bank receives water, than the third secondary block, and then his tertiary unit. And still he has to wait till his field in the tertiary unit receives water. Under normal conditions, which means under conditions that sufficient water is available in the river, there is no shortage of water. Conditions become more difficult during dry spells or periods of no rain. Dry spells may always occur, regardless of the season. Longer periods without rain happen more often during the dry season. In the dry season irrigation water supply is a must. Growing rice in the dry season without irrigation is impossible. Unless a pump is used to bring groundwater to the surface; farmers in e.g. East Java increasingly use pumps.

Access to water is one of the most important factors that contribute to a successful crop. Hence:

Challenge 5: How do I secure enough water for my crop at the right time?

Today increasing numbers of farmers have water pumps and pump up ground water to irrigate their crops. This allows them to grow even a third crop. This means that their fields always have a crop of rice standing. From a hydrological perspective using groundwater is not a save strategy. Farmers pump up as much water as they wish, but have no idea how much water is recharged to the underground layers that contain the water. Pumping up more ground water than is being replenished and do so season after season and year after year eventually causes serious environmental problems, as for example deep-rooting trees do not have enough water anymore and die, or areas further downstream or higher up become deprived of ground water all together. We do not know of any systematic attempt to assess ground water conditions and regulate ground water extraction on the basis of the findings.

To illustrate the risk that rice crop may whither because of drought conditions, the following figure is telling. During the dry season of 2015, 518,358 ha out of 5,427,179 ha planted, or 9.55%, suffered from drought (figures made available by P. Vlasbloem). If an average farm measures 0.5 ha, the total number of farmers experiencing drought problems is about 1 million. According to the same source, on 2.7% of the total area planted with rice the crop was completely lost (*puso*).

Farmers are always very alert at the end of the dry season to see whether the rains start. Traditionally they have all kind of ways to tell when the first rains, which can be erratic, have changed into more regular rainfall. However, with the climate changing, such matter become unreliable. Balitbangtan, the research department of the Ministry of Agriculture, with data input from BMKG, the meteorological service, prepares a Cropping Calendar with

the purpose to assist farmers with such decisions as when to start planting. The most up to date weather and season forecasting models are used. Starting at the most optimal moment definitely improves the chances on a good first crop, and even more so on a good second crop. This is because the second crop is harvested way into the dry season, and is growing under conditions of increased risk for drought.

The changing climate causes conditions to become more difficult for Indonesia's rice farmer. More heavy rains during the rainy season, prolonged dry seasons, such conditions do not help the farmer to safely grow crops. Moreover, traditional ways of predicting the weather in the coming days, or how the cropping season will unfold, become less certain. Which makes the Climate Field Schools and the Cropping Calendar more important than ever. To this is added the impact of El Nino.

Challenge 6. How do I apply seeds, fertilisers, pesticides?

The rice farmer exploits a set of resources to cultivate his crop. These include the resources land, water, capital, and labor, which were each discussed above. In addition he uses other inputs, like seeds, insecticides and pesticides, and fertilizers. The successful application of these inputs is directly related to the success of his crop at the end of the season. Top-quality seeds germinate better than seeds of sub-optimal quality. The correct amounts of fertilizer have a direct relation to the capacity of the rice plant to grow leafs, flowers and seeds. In the event of a pest of some sort, recognizing the pest and applying the correct pesticides helps to reduce the damage. Luckily specialist extension workers are available to assist the farmer with the correct application of these inputs.

We will not go into further detail to discuss how the rice growing farmer faces this challenge. That kind of detail goes beyond the purpose of this module. A challenge it is, however:

Challenge 6. How to acquire the best quality seeds, information about the correct application of fertilizers, insecticides, and pesticides?

It is obvious that it requires a well-informed farmer to make the right choices. How does the farmer acquire this expertise? Much of the knowledge he has inherited from his parents. Indeed by far the majority of the rice farmers in Indonesia have inherited their rice fields and have grown up a household where all the details of rice growing were constantly discussed. More information he gains from his fellow farmers, who he meets in his village. The third source of information are the agricultural extension workers (Penyuluh Petani Lapangan, or PPL), from the Ministry of Agriculture. Each PPL has his or her own territory, often consisting of several villages. They visit their villages regularly and discuss agricultural questions, transferring relevant information in the process. The specialist extension worker – PPL-S – was already mentioned above. Last but not least, the success of an individual farmer also depends on his experience, which allows him to weigh the factors, and on his motivation and drive.

The number of factors that have to be understood and manipulated to generate a successful crop is indeed substantial. On average Indonesian rice farmers, although subsistence farmers

in nature, are able to understand these factors and obtain good yields. They are truly professionals. Having said that, it is remarkable that Indonesian rice farmers have not received any kind of formal training (beyond occasion training sessions organized in their villages from time to time. Schools to systematically teach farmers all different aspects of rice cultivation, or more general, of plant cultivation, do not exist. It is hypnotized that yields could be even higher if young men and women with an aspiration to become farmer were systematically trained in plant cultivation.

Challenge 7. Who will succeed me when I retire?

Farmers have been asked whether they like to see one of their children to succeed them as a farmer; here is a quote by a professional aid worker who has raised this question wherever he was:

Across continents, the answer has hardly ever been 'yes'. That creates a bit of a problem for the 'peasant romantic' wing of the aid business, who are then forced to argue that either a) 'they don't know what they really want/they've been brainwashed by the media' – always a dangerous position for those who claim to listen to poor people, or b) 'ah yes, but that's because poor farmers have a crap life, and they would change their minds if they got land, access to markets etc etc', which kind of makes sense, but could do with a bit of evidence to back it up. (statement by Duncan Green, strategic adviser for Oxfam GB and author of 'From Poverty to Power'. His personal reflection is not intended as a comprehensive statement of Oxfam's agreed policies ; see https://oxfamblogs.org/fp2p/who-wants-to-farm-hardly-any-young-people-it-seems-shouldcould-that-change/ .

Indeed this is a challenge (challenge 7) for every rice farmer: Who will succeed me when I retire?

The above mentioned website explains the conclusions of a literature review with an analysis of focus group discussions and household case studies with almost 1500 people in 23 rural, urban and peri-urban communities in Asia, Africa and Latin America.:

The literature review shows an intriguing and unintended consequence of the spread of education – a 'generational break' in family and community traditions of smallholder and small-scale farming. This has been compounded by the spread of communications and media, which means that young rural people are more aware of the alternatives: 'young people speak movingly about the sorrow they feel witnessing their small farmer parents' often desperately hard struggles to earn a living'. Put bluntly, parents and kids alike think school is a way out of farming, not a way into it. This is particularly true where economies are creating lots of new jobs in factories and towns.

Young women seem particularly keen to escape. Here's Miss S, a 19 year old migrant job-seeker in Indonesia: 'I never want to be a farmer, ever … I don't to work under the sun; my skin will be darker. My mother said that I shouldn't be a farmer; the [earnings] are not enough to provide for life; it doesn't have a future; it'd be better to look for a job in the city. It is better becoming a factory worker; I don't have to work under the heat, it is not dirty.

The wage can be used to buy a cell phone, clothes, cosmetics, bags or other things needed by a teenager. It can be saved for parents, too.'

While not sharing the peasant romantic view of the world (heck, I live in Brixton), I do think there's a need to encourage more young people into agriculture, both because in most places the non-farm economy is not providing enough decent jobs, and because labour intensive agriculture remains a crucial initial path to equitable growth in many countries. What to do? One significant finding in the paper is that the standard policy recipes are not enough to do the trick; government and others somehow have to make it cool to farm: 'It was clear that people considered material assistance in accessing land and inputs, while necessary, would not be enough to make farming attractive to young people, citing a need for successful role models in agriculture. Not only that, there was a strong sense that government had a key role to play in creating the right signals that agriculture is a valued sector and farming a worthwhile profession.'

'Agriculture's lack of appeal to young people reflects i) lack of effective public investment in small holder farming and the public infrastructure needed to link to markets; ii) constrained access to land and uncertain access to inputs among young people, including land fragmentation in many countries in past few decades; and iii) social change resulting from rapid increases in mass education provision but which have often resulted in a perceived decline in the status of agriculture. But in digging deeper, the research also finds that agriculture could be made more appealing to young people, with the right kinds of measures and support. First, public policies need to improve the fit between the aspirations of young people and opportunities in agriculture and agri-food more broadly. This could include providing the right kinds of training at appropriate levels to reflect the demands of the job market and broader public investment. Second, an important factor in enabling young people to see the potential of different employment choices, in agriculture and other sectors, is the presence of positive, successful role models.

Finally, a strong message emerging from across all the countries in this research is that farmers, across all generations, need support for accessing markets and to improve productivity, such as access to inputs and in the uptake of modern technologies. It is clear that in a time when food prices are volatile, such policies would help to reduce or mitigate other areas of uncertainty in farming and would go some way towards creating the kind of dynamic agricultural sector that will drive poverty-reducing growth as well as attracting the 'talent' of future generations.'

Also in our research area we found that only a small number of farmer's children eventually became farmers as well (only 6% in Ngepeh village, East Java). They appear to prefer to work as servants, employees, factory workers and traders/entrepreneur. Having said that, a large number of young people state that they are (still) unemployed! The lack of interest by young people to work in the agricultural sector apparently has resulted in a shortage of farm laborers in rural areas, especially during peak season when the demand for agricultural activities is quite high. Although it remains to be seen whether this is indeed the case because young people are reluctant to work in the fields, or because at peak seasons all available man

power in already employed and there is an acute shortage of labor. This has always been the condition in rice farming in Indonesia.

Conclusions

By far the majority of the Indonesian rice farmer cultivate fertile soils, and many experience the blessings of irrigation. Hours of sunshine, an important growth factor, are plenty, climatological conditions in general are favourable, if not good or excellent. Nevertheless being a rice farmer in Indonesia is not an easy life. The challenges are numerous; 7 challenges stand out because they are the same for most rice farmers:

1. The rice farm is too small to generate sufficient income to live from; in consequence additional employment is needed
2. The rice farmer constantly works to achieve the best possible, not maximum, yield, without taking too many risks
3. (Almost) all rice farmers experience troubles finding enough cash to pay for labour, input, etc., during the growing season; this is especially a problem after a less successful previous harvest or some disaster in the family
4. At the beginning of the planting season the demand for agricultural labourers is high, and farmers face the challenge to secure sufficient labour from land preparation and transplanting in particular
5. Rice crops probably suffer most from insufficiencies in water supply; securing sufficient water is a continuous challenge, also for farmers in irrigation systems (2nd and 3rd crop in particular)
6. Probably the most farmers are those that can select the best seeds, apply enough and not too much fertilizer, are able to identify pests and know how to deal with them, and are constantly alert to nurture their crops as best as possible
7. After many years of farming the rice farmer faces to challenge to find a successor who will continue the farm; this is increasingly a challenge for farmers all around the world, including also the Indonesia rice farmer.

In general Indonesian rice farmers know how to handle these challenges. They have not had a formal education in rice farming, as such an education does not exist. Nevertheless they are professionals!

References

Data on irrigated versus non-irrigated land in Indonesia: https://en.wikipedia.org/wiki/Rice_production_in_Indonesia

Duncan Green for Oxfam GB, on farmers'perceptions on succession: https://oxfamblogs.org/fp2p/who-wants-to-farm-hardly-any-young-people-it-seems-shouldcould-that-change/

For data on main rice producing countries: http://www.indonesia-investments.com/nl/business/grondstoffen/rijst/item183

For general and detailed information on rice as a commodity: http://www.indonesia-investments.com/nl/business/grondstoffen/rijst/item183

For information on the BMKG Climate Field Schools: http://www.bmkg.go.id/BMKG_Pusat/Sestama/Humas/SEKOLAH_LAPANG_IKLIM_TAHAP_II_DI_SICINCIN_.bmkg

Hengsdijk. H, S. Conijn, E. Valkengoed, 2015, Field work campaign for assessing rice development and growth in Jombang, East Java, technical report G4INDO project http://www.agronomers.com/2015/10/laporan-pendampingan-upsus-pajale-2015.html

On rice cultivation in Indonesia and Indonesian agricultural support programmes: http://www.setneg.go.id/index.php?option=com_content&task=view&lang=en&id=8680; http://setkab.go.id/en/submit-thousands-of-free-tractors-president-jokowi-in-2-3-years-we-should-beve-rice-self-sufficiency/; and

Sanggar Kanto, 2015, Socio-economic conditions of households in rural East Java, baseline survey in three rice growing villages, research team Faculty of Social Sciences and Political Sciences Brawijaya University, research conducted under G4INDO Project, a cooperation framework between Government of Indonesia and Government of Netherlands

Schrevel.A., and W. Nibbering, 1981, Additional activities in rural Java: a case study of two villages, doctoral thesis, State University Utrecht

ORAL PRESENTATION

SENSORY AND FOOD PRODUCT DEVELOPMENT

Product Properness Analysis of Instant Uduk Rice (Study of Consumers Acceptance Reviewed by Fondness Level)

Dwi Kristiastuti Suwardiah[1] and Asrul Bahar[2*]
[1,2]Home Economics Department, Faculty of Engineering, Surabaya State University
Kampus Unesa Ketintang, Surabaya 60231
[*]E-mail : asrulbahar96@yahoo.com

ABSTRACT

Instant uduk rice is one of the products that was produced with the aim of presenting food products that are more efficient and quickly served (Dwi, 2013). Instant uduk rice was strived to fulfill the emergency food needs such as natural disasters, and also as supplies when traveling abroad such as the Hajj (pilgrimate), etc. This study was a follow-up that aimed to analyze the quality feasibility of instant uduk rice product that was associated with the consumer acceptance study in terms of the preference level. The type of this research was experimental. The research was conducted in the Home Economics Department of Surabaya State University, starting in March 2015 until October 2015. The production process was conducted in the Home Economics Department of Surabaya State University, and the data were collected from people that consisted of college students and housewives as the population in the study with the sample total of 137 people. The data collection method used observations by organoleptic tests that were related to the preference level to the quality of instant uduk rice including: color, smell, texture, and flavor. The instruments that were used to obtain the data in the organoleptic test were observation sheet on instant uduk rice quality with the criteria of color, smell, texture, and flavor. Data analysis techniques in this study used SPSS 18.00 with statistics descriptive and friedman analysis. The results based on consumer preferences in color uduk rice with the highest percentage values exist on the criteria very like by 45,4 %; Uduk flavored rice based consumer ratings on the criteria very like that is equal to 59,1 %; Flavor rice uduk based on assessment consumers on the criteria very like that of 57,7%. Texture rice uduk on the criteria very like of 42,3%. Uduk rice assessment results that include color, smell, flavor, and textur can be accepted by consumers with a higher percentage in the elections really like that expressed uduk instant rice products feasible and acceptable to consumers. Friedman test results showed that the four criteria which include: color, smell, texture and flavor affect the level of consumer acceptance based eligibility uduk rice.

Keywords: Instant uduk rice, the properness quality, and the consumers' preference level

INTRODUCTION

Rice is a staple food for most people in Indonesia which is consumed in the largest quantity each day. Based on this fact, Indonesian people are difficult to leave or to replace rice with other type of staple food with the same nutrition. Rice consumption as a staple food has psychologically stuck to Indonesian culture in wide area. Either in joy or sadness, people gather together in cultural events with symbols in forms of food whose main ingredient is rice that has been made to be various dishes such as steamed rice, *lontong* (a rice cake mostly in cylinder shape wrapped inside banana leaves), *ketupat* (a rice dumpling mostly in diamond shape wrapped inside woven palm leaves), porridge, etc. Those prove that Indonesian people are very dependent to rice as their main staple food, there is even a saying by Indonesian people that if one hasn't eaten rice for a meal, despite the fact that he/she has eaten other types of food and is already full, people will consider that he/she hasn't had a proper meal yet.This is an undebatable thing that rice has strongly gained its position in Indonesian people's mind as the most top staple food for every day consumption. Rice processing to be uduk rice/instant flavored rice is an effort to make rice serving to be a lot faster and easier, therefore this instant uduk rice can be introduced to people as a fast-serving food in emergency conditions such as in a natural disaster, it can also be served as a food supple in a long trip such as Hajj (Islamic pilgrimate), etc. From a rearch result that has been completed about this instant uduk rice, this product has several benefits: 1) It contains nutritions which are: Kh 62 %, Protein 12%, and fat 11%, 2) It has fast serving time which is five minutes, 3) It is packed in light packaging so it can be brought everywhere, 4) It is tasty because it contains seasonings and spices (Dwi, 2013).

A type of food that can increase human's energy is rice. Rice (*Oryza sativa*) is a cereal type of food which becomes Indonesian people's staple food. The high production capacity of rice is balanced by the high rate of domestic consumption. Rice consumption rate in Indonesia is 139,5 kg/capita/year in 2009. The high rice consumption rate in Indonesia makes rice as a commodity that has high opportunity to be exploited further in term of processing. Nowadays, rice is commonly processed as cooked rice or porridge. In traditional way, rice processing needs approximately 30 minutes of time to boil it until done. If it's added by the preparation time, this whole process can take up to approximately one hour.

Instant food products in markets nowadays very rarely use rice as their main ingredient. Instant food products that are sold all this time can only give the full feeling to the consumers, not the satisfied feeling. Other processing result from rice is uduk rice or flavored rice. Uduk rice has been known by Indonesian people especially Javanese ones and it is a traditional dish from Betawi (Jakarta). Consuming uduk rice is not something new for some people. Instant uduk rice has a potential to be accepted by consumers, thus thiis product is able to be developed further in bigger production scale. Instant uduk rice was produced with aim of creating a food product that is more handy, practical, and ready to eat. This instant uduk rice product is expected to change people's thought related to the uduk rice traditional making which takes a lot of time. This research was a further development that has goal of knowing consumers' acceptance and the product's properness level based on its color, smell, taste, and texture or softness, thus it is a necessary to hold a research by creating a bigger scale instant uduk rice product and still focuses on its nutrient content and good food safety.

This research was aimed to know consumers' acceptance based on the level of preference including: color, smell, taste, and texture. Therefore a conclusion about instant uduk rice product's properness level could be gained from the consumers' acceptance.

METHODS

The conducted research was an experimental one, which aimed to know the properness level of instant uduk rice product and consumers' acceptance based on the level of preference including: color, smell, taste, and texture. The population scale in this research was a big scale of people, and samples were randomly taken, and the total of samples werw 137 people as panelists. The research design was a plan created by the researchers where each step was identified as a reference of the research (Arikunto, 2010: 90). The research design showed one factor, which was the preferance level of instant uduk rice. The design is illustrated as a table below.

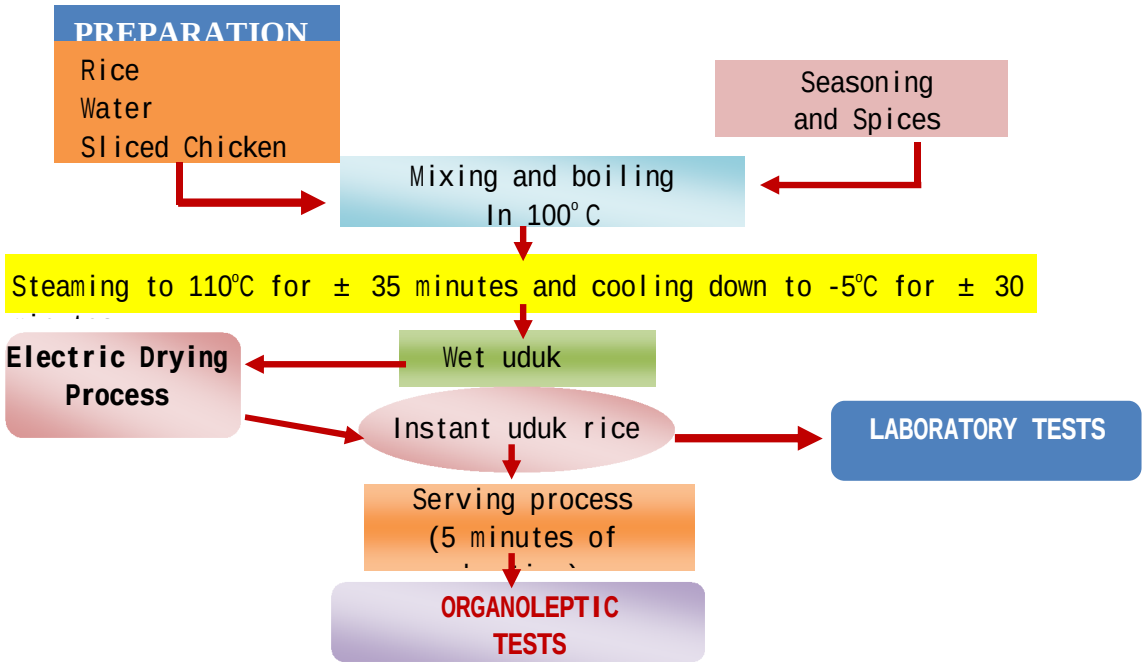
Table 1. Research Design of Instant Uduk Rice's Properness Analysis

Criteria / Sample	Y1 Color	Y2 Smell	Y3 Taste	Y4 Texture
(X)	XY1	XY2	XY3	XY4

Note:
X : The sample of instant uduk rice product
Y (1, 2, 3, 4): Consumers' preference toward the product

In this research, the data collection method used observation metod. Observation method is done by performing an organoleptic test to know the product's preference level including: color, smell, taste, texture. The data analysis technique was done by a help of SPSS 18,00 computer program. The data then were analyzed using descriptive statistics percentage and Friedman test to observe the properness level of this poduct through the consumers' preference level.

This is an illustration of the procedure of producing instant uduk rice:



Picture 1. The procedure of making instant uduk rice

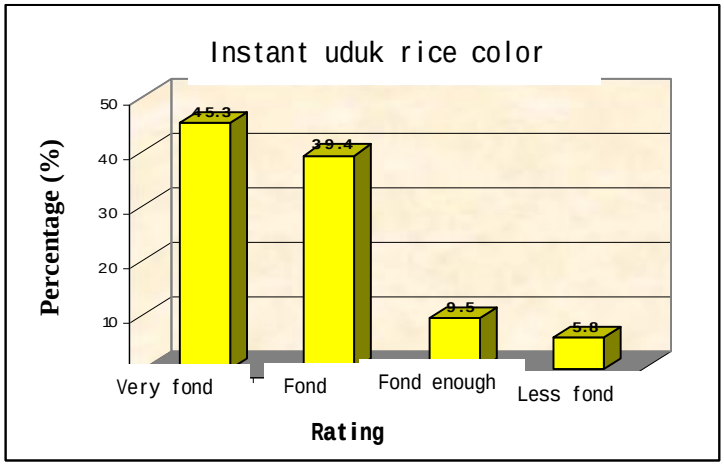
Picture 2. The result of instant uduk rice in packages

RESULTS AND DISCUSSION

Results Data

Consumers ratings of instant uduk rice's properness based on product preference level includes: color, taste, smell, and texture with the criteria of: very fond, fond, fond enough, and less fond. Results data are illustrated as follows:

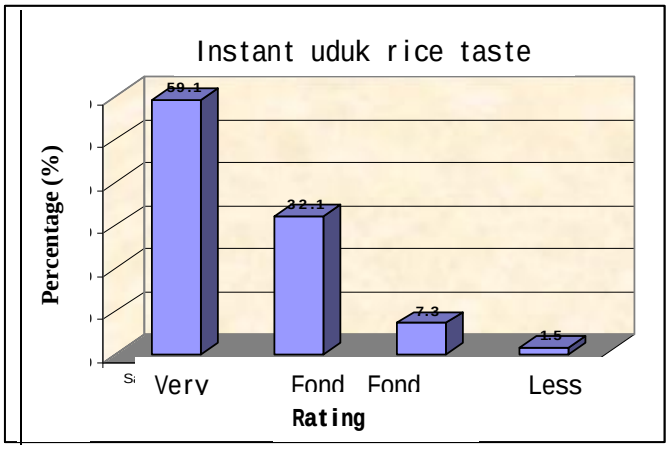
1. Color



Picture 3. Consumers rating for instant uduk rice's color

According to Picture 3, there are percentages of consumers' rating for instant uduk rice's color with the criteria of very fond 45,4%; fond 39,4%; fond enough 9,5%; and less fond 5,8%. The highest criteria was very fond which was 45,4%. This was because the color of dried instant uduk rice is yellowish that was caused of seasoning and drying process, the original rice's color without seasoning was whie, yet since the rice was mixed with seasoning, chicken broth, and coconut milk then it went through drying process (using an oven), the color turned to yellowish white. Seasoned rice which has gone through cooking process will experience color changing that's influenced by the seasonings, it is because that there is a process of starch browning in dried heating process.

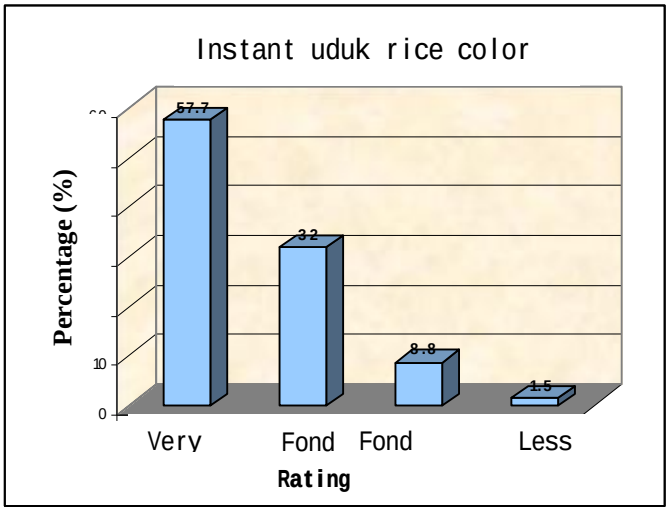
2. Flavor



Picture 4. Consumers' rating for instan uduk rice's taste

According to Picture 4 there are percentages of consumers' rating for instant uduk rice's taste with the criteria of very fond 59,1%; fond 32,1%; fond enough 7,3%; and less fond 1,5%. The highest criteria was very fond which was 59,1%. A taste of a food product both processed and non processed is the climax of that food. The taste of this instant uduk rice is influenced by the seasonings and spices that were added to it, such as chicken broth, coconut milk, and sliced chicken. Containing such ingredients, the instant uduk rice will have savory and delicious taste and will satisfy people who consume it.

3. Smell/Aroma

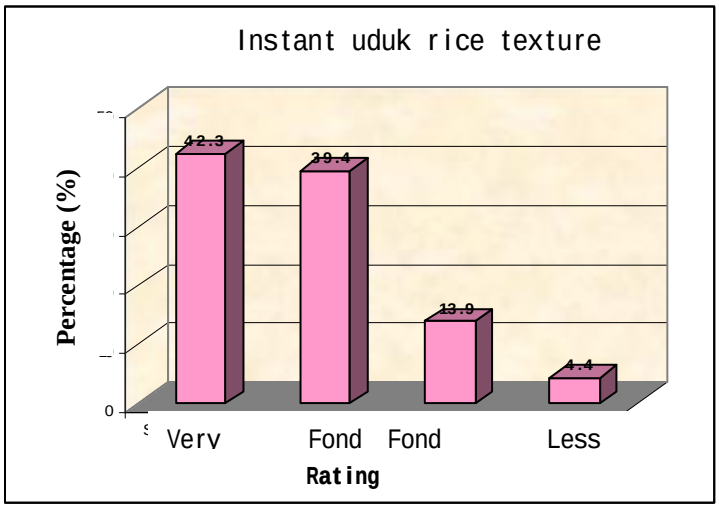


Picture 5. Consumers rating for instant uduk rice's aroma

According to Picture 5, there are percentages of consumers' rating for instant uduk rice's aroma with the criteria of very fond 57,7%; fond 32%; fond enough 8,8%; and less fond 1,5%. The highest criteria was very fond which was 57,7%. The aroma of instant uduk rice comes from the unique characteristics of uduk rice. In the making process, the rice was

added by spices and other ingredients such as sliced chicken, thus a strong aroma of those additional ingredients will dominate the product, such as the smell of spices, chicken, and coconut milk which are united to make a very nice and unique aroma that makes people to get interested in eating it.

4. Texture/Softness



Picture 6. Consumers rating for instant uduk rice's texture

According to Picture 6, there are percentages of consumers' rating for instant uduk rice's aroma with the criteria of very fond 42,3%; fond 39,4%; fond enough 13,9%; and less fond 4,4%. The highest criteria was very fond which was 42,3%. The instant uduk rice has soft texture and is easy to chew and swallow. The texture is a result of liquid addition (fresh mineral water) the re-cooking for serving, with the ratio of dried uduk rice and liquid 1 : 1,5. The liquid in re-cooking process is to wet and to increase the volume of starch granules inside the rice grains thus the grains will expand and become soft. This softening process is not too long, which is only three minutes, because rice grains have gone through twice of gelatinization process and the surfaces of the grains are already porious. Besides the amount f liquid, the amount of re-cooking time also determines the instant uduk rice's texture.

Organoleptic Tests Results

The result of organoleptic tests toward the properness quality of instant uduk rice including: color, taste, aroma, and texture/softness are as follows:

1. Descriptive Test

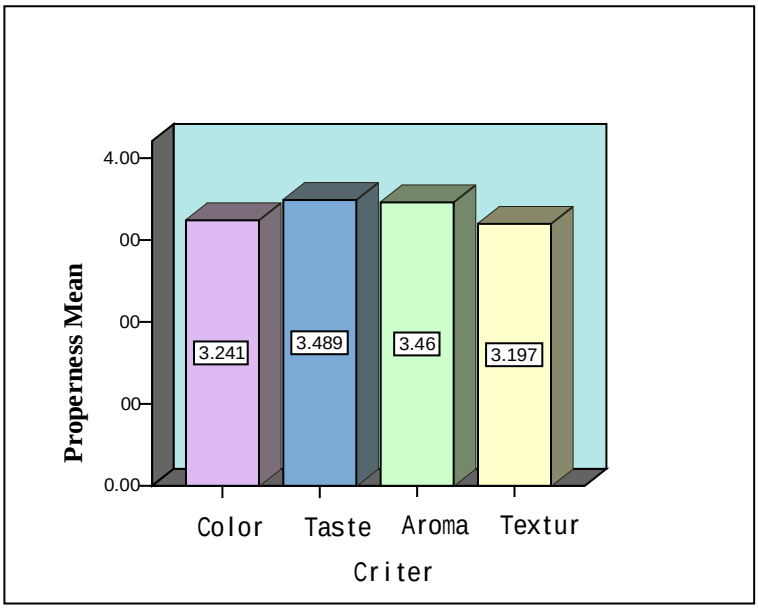
Table 2. Descriptive Test Results of Consumers' Ratings for Instant Uduk Rice's Properness Quality

Descriptives

Properness

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Color	137	3.2409	.85341	.07291	3.0967	3.3851	1.00	4.00
Taste	137	3.4891	.69786	.05962	3.3711	3.6070	1.00	4.00
Aroma	137	3.4599	.71758	.06131	3.3386	3.5811	1.00	4.00
Texture	137	3.1971	.83876	.07166	3.0554	3.3388	1.00	4.00
Total	548	3.3467	.78854	.03368	3.2805	3.4129	1.00	4.00

Mean of Instant Uduk Rice's Properness Level



Picture 7. Properness Mean Value of Instant Uduk Rice

According to Table 2 and Picture 7. above, the mean value of consumers' ratings for the criteria of color was 3,24; for the taste it was 3,49; for the aroma it was 3,46; and for the texture/softness it was 3,35. The highest mean value was for the taste category which was 3,49. It is because the taste of instant uduk rice is unique and savory because of the mixture

of spices including candlenuts, garlics, corinders, sugar, salt, lemongrass, orange leaves, bay leaves, chicken broth, and coconut milk. Those spices contain etheric substances that if they're heated, they will produce nice and delicious aroma.

2. Friedman Test

Table 3. Friedman Test Result of Instant Uduk Rice's Properness Level

Test Statistics[a]

N	137
Chi-Square	28.566
df	3
Asymp. Sig.	.000

The Friedman test result in Table 3. showed that all four criteria including color, taste, smell, and texure are influential toward the consumers' acceptance according to the properness level with Chi-Square value of 28,566 and the Asymp.Sig was 0,000 (under 5%). This means that the hypothesis which stated that there would be influences by all four criteria toward the consumers' acceptance based on the properness level of instant uduk rice.

CONCLUSIONS AND SUGGESTIONS

Conclusions

Organoleptic tests which was completed to know the properness and fondness level of consumers toward color, aroma, taste, and texture/softness for instant uduk rice resulted as follows:

1. Research result based on consumers' fondness level toward instant uduk rice's color had the highest percentage in very fond level which was 45,4%. The criteria of taste had the highest percentage very fond level which was 59,1%. The The criteria of aroma had the highest percentage very fond level which was 57,7%. The criteria of texture had the highest percentage very fond level which was 42,3%.. therefore the result of consumers acceptance according to the fondness level in color, taste, aroma, and texture resulted that the product is acceptable for consumers.
2. Friedman test results showed that all four criteria including color, aroma, taste, and texture/softness are influential toward the consumers' acceptance based on its properness level.

Suggestions

In order to accelerate the production of instant uduk rice in big scale of amount, it is suggested that the producers use electric drying oven with bigger size and capacity. Instant uduk rice products are already acceptable to be sold in large amount to people.

REFERENCES

Arikunto, Suharsimi. 2003. *Manajemen Penelitian.* Jakarta: Rineka Cipta

Hubeis, M. 1984. *Pengembangan Metode Uji Kepulenan Nasi*. Tesis, Pascasarjana IPB, Bogor.

Kristiastuti, Dwi. 2012. *Modul Bahan Ajar Pengelolaan Makanan Nusantara*. Unesa

Luh, B.S., R.L. Robert and C.F. Li. 1980. *Quick Cooking Rice. Di dalam Luh, B.S. (Ed). Rice Production and Utilization.* AVI Publ. Comp. Inc. Westport. Connecticus.

McNeely, Jeffrey A. 1992. *Economi dan Keanekaragaman Hayati*, Jakarta: Sinar Harapan.

Miles and A Michael Huberman. 1984. *Qualitative Data Analysis*. Beverly Hills: Sage Publications.

Rewthong, O., S. Soponronarit, C. Taechapairoj, P. Tungtrakul and S. Prachayawarakorn. 2011. *Effect Of Cooking, Drying And Pretreatment Methods On Texture And Starch Digestibility Of Rice Instan*. J Food Eng 103: 258-264.

Development of Mungbean Tempeh Production as Alternative Tempeh

Elsie Angelina, Elisabeth K. Prabawati, S.T.P, M.FoodSt and Maria D.P.T. Gunawan-Puteri, STP, MSc, PhD [1*]

[1]*Department of Food Technology, Swiss German University, EduTown, BSD City, Tangerang Selatan, 15339, Indonesia*

**Corresponding author. E-mail: maria.gunawanputeri@sgu.ac.id*

Abstract

Mungbean (*Vigna radiate (L.) R. Wilczek*) tempeh appeared to be a potential substitute for soybean tempeh with its comparable protein content. However, its sensory acceptability was considerably low when compared to soy tempeh. This study was conducted to evaluate the modifications required in the processing method of mungbean tempeh based on sensory preference level and protein content. Three different methods were developed to produce mungbean tempeh and the results were further evaluated for their sensory preference levels and protein content (Lowry's method). Ranking-preference test indicated that mungbean treated in hot water (± 98 – 60 ºC) for 15 minutes and reheated at ±75 ºC for 5 minutes resulted in tempeh (MBT II) with the most preferred overall sensory quality. Lowry's protein analysis showed that the protein content of MBT II (64.74 ± 2.058 mg BSA eq/ g dry base) was not significantly different with the two others. The production method of MBT II was therefore selected to be used to produce mungbean tempeh for further analysis. The mungbean tempeh produced turned out to have a comparable sensory acceptability compared to soy tempeh. Moreover, there was also a high retention in the total protein content (39.22% dry base) and protein digestibility (114 mg/100 g dry base) although process improvement was applied on the production method of mungbean tempeh.

Keywords: Tempeh, tempeh processing technology, alternative tempeh ingredient, tempeh-derived products

Introduction

Tempeh is a traditional fermented food originated in Indonesia. It has been widely consumed also and considered as a high protein food yet relatively cheap and easy to be obtained. In the year 2014, Badan Pusat Statistik Indonesia (2014) reported that the average consumption of tempeh in the country has reached 0.133 g per capita per week. However, despite of the high demand of tempeh, Indonesia has a very low productivity of local soybean that has the main role as the raw material of tempeh. Indonesia imported as many as 1,411,184 tons of soybeans while the local farmers produced only 779.992 tons of soybeans in 2013 (Direktorat Jenderal Tanaman Pangan, 2014). Importing soybean does not really solve the problem since it leads to the rocketing price of soybean in the domestic market. Responding to this issue, many researches aimed to found alternative ingredients for tempeh fermentation were recently conducted.

Mungbean is one of the local legumes that showed a great potential as an alternative ingredient for tempeh production. Beside of its high availability in the domestic market, mungbean processed into tempeh also showed a higher sensory acceptability compared to other non-soy tempeh, namely red kidney bean and cowpea tempeh (Dika, et al., 2014). Mungbean tempeh turned out to have comparable protein content to soybean tempeh and even showed a greater quality for several nutritional and functional characters such as protein digestibility, beta-carotene, total phenolic content and total antioxidant activity (Dika, et al., 2014; Belinda, 2015). Despite of its superior quality, the sensory acceptability of mungbean tempeh was still considerably low, particularly in its taste, aroma and texture (Belinda, 2015). Therefore, further development in its production method was necessary for sensory acceptability improvement. The production method of mungbean tempeh, however, cannot directly follow the production method of soy tempeh as different legumes have different physical and chemical characteristics. Boiling process, as it was usually done in the making of soy tempeh, was not applicable for mungbean as it might rapidly increase the moisture content of mungbeans, resulted in tempeh that will decay faster. Boiling munbeans might also turn them into pulp that cannot be used for making tempeh. Thus, the aim of this research is to further improve the production process of mungbean tempeh, mainly for improving the sensory quality and reproducibility as there was an indication of unstable result during the production trials for sensory character screening.

Materials and Method

Materials and equipment

Mungbean was the major ingredient used in this research and obtained in Pasar Modern BSD, Tangerang, Indonesia. The tempeh starter used was Raprima Ragi Tempe from Rumah Tempe Indonesia, Bogor, Indonesia. The chemical materials used in this research were 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany), Bovine Serum Albumin (BSA) (Sigma-Aldrich, Germany), Biuret reagent, Folin-Ciocalteu phenol (Merck, Germany), and Sodium carbonate (Na_2CO_3) (AnalaR BDH, England), Potassium dihydrogen phosphate (KH_2PO_4) (Sinopham Chemical Reagent, Shanghai), Potassium phosphate dibasic anhydrous (K_2HPO_4) (Sinopham Chemical Reagent, Shanghai),

Trichloroacetic acid (CCl_3COOH) (Merck, Germany), L-tyrosine (Sigma Aldrich, Germany), and Pancreatine (Sigma Aldrich, Germany).
The equipment used in this research were incubator C30 (Indonesia), thermocouple TM-902C (Lutron), blender Philips, UV-Vis spectrophotometer (Genesys 10 uv Thermo Electron Corporation), hot plate (Cimarex, Barnsteadthermolyne, USA), pH meter Hanna, Romania), centrifuge Rotina 35R (Hettich, Germany), moisture content analyser (Sartorius, Germany) and Vortex REAX (Heidolph, Germany).

Production of mungbean tempeh for sensory characterization
Mungbeans were soaked overnight and dehulled. Heat treatment for 15 minutes was applied by soaking the legumes1 in hot water (± 98 - 60 ^{0}C). The dehulled mungbeans was then re-soaked in tap water for 24 hours for acid fermentation. The acid fermented beans were then drained and dried. Starter inoculation (0.2%) was done and incubation was carried out under room temperature for 48 hours.

Production of mungbean tempeh with modified methods
Three equal portions of mungbean were soaked overnight for hydration. Heat treatment was applied by soaking the beans in hot water (± 98 - 60 ^{0}C). Subsequently, the mungbeans were dehulled and re-soaked in tap water for 24 hours with minimum exposure to oxygen to allow natural lactic acid fermentation. The acid fermented mungbeans were then reheated by soaking the first portion in hot water (± 98 - 60 ^{0}C) for 1 minute while the second and the third portion were soaked in water with temperature held at 75 ^{0}C respectively for 5 and 15 minutes. All the mungbeans were drained and dried afterwards. Starter inoculation (0.2%) was done and incubation was carried out under the temperature of ±30 ^{0}C for the first 24 hours and ±27 ^{0}C for the next 24 hours. The center temperature of tempeh was checked periodically and relative humidity was maintained at 70 – 85%. The complete production flow with process controls was best explained in Figure 1.

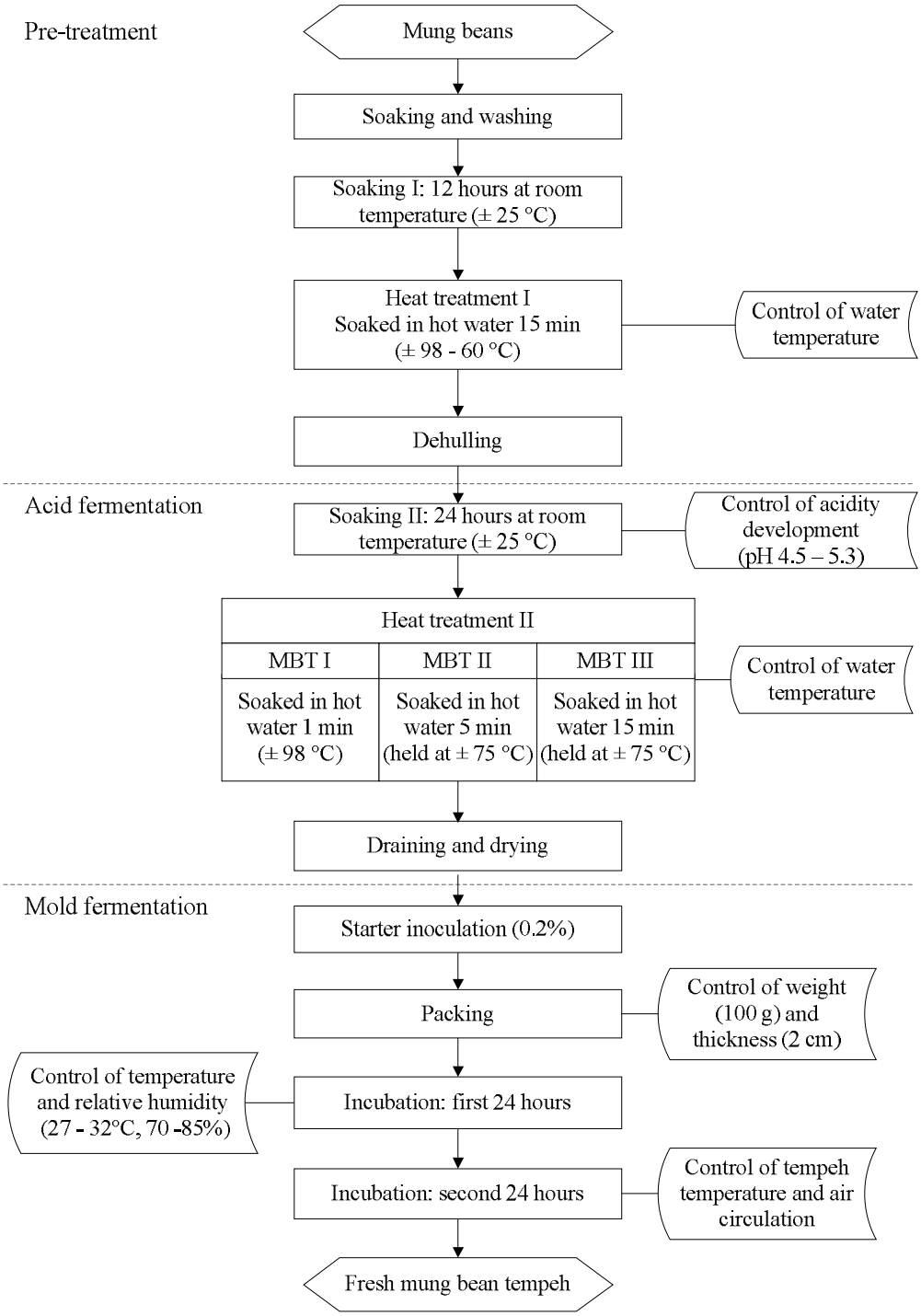


Figure 1. Modified production process of mungbean tempeh

Sensory evaluation

Mungbean tempeh produced from different methods was evaluated for its sensory preference level using ranking-preference test. The samples were prepared in the form of fried mungbean tempeh and 53 untrained panelists were asked to rank the tempeh based on the overall preference. The results of the sensory evaluation were further analysed using statistical analysis.

Affective-acceptance test was also conducted to evaluate the sensory acceptance level of mungbean tempeh produced from the selected method as a "prototype" while soybean tempeh as "control". Both types of tempeh were prepared in the form of fried mungbean tempeh and 50 untrained panelists were asked to evaluate the attributes (appearance, aroma, taste and texture) of the samples by giving score from 1 to 9 (1 = dislike extremely, 9 = like extremely).

Lowry's protein content analysis

The preparation of sample extract was required for protein content analysis. Fresh mungbean tempeh was stored overnight in freezer prior to extraction. The frozen samples were then grinded into powder by using blender. The solvent used for the extraction was distilled water and the ratio of sample to solvent was 2 : 5 (40 g of tempeh powder in 100 ml of distilled water). The extraction process was carried out at room temperature (±25 ^{0}C) and under constant agitation for 6 hours. The sample extracts were then filtered by using cheese cloth and centrifuged at 8000 rpm for 15 minutes. The supernatant was collected and stored in freezer until analysis was conducted.

The analysis was done by diluting 1 ml of each sample extract in 9 ml of distilled water. Then, in a test tube, 0.3 ml of the diluted sample was added with 1.5 ml of biuret. The solution was homogenized using vortex and incubated under room temperature for 10 minutes. After that, 750 µl Folin Ciocalteau (1:2) was added into the test tube and the solution was homogenized then left for 30 minutes. The absorbance value of each sample was read using UV-Vis spectrophotometer at 650 nm against a blank sample. Protein content was obtained by interpolating the absorbance values to BSA standard curve.

Soluble amino acid

Sample containing 9 mg protein/ ml was extracted in the same manner as in the sample preparation for Lowry's protein content analysis. The sample extract was prepared in a microtube and added with 250 µl buffer pH 8 and 250 µl distilled water, incubated at 37 ^{0}C for 5 minutes, and added with 750 µl of 10% TCA afterward. The solution was centrifuged at 10000 rpm for 10 minutes. The supernatant was collected and 300 µl of it was taken to be added with 1000 µl 0.5 M Na_2CO_3 and 200 µl Folin-Ciocalteu reagent (1 : 2). A blank solution was made with the similar procedure except the sample extract was replaced with distilled water. Same procedure was also applied for sample with enzyme treatment for protein digestibility analysis, but the distilled water was replaced by 250 µl pancreatin enzyme solution with concentration of 4 mg/ ml.

The absorbance of the samples with and without enzyme pancreatin was read using UV-vis spectrophotometer at 578 nm. Amino acid content of the sample extract with and without enzyme treatment was calculated by interpolating the absorbance value with tyrosine standard curve, which was constructed using tyrosine with concentration of 5.000, 2.500, 1.250, 0.625, and 0.500 mM. Protein digestibility was analyzed by referring to the change of the amount of soluble amino acid after enzyme treatment. The amount of amino acid released was calculated by subtracting the amino acid content with enzyme treatment with amino acid content without enzyme treatment.

Total nitrogen analysis

Kjeldahl method was applied for analyzing the total nitrogen content of mungbean tempeh. Sample weighing 2 g was prepared in a folded filter paper and put into 100 ml Kjeldahl flask. Catalysts such as z7 g of K_2SO_4 and 0.5 g of $CuSO_4$ were added into the flask together with boiling chips. Concentrated H_2SO_4 as much as 15 ml and 3 ml of H_2O_2 were added and the flask was then placed at fume hood for 10 minutes. Sample was destructed at 410 ^{0}C for ± 2 hours until it turned into clear solution and left to be cooled down to room temperature then 50 – 75 ml of aquadest was subsequently added. An Erlenmeyer flask containing 25 ml H_3BO_3 4% and indicators, which were 0.25 ml of Bromcresol green 0.1 % and 0.175 ml of methyl red 0.1%, were put in the outlet of the distillation set to hold the distillate. The distillation product was put in the distillation set and 50 – 70 ml of sodium hydroxide-thiosulfates was added. The solution was distilled until 150 ml distillate was obtained and titrated with HCl 0.2 N until its color turned from green to natural gray. Crude protein content (%) was calculated based on total nitrogen content using the formula below:

$$\% \text{ Protein} = \frac{(Va-Vb)VHCl \times NHCl \times 14.007 \times 6.25}{W \times 1000} \times 100\%$$

where,
Va= Volume of HCl used for titrating sample solution (ml)
Vb= Volume of HCl used for titrating blank solution (ml)
N= Normality
14.007 = relative mass of Nitrogen atom
6.25= Protein conversion factor for common food
W= Weight of sample (g)

Result and Discussion

Sensory Characterization

Mungbean tempeh was produced by following the method developed by Belinda (2015). The results, however, showed unstable formation of mungbean tempeh. Out of four trials, there was only one result that can be considered as a well fermented tempeh as the mycelium fully covered the whole surface. The other trials indicated that the growth of the tempeh mold was not optimal and even there was no mycelium formed in one of them. Two of the unsuccessful result also became slimy by the end of the fermentation period (48 hours). The result of the fermentation can be observed in Table 1 and Figure 2.

Figure 2. Mung bean tempeh produced for sensory characterization

Table 1. Sensory characteristic of raw mung bean tempeh

Trial	Sensory characteristic		
	Appearance	Aroma	Texture
First batch	Yellow beans, slimy, partly filled with mycelium	Acidic, ammoniac	Less compact
Second batch	Yellow beans, partly filled with mycelium	Neutral, tempeh like	Hard and compact
Third batch	Yellow beans, slimy, no mycelium	Acidic	Loose
Fourth batch	Covered fully with white mycelium	Neutral, tempeh like	Compact

Hedger (1982) stated that tempeh partly filled with mycelium indicates either the too high temperature by the end of the fermentation stage or contamination by bacteria. Therefore, this might explain why the growth of mycelium was not optimum in mungbean tempeh. Looking back to the processing steps, acid fermented beans went directly to draining and drying process as the preparation for inoculation and mold fermentation stage. It was necessary to understand that acid fermentation happened during the processing of mungbean tempeh was developed by lactic acid bacteria and the usage of tap water might also increase the number of contaminant bacteria.

Table 2. Sensory characteristic of fried mung bean tempeh

Trial	Sensory characteristic			
	Appearance	Aroma	Texture	Taste
Second batch	Yellowish	Neutral, tempeh like	Hard, gritty, grainy, starchy	Tempeh like
Fourth batch	Yellowish, murky	Neutral, tempeh like	Hard, gritty, grainy	A bit bitter

Figure 3. Fried mungbean tempeh (second and fourth batch) for sensory characterization

In terms of sensory quality, Belinda (2015) reported that fried mungbean tempeh had a relative low acceptance score compared to soybean tempeh, particularly for its texture, taste and aroma. Acidic or alcoholic smell as well as bitter aftertaste was detected in fried mungbean tempeh and the texture turned out to be mushy (Belinda 2015). In contrast, the mungbean tempeh produced in this stage indicated that fried mungbean tempeh processed from two different batches had hard, gritty and grainy texture. The sensory characteristic (Table 2) observed somehow resembled the fried mungbean tempeh evaluated by Dika et al. (2014). In terms of processing method, there was a slight difference in the pre-treatment stage between the two methods in which undehulled mungbeans were soaked for a longer time (24 hours) in the study conducted by Dika et al. (2014). However, the result of the sensory characterization indicated that the different soaking time did not affect the texture of the end product. In conclusion, modification in the processing method was needed to improve the sensory quality of mungbean tempeh, particularly to soften its texture. Control process might be also needed for producing mungbean tempeh with high reproducibility.

Modifying and Selecting Mungbean Tempeh Production Method

As it was showed in Figure 1, the modified production process of mungbean tempeh was mainly divided into three stages, which were the pre-treatment, acid fermentation and mold fermentation stage. The pre-treatment stage included soaking and heat treatment that aimed for hydration and to facilitate dehulling. Acid fermentation was naturally developed due to the presence of lactic acid bacteria. The low pH of the legumes might help to minimize contamination by spoilage bacteria. Acid fermentation was controlled by minimizing the exposure of the beans to oxygen as lactic acid bacteria are all facultative anaerob, so pH could be decreased optimally into the range of 4.5 – 5.3. In the end of the acid fermentation stage, heat treatment was applied in order to soften the beans and also for lowering the number of bacteria presented in the acidic beans. Heating acid fermented beans mildly allows sufficient mix flora of lactic acid bacteria and *Bacillus spp.* to survive and thus excessive spoilage can be prevented (Mulyowidarso et al., 1989; Nout et al., 1985; Nout & Rombouts, 1990). Mold fermentation was carried out at ±30 ^{0}C with 70 -85% relative humidity for the first 24 hours. In the second 24 hours, the tempeh was then placed in a better circulation site and the incubation temperature maintained at room temperature (± 25 ^{0}C).

Figure 4. Fried MBT I, MBT II, and MBT III

Table 3. Subjective profiling analysis of fried mungbean tempeh

Mungbean tempeh	Visual appearance	Aroma	Taste	Texture
MBT I	Yellowish, pale	Normal, tempeh-like	Bitter aftertaste (none), bland	Soft but a bit gritty
MBT II	Yellowish, bright	Normal, tempeh-like	Bitter aftertaste (weak)	Soft but a bit gritty
MBT III	Yellowish, murky	Normal, tempeh-like	Bitter aftertaste (strong)	Soft but a bit gritty

Table 4. Result of ranking-preference test of MBT I, MBT II and MBT III. The data are presented as mean rank value (N=53) ± standard deviation (SD)

Samples	Mean rank*
MBT I	2.15 ± 0.77^{b}
MBT II	1.66 ± 0.73^{a}
MBT III	2.19 ± 0.86^{a}

*Values with different letters indicated significant difference

Mungbean tempeh produced (MBT I, MBT II and MBT III) were then evaluated for their overall sensory preference level by using ranking-preference test. The result (Table 4) of the sensory ranking-preference test indicated that MBT II obtained the highest rank (1.66 ± 0.73) that was also significantly different ($p < 0.05$) to MBT I (2.15 ± 0.77) and MBT II (2.19 ± 0.77). Subjective profiling was also conducted to determine whether the target of the improvement has been reached before the products were evaluated by the panelists. The results of subjective profiling were presented in Table 3.

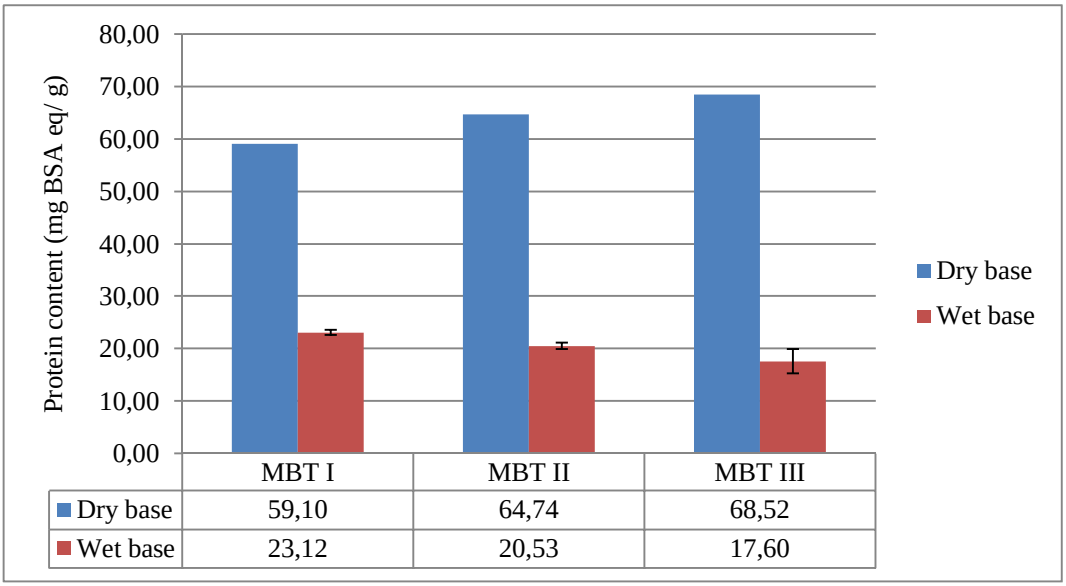


Figure 5. Protein content of MBT I, MBT II and MBT III

Protein content analysis using Lowry's method was also conducted to evaluate whether the different heat treatments of MBT I, MBT II and MBT III affect the protein content. The result showed that the highest protein content was found in MBT III with 68.52 ± 9.087 mg protein/ g dry base, followed by MBT II with 64.74 ± 2.058 mg protein/ g dry base and MBT I with 59.10 ± 1.201 mg protein/ g dry base. However, statistical analysis indicated that the three mungbean tempeh was not significantly different ($p < 0.05$) in terms of dry basis protein content. Moreover, the process improvement resulted in mungbean tempeh with comparable protein content to that observed by Belinda (2015) (62.72 ± 0.802 mg protein/ g dry base), hence it can be said that the additional heat treatment tend to be irrelevant with the protein content of the resulted mungbean tempeh.

Considering the wet basis protein content might also necessary for a better understanding about the effect of heat treatment to the overall protein content. Unlike the dry basis protein content, MBT I showed the highest protein content of 23.12 ± 0.470 mg protein/ g wet base, followed by MBT II with 20.53 ± 0.653 mg protein/ g wet base and MBT III with 17.60 ± 2.334 mg protein/ g wet base. Moreover, the statistical analysis also indicated that MBT III showed a significant different ($p < 0.05$) with MBT I and MBT II.

Looking back to the processing step, MBT III underwent the longest heat treatment compared to MBT I and MBT II, which means the legumes were exposed to hot water for the longest time. According to Iljas et al. (1973), soybean protein might leach out during the processing of tempeh, particularly during the immersion in excessive water. Soaking also lead to the increase of water content as the legumes were hydrated. Thus longer soaking time was likely to result in higher moisture content. The penetration of water into the legumes was also related to the temperature of the soak water, in which high temperature leads to more rapid penetration compared to lower temperature (Iswandari, 2006). Since the legumes were processed in water with temperature far above room temperature, the penetration of

water was more rapid compared to the earlier hydration process although the duration was shorter. Therefore, mungbean tempeh with higher proportion of water content was likely to have lower solid compounds, including protein. In addition, Kon (1979) also reported that increasing the temperature of soak water to 60 ^{0}C or above might cause a significant loss of nitrogen compounds compared to the temperature below 50 ^{0}C. In conclusion, the processing method of MBT II was suggested as the best selection as the mungbean tempeh produced has the highest sensory preference level and comparable dry basis protein content to the others.

Sensory Acceptance Level of Mungbean Tempeh

As an alternative form of tempeh, the sensory acceptance level of mungbean tempeh is very important for determining whether it can be considered as a substitute for soybean tempeh. Sensory acceptance test will provide a good indication of product's potential to be accepted by customer and marketplace. Therefore, affective-acceptance sensory test was conducted to evaluate and compare the acceptance level of appearance, aroma, taste and texture of mung bean tempeh (MBT) as "prototype" to soybean tempeh (SBT) as "control". Both samples were served as fried tempeh as it is the most common way to consume tempeh product. The samples were served to 50 untrained panelists to be evaluated and the results were further evaluated for the significant differences in each attributes by using statistical analysis (Wilcoxon test).

Table 5. Result of affective-acceptance test of SBT and MBT. The data are presented as mean value (N=50) ± standard deviation (SD)

Attributes	SBT	MBT	p-value*
Appearance	7.22 ± 1.11	6.90 ± 1.36	0.134
Aroma	6.58 ± 1.54	7.22 ± 6.58	0.028
Taste	5.28 ± 1.73	4.98 ± 2.03	0.241
Texture	6.50 ± 1.46	6.28 ± 1.40	0.257

*p-value < 0.05 in the same row indicates significant difference

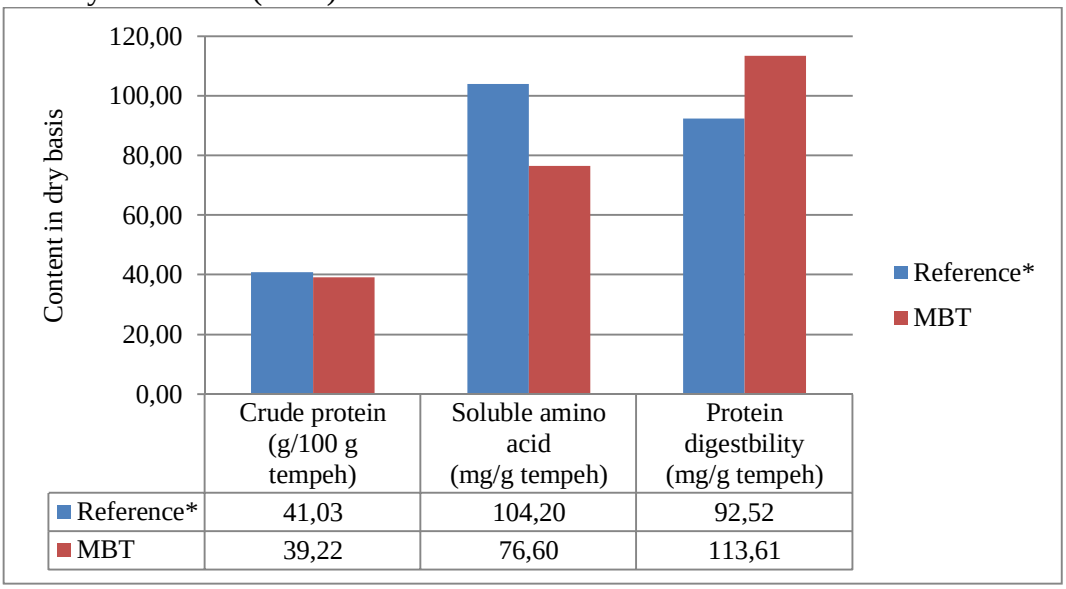
As it can be observed in Table 5, the result of the affective-acceptance showed that there was no significant difference ($p > 0.05$) between the appearance of MBT and SBT. The mean score of both MBT and SBT was around 7, which indicates that their appearance was "like moderately". However, in terms of aroma, SBT and MBT was significantly different ($p < 0.05$). MBT showed a higher mean value of around 7 which indicates that the aroma of MBT was "like moderately" while SBT showed a lower mean score around 6 which indicates that the aroma of SBT was "like slightly". This result also indicates the panelists prefer the aroma of MBT more than SBT. Several comments during the sensory test mentioned that MBT has strong savory aroma similar to shrimp. The acceptance level of SBT and MBT for taste turned out to have no significant difference ($p > 0.05$) with mean values around 5. This means that the taste of both products was "neither like nor dislike". Several panelists also left similar comment about the bitter taste of both SBT and MBT. Lastly, the acceptance level for texture indicates also no significant difference between SBT and MBT. The mean values of both samples were around 6 which indicates that the texture of both MBT and SBT was "like slightly".

Belinda (2015) reported that the acceptance level of mungbean tempeh was still low, particularly for its aroma (5.30 ± 1.83), taste (3.24 ± 1.85), and texture (4.56 ± 1.86). In terms of appearance, MBT (6.90 ± 1.36) showed quite a similar acceptance score to that observed by previously (6.74 ± 1.37). On the other hand, MBT showed a higher acceptance score for aroma (6.58 ± 1.54), taste (4.98 ± 2.03), and texture (6.28 ± 1.40). In conclusion, there is no significant difference ($p > 0.05$) for the appearance, taste, and texture of SBT and MBT. The only significant difference ($p < 0.05$) is for the aroma, in which MBT was more preferable than SBT. Hence, it could be said that MBT is comparable to SBT in terms of sensory acceptability. The result of the sensory acceptance test also indicates that the treatment applied for the process improvement led to the increase of the sensory acceptance level of mungbean tempeh.

Evaluation on Protein's Quality of Mungbean Tempeh

Tempeh is considered as source of protein. Dika et al. (2014) reported that even the initial total protein content of raw mungbean was lower than that of soybean, mold fermentation was found to be able to increase the total protein content to a similar level with soy tempeh. As the production method of munbean tempeh now was modified to improve its sensory quality, there might be a possibility that changes will occur to its protein content and quality. Therefore, analyzing the total protein content and quality of mungbean tempeh was necessary to evaluate whether mungbean tempeh fulfilled the requirement to be a high protein food as well as soy tempeh.

Total (crude) protein content was analyzed using Kjeldahl method and the result indicates mungbean tempeh showed a protein content of 13.95% (wet basis), which was 3.51% lower than observed by Dika et al. (2014). The protein content of the mungbean tempeh observed equals to 39.22% in dry basis (Figure 6) which turned out to be only 1.81% lower than that observed by Dika et al. (2014).



	Crude protein (g/100 g tempeh)	Soluble amino acid (mg/g tempeh)	Protein digestbility (mg/g tempeh)
Reference*	41,03	104,20	92,52
MBT	39,22	76,60	113,61

Figure 6. Result of protein content and quality analysis
(*source: Dika et al. 2014; Belinda 2015)

Protein content tends to decrease during the processing of tempeh. Dika et al. (2014) reported that the protein content of mung beans tend to decrease by 20 to 38% after acid fermentation which might be due to the microbial activity of the lactic acid bacteria or also possibly caused by the heat treatment prior to the acid fermentation (Dika, et al., 2014; Belinda, 2015). Hence the subsequent heat treatment after acid fermentation was likely to decrease the protein content of the mungbeans as the protein might further leach out and dissolved in the soak water. Hefnawy (2011) also reported the similar finding, in which lentil seeds underwent a decrease in total protein and non-protein nitrogen content after heat being boiled.

The lower percentage of protein content might also due to the increase of moisture content. As the heat treatment was done by soaking the beans in hot water, therefore there was a possibility that the moisture content increase by the end of the soaking period. Temperature of soak water, obviously, also played a significant role. As it has been previously discussed, higher temperature of soak water causes a more rapid penetration of water into the beans (Iswandari, 2006). Therefore, as the proportion of water was increased, it was very possible that the proportion of other solid compounds decreased.

Although the protein content of tempeh was actually lower than the fresh legumes, it could be said that the quality of tempeh protein was greater than that of raw beans. Raw legume seeds provide only a small amount of protein that is readily digested. Fermentation of the beans helps to breakdown protein macromolecules in legumes into amino acids and small peptides. Therefore the protein in tempeh was more readily absorbed by human body.

The amount of amino acid presented within mungbean tempeh was quantified using spectrophotometry method, based on the ability of Folin's reagent to form blue color complex when bound with several amino acids such as trypsin and tryptophan. The result indicated (Figure 6) that the amount of mungbean tempeh soluble amino acid was 76.60 ± 2.167 mg Tyrosine eq/ 100 g dry base, which was lower than that observed by Belinda (2015) (104.20 ± 12.08 mg Tyrosine eq/ 100 g dry base). The decrease of soluble amino acids might be due to several factors. To begin with, the addition of second heat treatment in the processing might lead to more severe denaturation of protein structure compared to that ocurred to mungbeans that only heated once. As a result, more hydrophobic amino acid residues were exposed and thus the compounds become less soluble. Moreover, the soluble amino acid might also leached out into the soak water during the heat treatment. It was also observed that the increase and decrease of amino acids during tempeh fermentation was related to microbial activity (Belinda, 2015). Belinda (2014) suggested that although proteases were released to break down protein molecules, the secretion of the enzyme by microorganisms might be stopped once enough amino acids were released because microorganisms tend to take up the available resources first as efficiently as possible.

Despite of the lower amount of amino acids, mungbean tempeh still showed a comparable protein digestibility to that observed by Belinda (2015). The evaluation of protein digestibility was based on the ability of protease enzymes to break down protein into amino acids and generate TCA-soluble peptides. Several amino acids were known to have the ability to form blue color complex with Folin's reagent under alkaline condition. When protease, in this case pancreatin enzyme, was added, amino acids were released as a result

of protein digestion and the color complex formed could be interpreted by using spectrophotometer (Belinda, 2015). Figure 6 indicates that the increase of amino acid in mungbean tempeh extract after the addition of pancreatin enzyme was 114.64 ± 8.12 mg Tyrosine eq/ 100 g dry base, which was actually higher than that observed by Belinda (2015) (92.52 ± 9.05 mg Tyrosine eq/ 100 g dry base).

The increase and decrease of protein digestibility was related to anti-nutritional compounds and food processing. One antinutritional compound, which is known to decrease protein and amino acid digestibility in soybean and other legumes, is trypsin inhibitor. Thus the inactivation of trypsin inhibitor might lead to the increase of digestible protein. One of the most effective ways to reduce the level of trypsin inhibitor was by applying heat treatment although severe heat treatment might also reduce the nutritional value of the seeds (Iljas, et al., 1973). Hefnawy (2011) proved that boiling lentil seeds effectively removed trypsin inhibitor up to 80% while phytic acid were decreased for more than 30%. Thus, it could be suggested that the high digestibility of protein in mungbean tempeh was likely due to the subsequent heat treatment of mungbeans, that might resulted further decrease the amount of anti-nutritional compounds presented within mungbeans.

In conclusion, mungbean tempeh still showed a high value of protein's quality although the improvement of the production method of mungbean tempeh tend to decrease the amount of the total protein content and soluble amino acid compared to the previous study by Dika (2014) and Belinda (2014), in which mungbeans were only heated once during the processing.

Conclusion

The addition of heat treatment after the acid fermentation stage in the production process of mungbean tempeh gave a more reproducible and stable result. Heating the beans for 5 minutes in water with temperature maintained at ± 75 ^{0}C prior to starter inoculation was the most preferable treatment as it resulted in mungbean tempeh with the most preferred overall sensory characteristic and comparable protein content to the other methods. In terms of sensory acceptance level, mungbean tempeh showed a comparable acceptability compared to soybean tempeh, even it has more preferable aroma, which indicates that the target of improving the sensory quality of mungbean tempeh has been achieved. Mungbean tempeh also showed a good retention of total protein content and protein's quality although the legumes underwent additional heat treatment. Therefore, mungbean tempeh can be considered as a potential substitute for soybean tempeh in terms of protein's quality and sensory acceptance.

References

Belinda, A. (2015). *Evaluation of Bioactive Compounds and Functional Properties of Mung Bean Tempeh as an Alternative Form of Tempeh*. Thesis. Swiss German University.

Dika, P., Prabawati, E., Kartawiria, I. and Gunawan-Puteri, M. (2014). Exploration of Non-Soy Tempeh and Characterization of Its Microbial and Nutritional Development. In: *The 3rd*

International Symposium on Processing of Foods, Vegetables, and Fruits. Kuala Lumpur: University of Nottingham Malaysia Campus.

Hedger, J.N. 1982. Production of Tempe, an Indonesian Fermented Food. In: S.B.

Primrose and A.C. Wardlaw, eds. 1982. *Sourcebook of Experiment for the Teaching of Microbiology*. New York and London: Academic Prexx xvii, pp. 599-600

Hefnawy, T. (2011). Effect of processing methods on nutritional composition and anti-nutritional factors in lentils (Lens culinaris). *Annals of Agricultural Sciences*, 56(2), pp.57-61.

Iljas, N., Peng, A. and Gould, W. (1973). *Tempeh, An Indonesian Fermented Soybean Food*. Ohio: Holiculture Series, pp.18-19.

Iswandari, R. (2006). *Studi Kandungan Isoflavon pada Kacang Hijau (Vigna Radiata L.), Tempe Kacang Hijau, dan Bubur Kacang Hijau.* Thesis. Institut Pertanian Bogor.

Kon, S. (1979). Effect of Soaking Temperature on Cooking and Nutritional Quality of Beans. *Journal of Food Science*, 44(5), pp.1329-1335.

Mulyowidarso, R., Fleet, G. and Buckle, K. (1989). The microbial ecology of soybean soaking for tempe production. *International Journal of Food Microbiology*, 8(1), pp.35-46.
Nout, M.J.R. and Rombouts, F.M. (1990). Recent developments in tempe research. *Journal of Applied Bacteriology*, 69(5), pp.609-633.

Nout, M.J.R., Bonants-Van Laarhoven, T.M.G., de Dreu, R. and Gerats, I. (1985). The Influence of Some Process Variables and Storage Conditions on The Quality and Shelf-life of Soybean Tempeh. *Antonie van Leeuwenhoek*, 51(5-6), pp.532-534.

FOOD QUALITY AND SAFETY

The application of "Bioextract" or "Bio-fermented solution" for treatment of domestic wastewater

Viyada Kunathigan[1]*and Suchawadee Wiratthikowit[2].

[1]Department of Food Biotechnology, School of Biotechnology, Assumption University, Huamak campus, Bangkapi, Bangkok 10240 Thailand
[2]Department of Agro-Industry, School of Biotechnology, Assumption University, Huamak campus, Bangkapi, Bangkok 10240 Thailand

ABSTRACT

Bio-fermented solution or "Bioextract" is a solution composes of a diverse variety of microorganisms coexisting together or so called "liquid microbial consortium". Nowadays bioextract is widely used with one of the recent application for treatment of organic waste and wastewater. The application of bioextract to treat domestic wastewater was investigated by collecting samples from different establishments in urban areas including restaurants, medium size food factories, condominiums and hotels. The result showed that the addition of bioextract could reduce total solid (TS) content up to 53.33% compared with initial TS in the wastewater collected from restaurant in the first 24 hours in most of the treatment. The addition of bioextract also demonstrated ability to reduce greases and oils in the wastewater samples and the reduction of up to 80.43% was observed in wastewater collected from medium size food factories that were treated with 0.5 ml/L of bioextract and kept in light condition with air after 48 hours. Despite total solid and greases and oils contents, other quality was not affected significantly by addition of bioextract to wastewater samples. The index microorganisms including *E. coli*, coliform and fecal coliform bacteria was also monitored and found that addition of bioextract to wastewater did not increase the population of these bacteria. On the contrary number of these bacteria indicated reduction trend of these bacteria.

Keywords: bioextract, bio-fermented solution, wastewater treatment, greases and oils

Introduction

Bio-fermented solution or "Bioextract" is a product that produced by fermentation of agricultural raw materials mostly waste from other processes for example fruit rind and/or vegetable peels. The solution usually have brownish color range from light to dark brown depending on raw materials. Bioextract was originally produced as liquid biofertilizer or microbial inoculants for agricultural purposes. This bioextract containing mixture of different species of microorganisms which could also be called as consortium of microorganisms. The well-known bioextract is EM™, the effective microorganisms, that produced by EMRO™ (EM research Organization, 2016) that founded by Dr. Teruo Higa. The liquid biofertilizer or bioextract mostly containing consortium of microorganisms became popular and can be produced on site with local raw materials that usually be agricultural waste. Source of microorganisms may be either the concentrated biofertilizer e.g. EM-1, (EM research Organization, 2016)) or mixture of microbial consortium e.g. Microbial activator Super LDD 2 (Land Development Department, 2014) or using endogenous microorganisms from raw materials or environment as inoculum for fermentation.

Microbiological analysis of bioextract from different sources revealed that they were usually consisted of mixture of photosynthesizing bacteria, lactic acid bacteria, yeasts, actinomycetes and fermenting fungi (Bunchoo, 2002) (Ngampimol & Kunathigan, 2008) (Nitsuwat, et al., 2012). The number and diversity of microorganisms may vary according to the raw materials used as substrates in the fermentation. The consortium microorganisms exist within the bioextract was found to contribute to restore soil ecosystem and facilitate the conversion of materials in soil into forms that can be easily used by plants (Boraste, et al., 2009). Application of biofertilizer extended to other purposes like for odor treatment of animal pens and to use for organic waste management (Shalaby, 2011) (Feunganksorn, et al., 2011) (EM research Organization, 2016). The application of bioextract to solve wastewater problems was reported in Okinawa during the 1970's, in which the microorganisms in bioextract showed ability to reduce biological toxicity as well as remove the foul odor in wastewater (Okuda & Higa, 1995). There were other aspects of application for the bioextract that have been suggested by producers that some still need more evidences to support e.g. to be used as cleaning agents instead of chemical cleaning agents or add to unclogged sewage pipe (EM research Organization, 2016). The popularity of bioextract application was increased due to the awareness regarding the excess utilization of chemicals in every aspect of life. Therefore, bioextract became an alternative way of non-chemical and eco-friendly multipurpose tools for everyday life as the bioextract itself is naturally produced using natural microbiota or inoculum that originally isolated from ecosystem (EM research Organization, 2016). However, many of these suggested applications will need further research to address the effectiveness of bioextract to perform each task.

As mentioned above, one aspect of bioextract utilization is for pre-treatment or treatment of organic waste including wastewater. The problem of water quality in natural resources have become more and more problematic especially in urban area. Large amount of water is being used every day for purposes ranging from drinking, cleaning to agricultural use. This rate of water utilization created large amount of wastewater discharged including sewage contaminated with chemical, biological and microbiological materials (EPA, 2016).

Generally, the domestic wastewater will be collected and treated at municipal wastewater treatment plant. For the sewage waste, most will be pre-treated in place using household septic tank before release into public drainage to be collected to the wastewater treatment plant (EPA, 2016). There were number of works conducted to assess the ability of bioextract in wastewater treatment. There was a study published in 2003 (Szymanski & Patterson, 2003) to apply effective microorganisms (EM) in domestic wastewater treatment plant for reduction of sludge in wastewater plants and also domestic septic tank. However, the result from Szymanski and Patterson did not showed the ability of EM to remove the sludge from the system. In 2008, EM were used to produce EM mud ball and used in the rivers in Malaysia in attempt to improve the quality of water in those river (Zakaria, et al., 2010). The result from the application of mud ball indicated in this report was successful. Recently there was a study showed application of bacterial consortium that could reduce greases and oils in lipid-rich wastewater in aerobic treatment system up to 80% (Fadile, et al., 2011).

In Thailand during the 2011 flooding, water pollution became a common problem as the flood water became stagnant with a lot of organic materials in the flood area. Bioextract became one of the proposed solution that was brought in to lessen the problem especially for the odor problem (MacKinnon, 2011). During the flooding, the bioextract from different sources/manufacturers were used to produce mud balls which initially formulated by EM™ producer (currently known as EMRO™) and added into the body of flooded water. However, due to the size of the flooding area, volume of flood water and also the movement of water, the result was not conclusive. This research was an attempt to provide more scientific information about the utilization of bioextract in wastewater treatment. The bioextract was produced by NAVA social enterprise and have been freely distributed among some community in Eastern area of Bangkok. The experiment aimed to observe the effect of bioextract to quality of different types of domestic wastewater. The wastewater samples used in this experiments were collected from 4 different types of establishment in commonly found in Bangkok Metropolitan area including restaurants, medium size food factories, condominiums and hotels. Wastewater samples were collected from 2 different sources for a similar type of establishment and used as physical replication. The result from this experiment will provide more information for those who considering using bioextract as an alternative way for improvement of wastewater quality.

Materials and Methods

Wastewater sample was collected from 2 of each restaurants, medium size food factories, condominiums and hotels using Nansen bottle. The wastewater sample was collected twice from each source. Then the wastewater sample was treated using factorial design by varied the amount of bio-extract (0 ml/L, 0.25 ml/L, 0.5 ml/L and 1 ml/L), time (24 hours, 48 hours), oxygen (with oxygen, without oxygen),and light (with light, without light). The bio-extract were produced and provided by NAVA social enterprise. After treatment, the treated wastewater was measured for the quality of water. The parameters for measurement are total solid (TS), total suspended solid (TSS), total dissolved solid (TDS), dissolve oxygen (DO), biochemical oxygen demand (BOD), pH, total plate count, MPN and greases and oils. Then results from each parameter were statistically analyzed using Tukey's multiple comparison tests in SAS 9.2 program.

TS and TSS was analyzed using the drying method to find the weight difference (AOAC, 1990). TDS, DO, and pH were measured by using multi-parameter meter (AMTAST EC-900). BOD5 was found from subtraction of DO0 and DO5 (AOAC, 1990). Greases and oils were determined by liquid-liquid extraction (Mohrig, et al., 2006). Total plate count was used the drop plate technique on nutrient agar. MPN method were testing by using LT (Lauryl Tryptose) broth with bromocresol purple, EC (*Escherichia coli*) broth, EMB (Eosin-methylene Blue Agar), and LST broth (American Public Health Association, American Water Works Association, and Water Environment Federation, 1999).

Results and Discussion

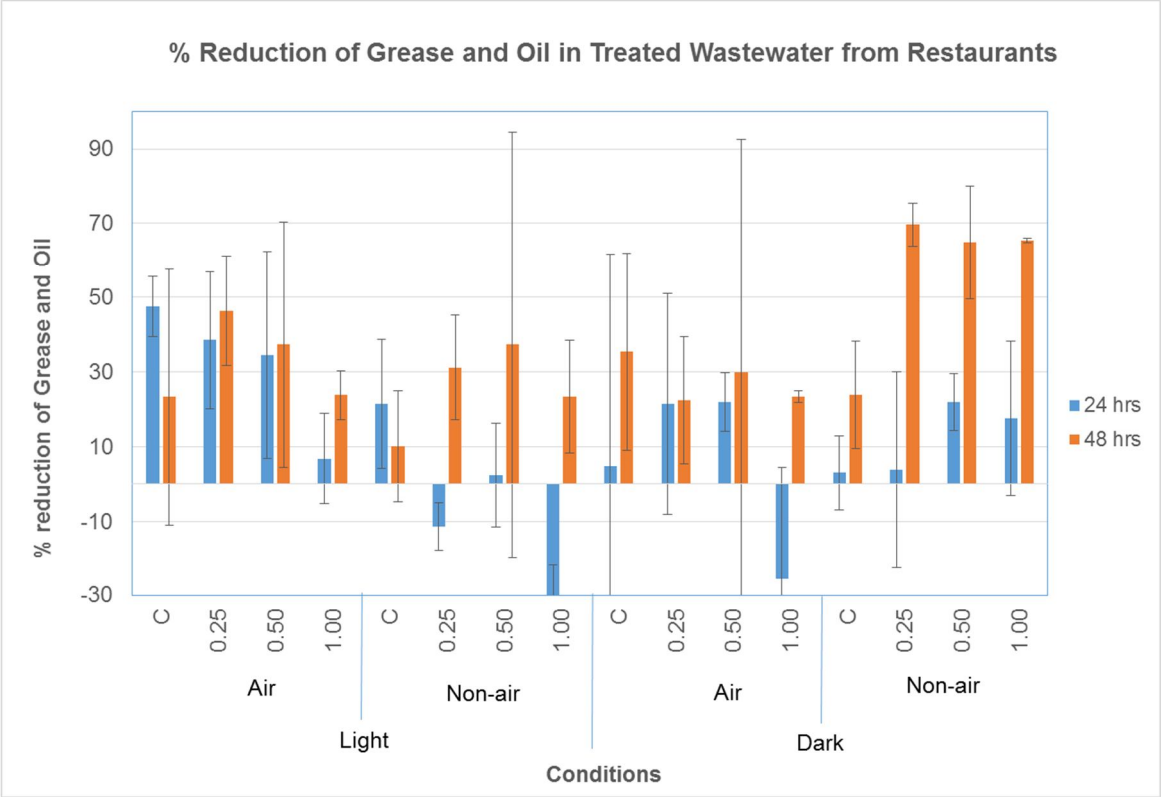


Figure 1 % Reduction of greases and oils in wastewater collected from 2 restaurants before and after treatment in different condition analyzed at 24 and 48 hours after treatment. % reduction was calculated in relative to the raw wastewater sample. The negative value in the figure indicate increase amount of greases and oils in the sample.

Note: C = control (not add bioextract), 0.25, 0.5 and 1.00 indicated amount of bioextract added to the wastewater sample in the unit of ml/L. Air indicated that the wastewater was treated under the condition that have regular air flow, Non-air indicated the condition where air flow was limited. Light indicated that the treatment was done with exposure to normal sunlight during the day while Dark indicated that the treatment was done in the dark closet to prevent light along the treatment period.

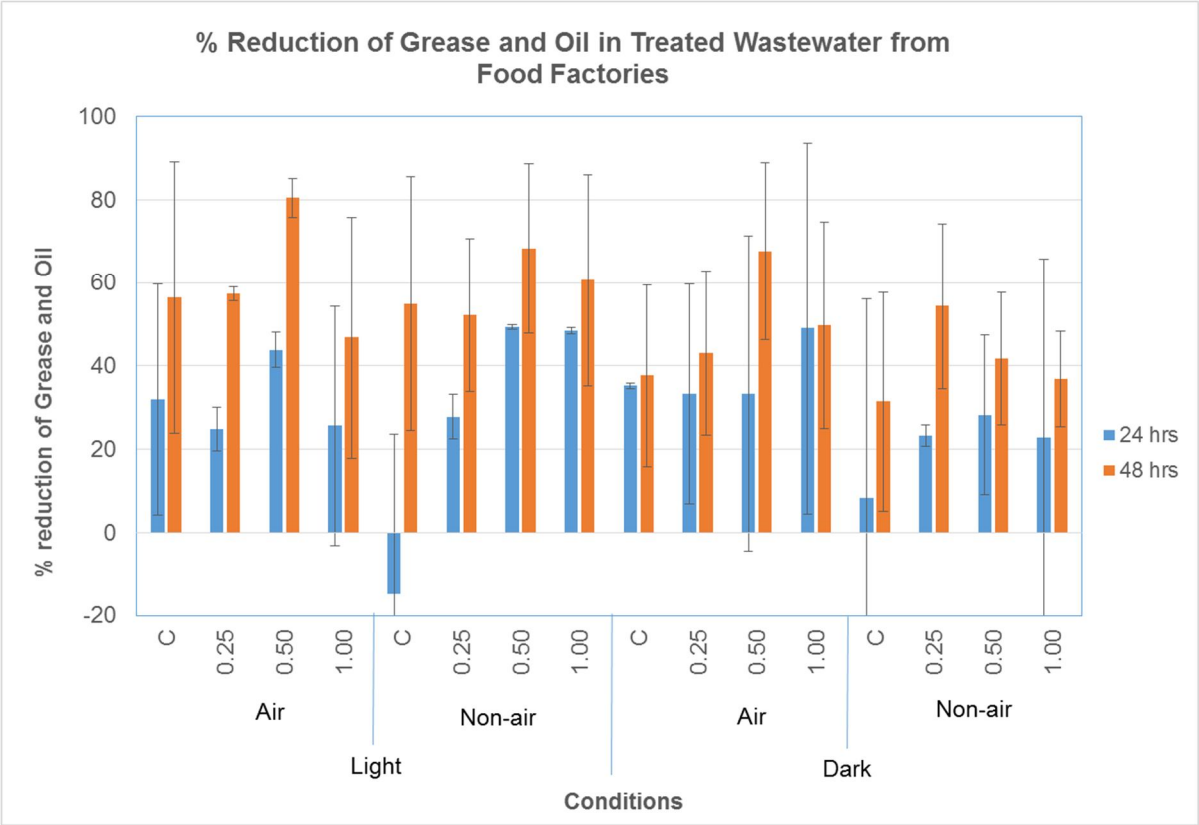


Figure 2 % Reduction of greases and oils in wastewater collected from 2 medium size food factories before and after treatment in different condition analyzed at 24 and 48 hours after treatment. % reduction was calculated in relative to the raw wastewater sample. The negative value in the figure indicate increase amount of greases and oils in the sample.

Note: C = control (not add bioextract), 0.25, 0.5 and 1.00 indicated amount of bioextract added to the wastewater sample in the unit of ml/L. Air indicated that the wastewater was treated under the condition that have regular air flow, Non-air indicated the condition where air flow was limited. Light indicated that the treatment was done with exposure to normal sunlight during the day while Dark indicated that the treatment was done in the dark closet to prevent light along the treatment period.

Wastewater samples collected from different types of establishment was analyzed and showed different physical and chemical properties. The differences reflected human activities within those establishment. Wastewater samples collected from condominiums and hotels may categorized into the same group which were the least contaminated compare to the rest of the establishment usually have low level of total solid as well as low in greases and oils. Wastewater collected from restaurants was considered to have quite high contamination with high amount of total solid and also greases and oils level. The wastewater from medium size food factories is the grimiest one with high level of total solid and greases and oils. The result from each sources of wastewater then will be discussed separately in this section. In all treatment, even though physical and chemical analysis was done to address all the parameter that mentioned above in the materials and methods, only the result of total

solid (TS) and greases and oils will be focused here in the result and discussion as these was the most affected quality in this experiment.

Wastewater from Hotels

Wastewater samples were collected from the 2 hotels that have similar capacity (300 rooms). Both hotels offered room services as well as restaurant services to their guests. The wastewater was collected from the first wastewater collection tank of the hotels' wastewater treatment system. Due to the limitation of the hotel wastewater treatment system capacity that have a treatment period before wastewater was released into nearby waterway of not more than 24 hours during the normal season and could reduce to less than 8 hours in high season. The result for treatment of hotels' wastewater were observed at 6 and 24 hours instead of 24 and 48 hours.

Wastewater from hotels showed low total solid (TS) content at 5.28×10^{-3} mg/L which considered quite low (Bangkok metropolitan regulation for released wastewater TS to be less than 135 mg/L). While the original greases and oils level in wastewater collected from the hotels was 1.17×10^{-2} mg/L which also lower than 20 mg/L that require by law. The addition of bioextract to the wastewater did not showed significant different in the changes of all parameter analyzed from the untreated control (not added bioextract) within 24 hours of experiment. In addition, all parameters tested was below the level that enforced by law for both before and after the treatment. This could resulted from the fact that the original wastewater was already have low level of contamination.

Wastewater from condominiums

Wastewater from 2 condominiums was collected from the collection tank of the wastewater treatment system of the condominiums. Percentage of residency in both condominiums is about 60-80% of total capacity. Wastewater collected from the condominiums had quite similar characteristic to the wastewater from hotels that have low level of TS and greases and oils. The wastewater TS content was 2.30×10^{-2} mg/L and greases and oils content was 1.77×10^{-2} mg/L. The result after addition of bioextract showed reduction of TS up to 50% reduction from original level in 24 hours (data not showed). However, the reduction was not statistically different among the treatment and untreated control that may due to the limitation of detection method and low level of TS in the wastewater sample. As for greases and oils, there was no significant different changes in the level of greases and oils observed before and after treatment.

Wastewater from restaurants

Wastewater was collected from 2 location, one was a western style full services restaurant located the shopping mall while another was the self-services food center opened nearby local fresh market. Wastewater from the restaurants contain high TS content at 3,195 mg/L and greases and oils content at 2,255 mg/L. After treatment with different amount of bioextract and varied treatment condition, the result showed reduction of TS in all treatments. The average reduction of TS was up to 53.33% reduction regardless of condition used especially in the first 24 hours but became higher at 48 hours. However the reduction of TS was not significantly different between the treatment and the control (not added bioextract) at the same time point (data not showed). For greases and oils, the result indicated a significant reduction ($p < 0.05$) of greases and oils in the treatment that added different

amount of bioextract and kept for 48 hours in the dark and no-oxygen condition (Figure 1). While in other conditions the reduction was also observed but with no significant different from raw wastewater and control (not added bioextract). Despite a reduction of greases and oils up to 70% reduction in the treatment that add different amount of bioextract and kept for 48 hours in the dark and no-oxygen condition the remaining greases and oils content was still above the regulation which is ≤100 mg/L at the amount of 680.00±113.14 – 785.00±318.20 mg/L (Ministry of Natural Resources and Environment, 2005). Therefore further treatment or longer treatment period may be needed to reduce more greases and oils. The reduction trend for greases and oils in all treatment were observed however the degradation took more than 24 hours. The delay degradation of greases and oils could be a result from the nature of restaurant wastewater that usually contain high nutrients that easier to utilized by microorganisms (Barnstable County Department of Health and Environment, 2016). The amount of chemical contamination especially detergents in wastewater from the restaurant could also affected some group of microorganisms that reside in the bioextract.

Wastewater from medium size food factories

Wastewater in this categories was collected from two different food factories: one was a frozen seafood factory, another was glass noodle factory. Wastewater samples was collected in the wastewater collection well of the factory that immediately accepted wastewater from production line. Quality of wastewater from the two factories was quite different in the two parameters namely total solid (TS) and Greases and oils. Wastewater from frozen seafood factory had higher content of greases and oils (1,710 mg/L) but lower TS (840 mg/L) compared to the grass noodle factory that was 980 mg/L of greases and oils and 4,100 mg/L of TS. Therefore the analysis was done using %reduction in both TS and greases and oils to address the efficiency of bioextract. The result demonstrated that in all treatments the TS contents can be reduced up to 25% from the original content. However when compare with control (not added bioextract) there was no significant different in % reduction. For greases and oils, the result showed that there were a reduction of greases and oils in all treatment up to 80.43% reduction in the treatment that add 0.5 ml/L of bioextract for 48 hours with light and air (Figure 2). The other treatment showed that the reduction of Greases and oils content increase over time. Despite the reduction observed in greases and oils in the wastewater samples, the remaining amount of greases and oils in all samples was still above the regulation issued by Ministry of Industry, Thailand (maximum not more than 15 mg/L of greases and oils) (Ministry of Industry Thailand, 1992). therefore longer treatment period of additional treatment will be needed.

Besides the wastewater quality in term of TS and greases and oils that presented above, other parameter including pH, BOD, TSS, and also TDS was also analyzed for all the wastewater before and after treatment. However, these quality was not change significantly and the level of all these parameter after the treatment were compile with the regulation therefore will not discuss in this report. Additional issue that addressed in this report is to address the existing of index microorganisms in the wastewater before and after utilization of bioextract. This analysis was done to answer one of the concern about the possibility that the addition of bioextract hence living microorganisms in the wastewater will affect the microbiological quality of water after release into the environment especially the contamination of pathogens. In this research the amount of Total Coliform Bacteria, Fecal Coliform Bacteria and

Escherichia coli were analyzed as an indicator to indicate pathogens contamination in water samples (Bitton, 2005) as well as total aerobic bacteria.
The result from Total aerobic plate count indicate increasing number of total bacteria in all treatment from the original wastewater sample. However, there was no significant different in number of total count between the treatment that added or not added bioextract. This indicated that the indigenous microorganisms that already reside in wastewater sample were also increase during treatment period. In all sample the increasing of total bacteria at the average of 3 log was observed within the first 24 hours and reduced within 48 hours. The increase and decrease of microbial population could resulted from the depletion of nutrient after the first 24 hours.

Analysis of index microorganisms indicated that in all wastewater sample before treatment most probable number of total coliform bacteria and fecal coliform bacteria was more than 1.6×10^4 MPN/100 ml. The amount of Total coliform bacteria and fecal coliform bacteria was reduce in small amount after treatment with bioextract. For *E. coli*, original amount of *E. coli* in wastewater samples was averagely less than 500 MPN/100ml and after addition of bioextract the number reduce to less than 100 MPN/ml.

One of the issues that could be observed from this experiment is the variation in the properties of wastewater that came from different or even the same type of establishment and also the sampling periods. These variation resulted in high deviation among the data collected therefore the conclusion is still not clear enough to support the utilization of bioextract exclusively for wastewater treatment. Despite those variation, however, the trend of the data showed in this work provide a positive support toward the utilization of bioextract as an alternative or pre-treatment.

Conclusions
In this research the bioextract was used at different concentration to add to wastewater from different establishments that commonly found in urban area in attempt to study the ability of bioextract to treat wastewater by varied the exposure to light, oxygen and length of treatment. The result demonstrated that the efficiency of bioextract to treat wastewater was dependent mainly on the length of treatment in which in this study 48 hours of treatment showed better result. The result also demonstrated that bioextract can be used to treat wastewater that have high amount of total solid and grease. The reduction ability was more pronouced in the sample that have high initial load of TS and greases and oils. In addition to chemical quality, the analysis of total aerobic bacteria and index microorganisms showed a consistent data that the application of bioextract did not result in the increasing of microbial population in the sample. The result also demonstrated the reduction in number of *E. coli* which is the indicator of human fecal contamination and pathogen contamination.

Acknowledgements
Special thanks to NAVA Social Enterprise who providing funds and resources. Sincere appreciation to Mr. Pei-Fu Chiu, Ms. Supatchayaporn Nitsuwat and Ms. Tongkon Wachirasakun previous undergraduate students who contributes to this work.

References

American Public Health Association, American Water Works Association, and Water Environment Federation, 1999. Standard Methods for the Examination of Water and Wastewater. 20th ed. s.l.:American Public Health Association.

AOAC, 1990. In: H. K., ed. AOAC Official Methods of Analysis. 15 ed. Washington D.C.: Association of official Analytical Chemistry.

Barnstable County Department of Health and Environment, 2016. Greases and oils in Restaurant Wastewater. [Online] Available at: http://www.barnstablecountyhealth.org/resources/ publications/ compendium-of-information-on-alternative-onsite-septic-system-technology/grease-and-oil-in-restaurant-wastewater [Accessed June 2016].

Bitton, G., 2005. Wastewater Microbiology. 3rd ed. New Jersey: John Wiley and Son Inc.

Boraste, A. et al., 2009. Biofertilizers: A Novel Tool for Agriculture.. International Journal of Microbiology Research, 1(2), pp. 23-31.

Bunchoo, M., 2002. The study of microorganism isolated from EM, Bangkok: s.n.

EM research Organization, 2016. EMRO. [Online].

EPA, 2016. EPA (Environmental Protection Agensy): Domestic Wastewater Advice and Guidance. [Online] Available at: http://www.epa.ie/water/wastewater/ [Accessed June 2016].

Fadile, A. et al., 2011. Aerobic Treatment of Lipid-rich Wastewater by a Bacterial Consortium. Afr. J. Microbiol. Res, pp. 5333-5342.

Feunganksorn, P., Akeprathumchai, S. & Tripetchkul, S., 2011. Effect of bio-extract as microbial inoculum on composting of cassava leaves and stems. Thai Journal of Agricultural Science, 45(5), pp. 236-241.

Land Development Department, 2014. Soil Biotechnology: Microbial activator super ldd-2. [Online] Available at: http://www.ldd.go.th/ldd_en/en-US/microbial-activator-super-ldd-2/ [Accessed 7 Jun 2016].

MacKinnon, I., 2011. The Telegraph. [Online] Available at: http://www.telegraph.co.uk /news /worldnews/asia/thailand/8902448/Thailand-floods-half-a-million-mud-balls-being-used-to-purify-water.html [Accessed Jun 2016].

Ministry of Industry Thailand, (1992), Official Announcement of Ministry of Industry Thailand, Issued no. 2, Issued under the Factory Act 1992
Ministry of Natural Resources and Environment, 2005. Control Standard Specification: Wastewater Discharged from Buildings of Certain Size. Bangkok: Royal Thai Governmenr Gazette.

Mohammad, A. A., Al-Farhan, A. A., Al-Haddad, A. A. & Al-Kholosy, S. A., 2011. he Effect of Suspended Solid Concentration and Oil Droplet Size Distribution on Particle Coalesces and Emulsion Stability. Al-Khobar, Saudi Arabia , SPE/DGS Saudi Arabia Section Technical Symposium and Exhibition.

Mohrig, J. R., Hammond, C. N. & Schatz, P. F., 2006. Techniques in Organic Chemistry. 2nd ed. s.l.:W. H. Freeman.

Ngampimol, H. & Kunathigan, V., 2008. The study of shelf life for liquid biofertilizer from vegetable waste.. AU J.T., 11(4), pp. 204-208.

Nitsuwat, S., Sanjaya, S., Wiratthikowit, S. & Kunathigan, V., 2012. The study of the biodiversity in local bio-fermented solution and the treatment of community wastewater at laboratory scale: wastewater from restaurants. Bangkok, Thailand, s.n.

Okuda, A. & Higa, T., 1995. Purification of Waste Water with Effective Microorganisms and its Utilization in Agriculture. University of the Ryukyus, s.n.

Shalaby, E. A., 2011. Prospects of effective microorganisms technology in wastes treatment in Egypt. Asian Pac J Trop Biomed, 1(3), pp. 243-248.

Szymanski, N. & Patterson, R., 2003. Effective Microorganisms (EM) and Wastewater Systems in Future Directions for On-site Systems: Best Management Practice.. Armidale, Lanfax Laboratories Armidale.

Zakaria, Z., Gairola, S. & Shariff, N., 2010. Effective Microorganisms (EM) Technology for Water Quality Restoration and Potential for Sustainable Water Resources and Management. Ottawa, Canada, s.n.

Effect of Carageenan from Local Seaweeds on Bread Quality

Sri Widyastuti[1], Nazaruddin[2], Baiq Rien Handayani[3], Wiharyani Werdiningsih[4], Mutia Devi Ariyana[5] and Novia Rahayu[6]

[1]Faculty of Food Technology, Mataram University, (sriwidyastuti@unram.ac.id; sriwidyastuti10@yahoo.co.id)
[2]Faculty of Food Technology, Mataram University, (.....)
[3]Faculty of Food Technology, Mataram University, (baiqrienhs@yahoo.com)
[4]Faculty of Food Technology, Mataram University, (wiharyani@gmail.com)
[5]Faculty of Food Technology, Mataram University, (mutiadevi0705@gmail.com)
[6]Faculty of Food Technology, Mataram University, (noviarahayu1185@gmail.com)

ABSTRACT

Increasing awareness on the health safety issues has highlighted the need for safer food additives. The use of chemical substances in bread making has been correlated to many health problems, therefore it is neccesary to explore potential natural compounds as additive in bread making. This work reports the potential use of carrageenan, phycocolloids from Red Algae (*Halymenia* sp.and *Eucheuma cottonii*), as natural bread improvers. Results showed that the extracted carrageenan from *Hallymenia* sp. at 0.4% and that from *E. cottonii* at 0.6% improved the texture and structure of bread crumb, increased bread volume by 30%-50%, delayed the water evaporation by 2%-6% and maintained crumb elasticity by 5%-15% during 96 hours storage at room temperature. Organoleptic tests showed that addition of carrageenan in bread formulation improved overall acceptability compared to those untreated.

Keywords: red algae, phycocolloid, bread, safety, carrageenan.

Introduction

Bread is consumed widely as an alternatif foodstuff for Indonesian people. Increasing demand for quality and also quantity of bread has lead to improvement of technology in baking process. The use of synthetic food additives such as *bread improver* in bread making is aimed to meet the consumer need of desired organoleptic properties and longer shelf life products. Unfortunately, several bread improvers such as Potasium bromate (E 924) dan Calcium propionate, which are used in dough mixture to improve volume and texture of the crumb, have been banned in Europe, Canada also in Indonesia. as related to the health issues as stated by *Enviromental Protection Agency* (EPA). Unfortunately, not all producers especially home industry processors, aware of the consumer health consequencies caused by sunthetic food additives. However, in developing countries like Indonesia, many producers are still add calcium propionat in bread making.

Increasing awareness of the use of synthetic food addities have highlighted the need for safer bread improvers. Research on the use natural substances for food additives in bread making has been mentioned by several authors. Rosel *et al*., (2001) stated that carrageenan, hydrocolloid extracted from seaweed could be used as *bread improver*, increasing dough volume and improving crumb texture. Cindy (2006) also reported that carrageenan has ability to retain water in food products. Other researcher also found that natural hydrocolloid has been reported improved dough characteristics and extended bread shelf life (Mandala *et al*., 2007), also improve texture of the bread crumb (Rodge *et al*., 2012). Moreover September (2007) and Sciarini *et al*. (2012) wrote that carrageenan inhibited growth of spoilage fungi and slowed staling of bread. This paper reported the effect of addition carrageenan extracted from locally grown seaweed (*Halymenia sp. and Eucheuma cottonii*) on the qality and safety of bread.

Materials and Methods

Research Design

Completely Randomized Design (CRD) was used througthout the study with three replicates. The principle factor was concentration of carrageenan. Data were then analyzed statistically at 5 % significant level. Data showing significant differences were then further analyzed using Tukey's HSD test. Parameter measured were rate of water loss, organoleptic tests, bread volume , shelf life, staling and fungal growth.

.

Preparation of Carrageenan

Carrageenan is extracted from *Eucheuma cottonii* from Grupuk Central Lombok, and *Halymenia* sp. harvested from the coastal area in Pantai Pendawa Bali (Fig.1 and 2). The extraction process was conducted modified methods of Indriani dan Suminarsih (1996) dan Handito (2011) using hot alkaline method followed by precipitation using Isopropil alkohols. Small pieces of sun dried seaweed were boiled for 1 hour in hot water (1: 40) at 80-90°C and pH was adjusted to pH 8using 0.1 N NaOH. Filtratwas then collected and added by 10%NaCl at a concentration of 5% (v/v) which then reheated to reach halfed of the original volume and was settled down for 10-15min.Precipitation was done by addition of IPA (2 x

volume) with manual agitation for 10-15 min resulted in a thin layer prepitate which then ready for drying in a cabinet drier (Memert –GermanyUNB-800 set at 40-50°C for 12 hr.).


a


b.

Figure 1 a. *Halymenia fluoresii*, b. *Eucheuma cottonii*

Bread making

Basic formula of bread was made up by mixing 75 g wheat flourwith other weighed ingredients : yeast (2%), sugar (20%), milk (5%) , margarine (20%) by weight of wheat flour, 3 egg yolks, salt dan 172.5 ml of water. Firstly, wheat flour and milk was mixed in a bread maker (Oxone stand mixerOX-855) at the lowest speed. Yeast was diluted in warm water while adding gradually egg yolk with slow agitation to get a sandy texture dough. The dough was divided according to the treatments. Carrageenan was added at various concentration i.e. 0.2%, 0.4%, 0.6% and 0.8% based on the flour weight followed by addition of water while kneaded at a slow speed. Margarine was then added with high speeduntil dough become not sticky. The dough was allowed to ferment in a bowl covered with plastic wrapp at room temperature for 30 min. The dough was then punched and weighed to 70 gdough balls. The pieces of dough were wrapped in aluminium foil and proofed for 1 hr in a proofingchamber (Getra Fx 15 S) set at T 30°C in 85% relative humidity and baked in anoven set at 180°C for 20 min.The bread were then cooled at room temperature and assesed for loaf volume, crumb structure was examined under microscope, texture, color of crumb , taste, flavor, texture and overal aceptability.

Bread characteristics

Loaf volume were measured after removal of the bread and cooled down for 1 hr. Loaf volume was measured as a modified procedureof seed replacement method. A 100 ml *Beaker glass was* hard filled with rice grain and shaken vigorously to make sure the grain settled down properly filled the space in the beaker then overfilled and pressed across over the top using a ruler let the overspill fell down out the beaker.The grain was then re-poured and re-weighed for three times to get the value of grain weight. A weighed loaf was placed in the beaker and filled with the weighed grain and pressed off as above. The overspill grain was weighed and the difference grain weight is the volume replaced by the loaf. Measurements of the loaf volume were done after proofing and also after baking.

Observation of crumb pores

Pieces of bread *crumb* were made by slicing thinly in the middle part of the crumb using regular knife. The pieces were placed inbetwen the microscope slide and a cover glass for observed under a microscope at a maginification of 10 x 40 and foto graphed.

Measurement of bread crumb elasticity

Bread elasticity was determined as a relative elasticity of 3 cm height pieces of crumb (measured from the bottom part), placed on the flat glass and was then pressed by hand with a 50 ml beaker glass untill down to half of the original height (1.5 cm) and hold for 1 min. The height of the crumb after pressed for 1 min. was assumed as the relative elasticity.

Bread elasticity = height after pressed /height before pressed x 100%

Measurements of water content

The crumb was grinned finely using a manual grinder and weighed to 3 g portions then were placed in pre-weighed moisture bottles for drying in the oven set at 105°C for 4 hr. The bottles containing samples were then cooled in a desicator for 15 min. which then were weighed. The samples were placed back into the oven set at the same temperature for another 1 hr then colled as before then weighed. This procedure was repeated until reach a constant weight (differences between the weighing is less than 0.2 mg). The difference of weight before drying and after drying was considered as the amount of water loss form the material referred as water content of the samples.

Water content = weight before drying- weight after drying/ weight before drying x 100%

Determination of shelf life (stalling) and observation for fungal growth

Shelf life of the bread sampels were determined by observing physical characteristic of the whole bread. Breads were packaged in an *Oriented Polystyrene* (OPP) plastic bag and stored at room temperature for several range of times. The observation was done by observing dryness of the surface part of bread and inside crumbs. Microbiological observations of the bread were done by visually detect the sign of fungal growth at the interval of 0, 24, 48, 72 and 96 hr. during storage at room temperature.

Organoleptic tests

Organoleptic testing were conducted according to Hedonic and Scoring Methods. Twenty five trained panelis asked to fill the provided questionare tosubjectively evaluate sensory characteristics of the bread samples including taste, texture, colour, flavour, physical appearance and overall acceptability.

Results and Discussions

A. Effect of addition carrageenan extracted from Halymenia on the development loaf volume

Carrageenan extracted from *Halymenia* sp. mostly are lamda type (Freile-Pelegrin et al. 2011). The results show that the addition of carrageenan extracted from *Halymenia* sp. Into the dough mixture significantly increase development of loaf volume, crumb characteristics such as structure, texture, diameter pores, elasticity, water content extend stalling time. The highest loaf volume was obtained from dough added with 0.4% carrageenan, while at 0.6% the loaf volume only increase at about 30% and the other treatments did not show signifant effects (Fig. 2). Development of loaf volume occurss when stucture of dough is strong and stable to retain gas. Although there is no spesific report yet on the effect of Lamda carrageenan on the development of dough volume, but according to Raychaudhuri dan Chakraborty (2013), carrageenan generally has an ability in improving gel network such as gluten, which in turn is related to increasing viskoelastisitas, increasing gas retention and optimum loaf development.

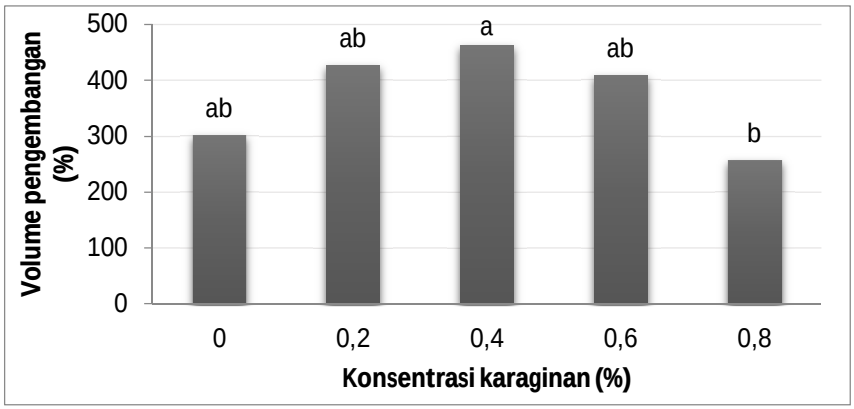


Fig 2. Effect of concentration of λ-carrageenan on the development of bread loaf volume

Chemically, λ-carrageenan contain high sulphated ester which can not form gel but only a highly viscous solution in water. This characteristics might contribute to the increasing loaf volume as noted by Delcour *et al.* (1991) in. Lazaridou *et al.* (2007) that increased dough viscocity played important role in the gas holding capacity of the which then in turn lead to improvement in the dough development. he addition of λ-carrageenan at a concentration of 0,6% might be the right portion to increase the viscocity of the dough mixture but does not for gel network so elasticity od the dough is maintain. Menurut Bell *et al.* (1990) in. Lazaridou *et al.* (2007), mentioned that high elasticity of dough improved its ability in maintain pores stability therefore the development of loaf volume during baking.

Effect of λ-carrageenan on Texture, structure and pores diameter of bread crumb

Microscopic observation on slices of crumb showed that addition of λ-carrageenan gave crumb with better texture and structure. Without the addition of λ-carrageenan, crumb was dry and big pores in high variety sizes (Fig. 7). The low viscosity of the dough mixture might caused weak gel network so the dough development was not optimal, pores are big and high variety pores with lower gas holding capacity which then reasulted in dry and coarse crumb. While addition of λ-carrageenan gave crumb with small pores size in homogen size resulted in smooth and moist crumb compared to control.

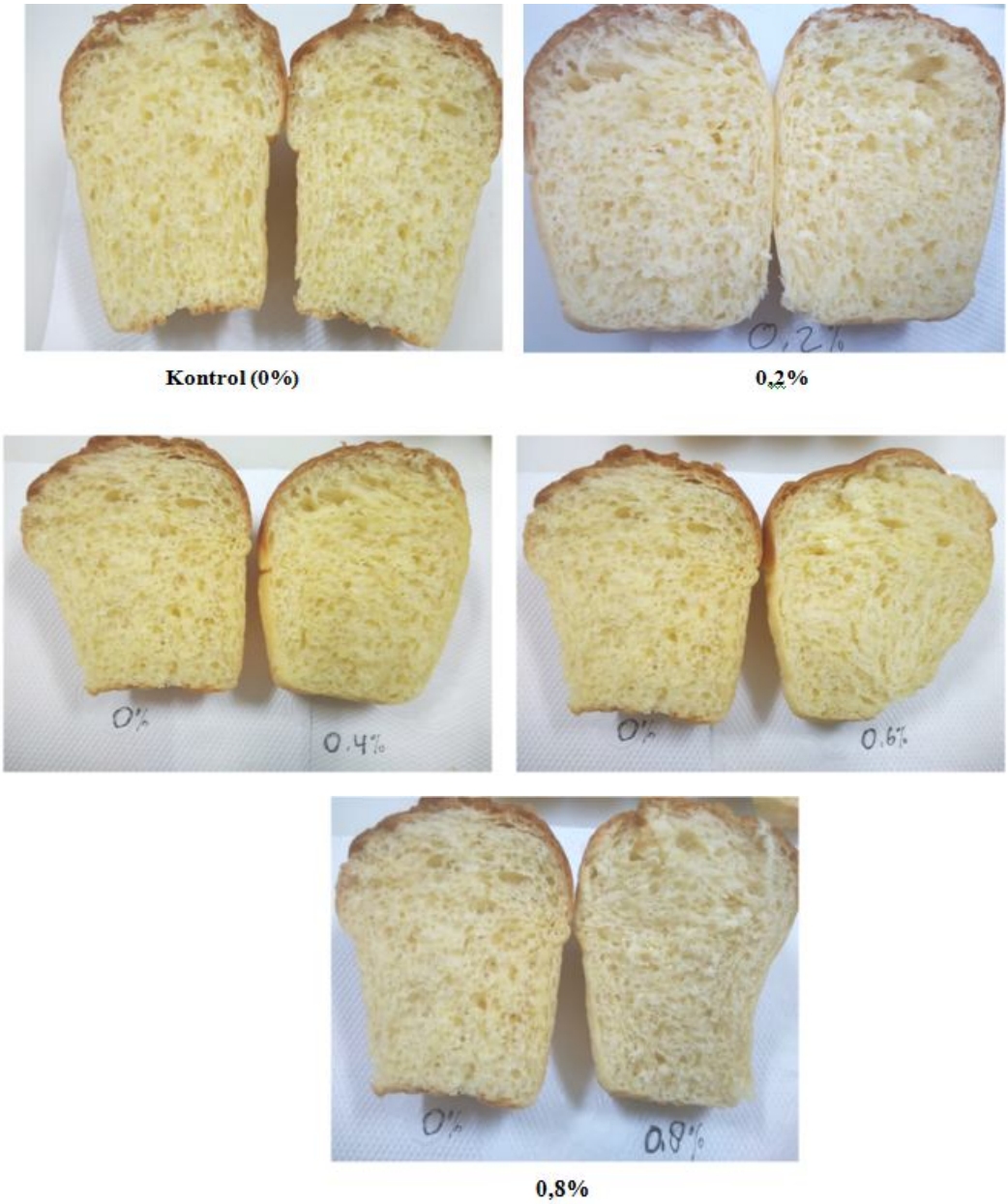


Fig 3. Cross section of crumb without λ-carrageenan dan crumb with λ-carrageenan 0.2-0,8%).

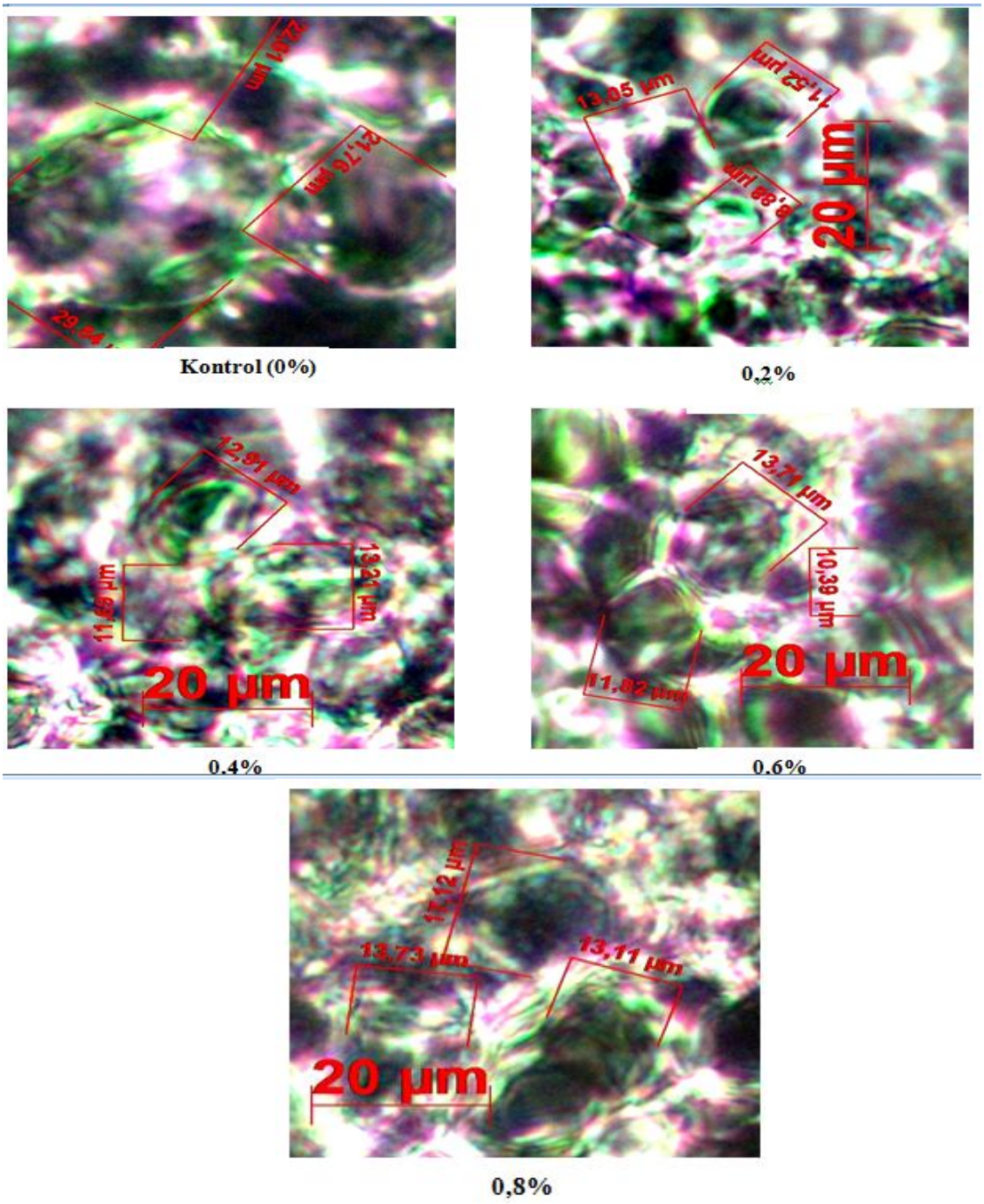


Fig 4. Pores of untretaed crumb and crumb of samples treated with λ-carrageenan at 0.2% - 0.8%

Results of Raychaudhuri dan Chakraborty (2013) also specified that the presence of hydrocolloid in dough might inhibit the occurence of big pores which contribute to the coarse crumb. Increasing the number of small pores might contributed to formation of an homogenous matrix improved CO2 holding capacity of the crumb during baking process. Microscopic or visuall obsevation that crumb made up with addition of carrageenan mixture

tend to have smaller pores (Fig 4.). In fact, as stated by Hartajani dan Anjarsari (2010), increased addition of synthetic bread improver in bread making resulted in increasing volume and number of pores with smaller size.

Effect of λ-carrageenan type on bread elasticity and water content.

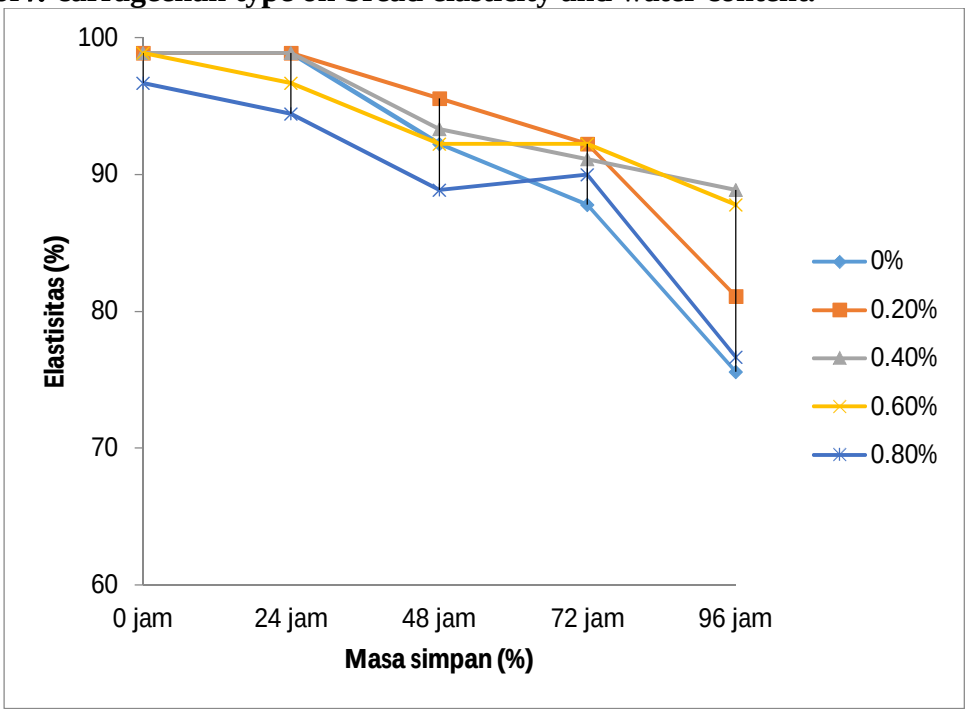


Fig. 5. Effect of λ-carrageenan at a concentration of 0% - 0.8% on bread elasticity during storage for 96 hr.

Elasticity of bread is reduced during storage at room temperature. In general , the addition of λ-carrageenan at 0.4% was the best treatment in maintaining elasticity of bread among the treated samples during storage (Fig. 5). Percentage of elasticity of bread added with 0.4% λ-carrageenan reduced only by 10% at the 96 hr storage, while the control and addition of 0.8% resulted in reduction of elasticity up to 25%. In other words that λ-carrageenan at 0.4% could inhibit elasticity reduction of bread by 15% during 96 hr of sorage.

The addition of λ-carrageenan also slowed down water lost of bread during 96 hr of storage (Fig. 6). Incorporation of 0.4% λ-carrageenan in dough making slowed down water evaporation of bread by 6% as in lined with that stated by Sharadanand and Khan (2003) In. Raychaudhuri and Chakraborty (2013) stated that bread added with hydrocolloid have higher water content during storage compare to the control. This phenomenon revealed that λ-carrageenan might have ability to improve water holding capacity of the crumb. Kahajdova and Karovicova (2008) added that hydroxyl groups increases interaction of water molecules and hydrocolloid through hidrogen bonds. The better retention of water in crumbs the slower water movement and evaporation and elasticity lost of bread. Similar data reported by Ghanbari dan Farmani (2013), that the addition of hydrocolloid in bread preparation resulted in reduction of water dehydration of crumb and slowed the reduction of bread elasticity.

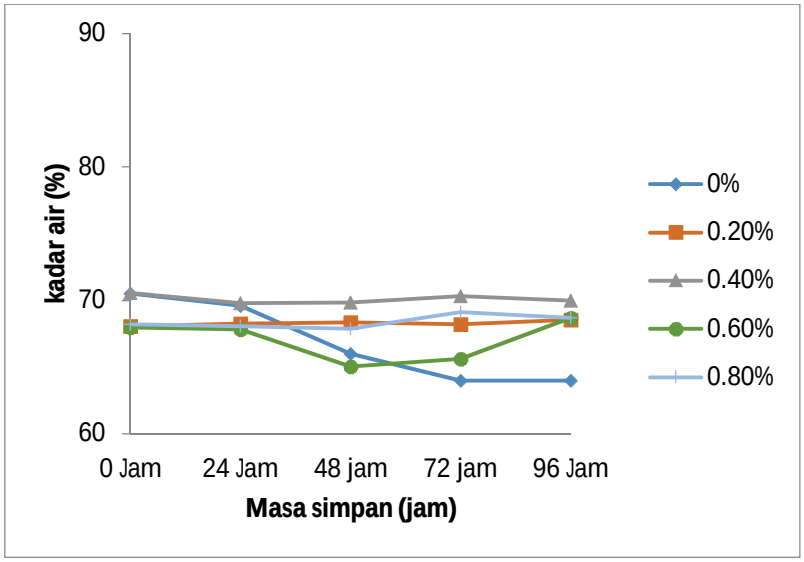


Fig.6. Effect of carrageenan λ-carrageenan at a concentration of 0-0.8% on the water content of bread during storage for 96 hr.

Effect of λ-carrageenan on the shelf life of bread

Bread staling occur during storage is very complicated process, which is then followed by fungal growth. *Staling* of bread characterised by dried, hard and rigid crumb with soft, leatherly texture, and uncrispy crust. Observation by hand touching visualised that bread added with carrageenan staled slower than the untreated samples. Without addition of carrageenan, staling observed at 72-96 storage time with characterised by dry, hard britle and bad elasticity crumb. Water content of untreated bread also significantly reduced. On the other hand, crumb with added carrageenan staled started at 96 hr of storage.

During staling process, distribution of water in crumb changes with movement of water from crumb to crust. According to Sciarini *et al.* (2012) hydrocolloid has capability to slow down humidity movement from crumb to crust which might inhibited crust hardening. Bread without carrageenan lost water faster than untreated samples, caused reduced elasticity. The lower water content of bread the higher formation of cross link network caused crumb become hard and lost elasticity (Raychaudhuri and Chakraborty, 2013).

It is stated that carageenan potentialy inhibited fungal growth on bread during storage and extend shelf life (September, 2007). However, in this experiment have not prove that carrageenan inhibited fungal growth of bread since both of carrageenan treated and untreated showed fungal growth at 96 hr storage.

B. Effect of addition κ-carrageenan on sensory qualities of bread

Organoleptics test showed that addition of **κ-carrageenan** increased acceptability of panelis in taste, texture, flavour and performance (Table 1). Carrageenan treated bread considered has better taste like “umami” taste with spesific bread taste. The texture of treated samples were softer, easier to chewed swallowed compared to the control samples. Colour of bread crust were accepted similarly either the treated or untreated samples.

Tabel 1. Organoleptic data of taste, texture, colour and flavour of Bread added with λ-carrageenan at various concentration

Carrageenan (%)	Taste	Texture	Colour	Flavour	Appearance
0%	5.98 abc	5.19 a	4.16 a	6.15 a	6.26 a
0.2%	6.24 ab	5.53 a	5.01 a	6.21 a	6.66 a
0.4%	5.81 bc	5.31 a	4.38 a	6.10 a	6.45 a
0.6%	6.44 a	5.28 a	4.86 a	6.20 a	6.54 a
0.8%	5.71 c	4.28 b	3.96 a	5.90 a	5.61 b

*values are mean of three replicates

* mean values followed by different letters are significantly different at (P<0.05)

Effect of addition κ-carrageenan on the loaf volume

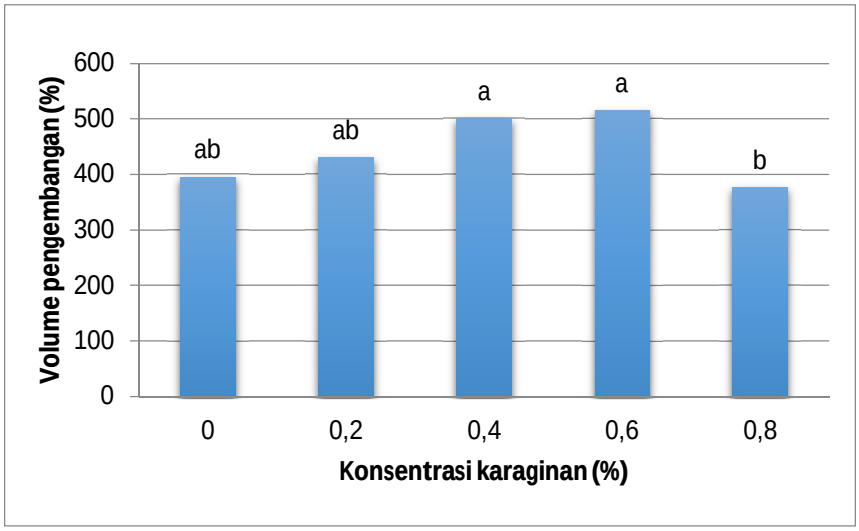


Fig 8. Effect of concentration of κ-Karagenan on the development of loaf volume

Addition of hydrocolloid extracted from *E. cottonii* which is mainly consisted of kappakaragenan had sifnificantly affected development of loaf volume, structure and texture of bread crumb, pori diameter , elasticuty. Eater content, time staling and also organoleptic values of the bread. At a concentration of 0.6% ., addition of carrageenan resulted in increased loaf volume up to 0% (Fig. 8). However, addition of carrageenan more than 0.6% showe reduced bread volume. Similar report of Lazaridou et al. (2007), that addition of carrageena n at low level could increased loaf volume but increased concentration of carrageenan could reduced the development of bread volume.

Effect of addition of kappa carrageenan on texture, struxture and pores diameter of bread crumb

Observation on crumb slices visually and microscopically showed that the carrageenan treated crumb samples have similar structure with the control samples. However, treated bread has crumb with softer crumb with smaller pores (Fig. 9). Bread with addition carrageena nhas soft crumb but with bigger unsimilar sizes pores which might be caused by unique brittle characteristic of kappa carrageenan gel .

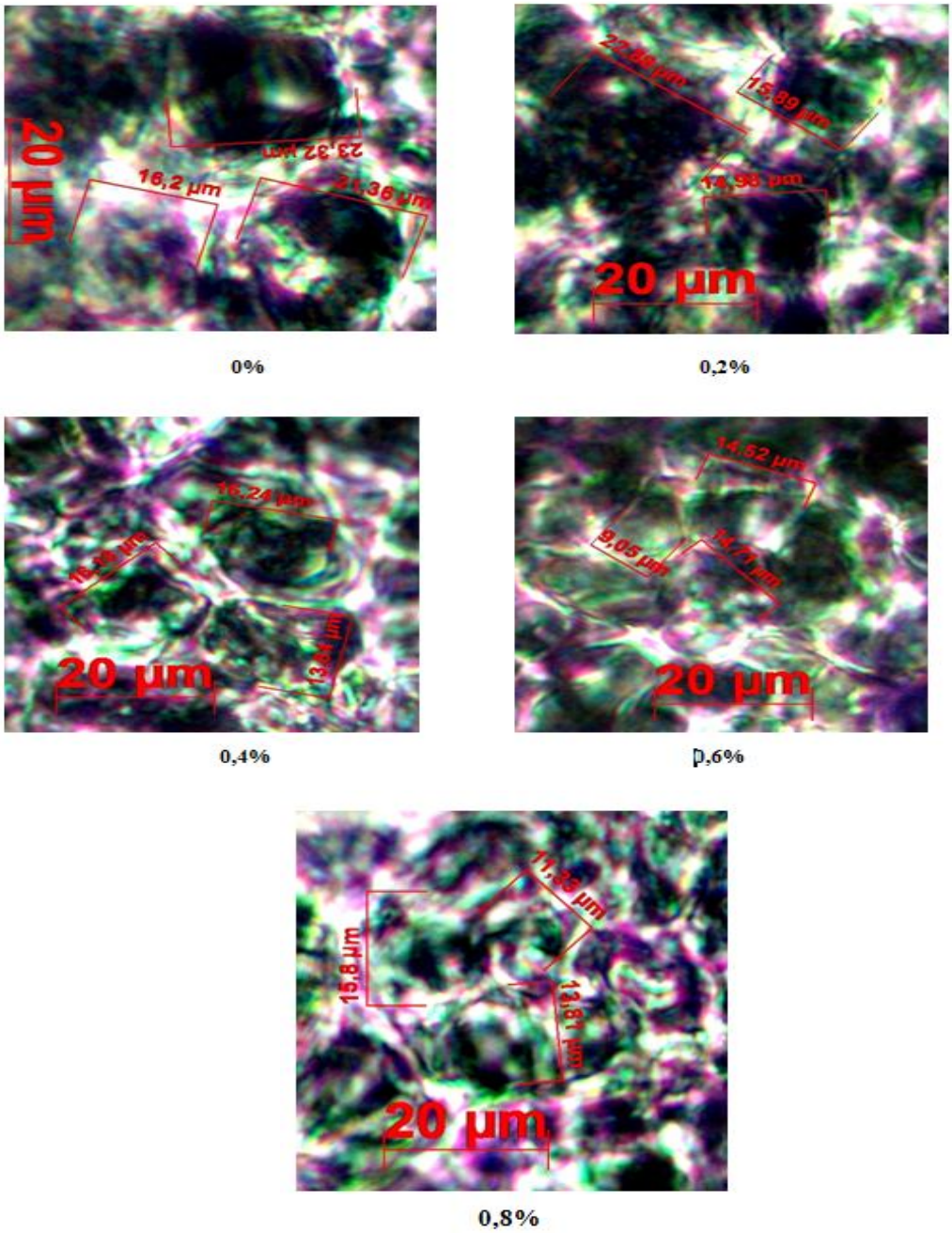


Fig 9. Pores of Untreated Crumb and crumb with κ-carrageenan at the concentration of 0.2% - 0.8%

Effect kappa carrageenan on elasticity and water content of bread

Addition of kappa carrgeenan at a concentration of 0.2 and 0.4 % resulted in hihest elasticity values than othe samples (Fig.10). Elasticity of those two samples reduced only 20% at 96 hr storage While at a concentration of 0.8%, carrageenan caused reduction of bread elasticity which was started at day 4 and day 5 during storage.

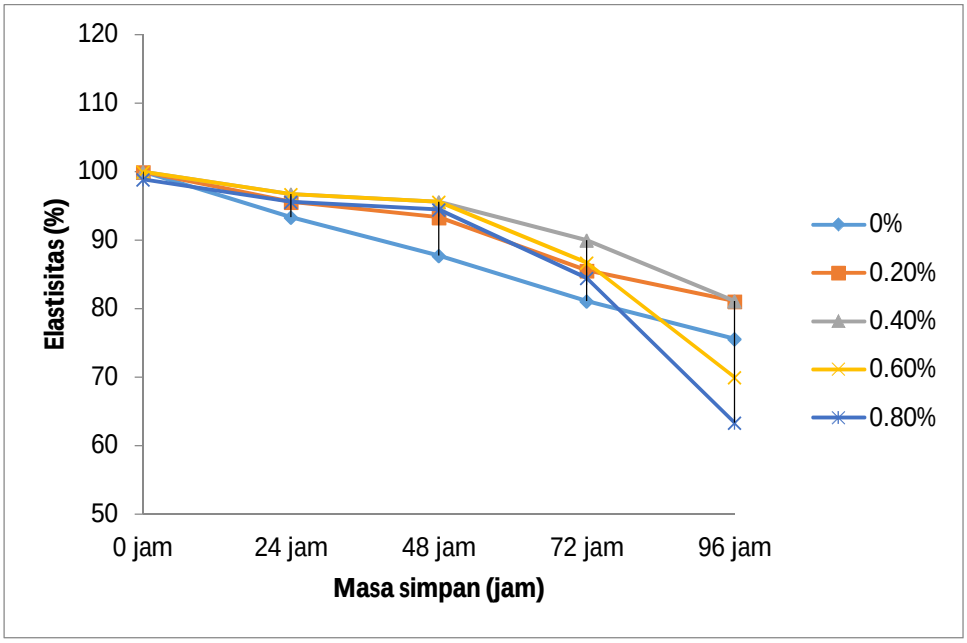


Fig 10. Elasticity of bread added and without κ=carrageenan at 0.2-0.8% during storage at room temperature for 96 h .

Kappa carargeenan however could retain water in crumb resulted in maintain elasticity of bread (Fig. 11) . Reduction of water content related to aceleration of crumb dehidration which lead to reduction of elasticity.

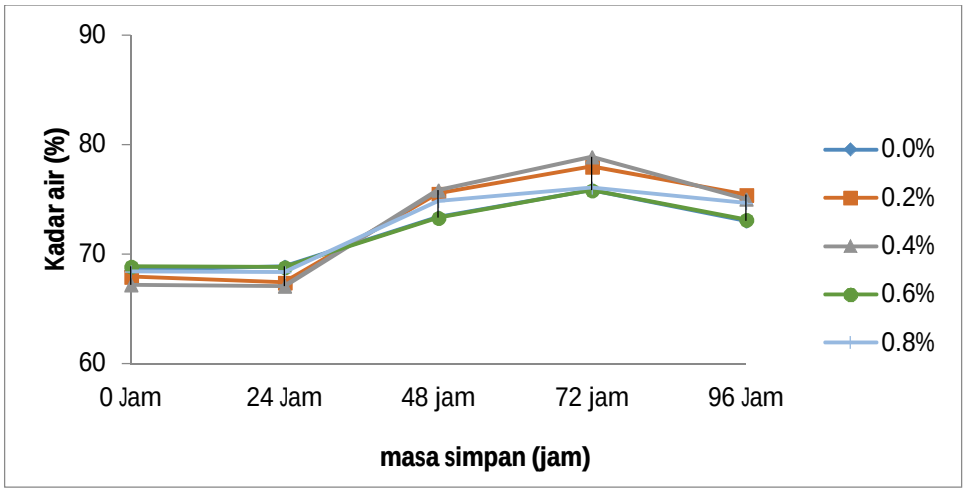


Fig 11. Water content of bread with and without κ-carrageenan during 9 h storage at room temperature

Effect of κ-carargeenan on the shelf life of bread

Visual observation revealed that the bread samples without addition of carrgeenan showed stalling i.e dry and hard crumb at 72h storage. While bread made with th eaddition of carrageenan showed stalling at 96 hr of storage. However, there was no sign that kappa carrageenan affected spolilage fungal on bread, as the both samples were not show any fungal growth.

Effect κ-carrageenan on the sensory qualities of bread

Results of organoleptic test did not show significant differences among the samples (Table 2), although researchers felt the difference of the elastisity of between the treated and untreated samples.

Tabel 2. Organoleptic data of taste, texture, colour and flavour of Bread added with κ-carrageenan at various concentration

Carrageenan (%)	Taste	Texture	Colour	Flavour	Appearance
0%	5.92 a	5.62 a	4.43 ab	5.95 a	6.60 a
0.2%	6.43 a	5.85 a	4.58 a	6.32 a	6.88 a
0.4%	5.58 a	5.58 a	4.31 ab	5.83 a	6.45 ab
0.6%	5.52 a	5.23 a	4.17 b	6.00 a	5.77 b
0.8%	6.13 a	5.62 a	4.37 ab	6.25 a	6.60 a

*values are mean of three replicates

* mean values followed by different letters are significantly different at (P<0.05)

Conclusions

In general , carrageenan could improved bread ualities at low concentration. Further addition of carrageenan might affectedformation of small pores then reduced loaf development and also reduced elasticity of crumb due to formation of gel network. The best treatment was the addition of 0.4% lamda carargeenan resulted in bread with soft moist crumb with homogen small sizes pores, loaf volume was development for 50% more than untreated samples, delayed loss of water up 6%, maintain crumb elasticity to 15% untill 96 hr of storage. Moreover, the λ-carrageenan treated bread had high acceptability in term of tatse, teture, color, flavor and aslo appearance compared to untreated samples.

Similar to the above phenomena but to a lesser extend also found on bread add with 0.6% κ-carrageenan. The treatment resulted to the development of loaf volume up to 30% more than control but did not significantly improved the other quality parameters. While at the concentration of 0.2 and 0.4 %, kappa- carrageenan also reduced water loss up to 2%, and maintain crumb elasticity to 5% at the 96h storage. Treated crumb samples were also moist dan softer than the control although the pores of crumb, appearance of crumb also all sensories characteristics were difficult to diffrentiate with the untreated control sampel.

References

Ananingsih, K. dan Y. Wirawan. 2006. Optimasi Proses Produksi Tepung Karagenan (*Processed Eucheuma Seaweed*/PES) yang Diekstrak dari Eucheuma Cottonii dengan Penggunaan Berbagai Jenis Alkali dan Bleaching Agent. Skripsi Program Studi Teknologi Pangan Fakultas Teknologi Pertanian UNIKA Soegijapranata Semarang.

Ćuric, Duška; Novotni, Dubravka; Tusak, Dubravka; Bauman, Ingrid; Gabric, Domagoj. (2007). Gluten-Free Bread Production by the Corn Meal and Soybean Flour Extruded Blend Usage. Agriculturae Conspectus Scientificus University of Zagreb, Faculty of Food Technology and Biotechnology Vol. 72 No. 3 Hal. 227-232.

Dengate S. and Ruben A.: Controlled trial of cumulative bahavioural effects of a common bread preservative. J. Paediatr. Child. Health 2002, 38, 373-376.

Freile-Pelegrin, Y., Azamar, J.a., Robledo, D. 2011. Preliminary characterization of carrageenan from the Red Seaweed *Halymenia floresii*. *Journal of Aquatic Food Product and Technology*. 20: 73-83.

Fennema. 1976. Principle of Food Science I. Food Chemistry. Marckel Dekker Inc. New York.

Florian M. Ward, William H. Hanway, Richard B. Ward. 2006 Food Gums Functional Properties and applications In Handbook of Food Science, Technology, and Engineering Ed. Y.H.hu AB.i. Vol 3. Cpt 139. Pgs 139-11CRC Press. USA

Gaman, P. M. dan Sherrington, K.B. 1992. Ilmu Pangan, Pengantar Ilmu Pangan, Nutrisi dan Mikrobiologi. Yogyakarta: Gajah Mada University Press

Ghanbari, M., and J. Farmani.2013. Influence of Hydrocolloids on Dough Properties and Quality of Barbari: An Iranian Leavened Flat Bread. *J. Agr. Sci. Tech, Vol. 15: 545-555*

Giami S.Y. Amasisi T and Ekiyor, G. 2004. Comparison of bread making properties of composite flour from kernels of roasted and boiled African breadfruit (Treculia Africana decne) seeds. *Journal of Raw Material Research* 1: 16-25.

Glicksman M. 1983. *Food Hydrocolloids, Volume II*. New York: CRC Press. Inc.

Kohajdová, Z. and J. Karovicová.2008. Influence of Hydrocolloids on Quality of Baked Goods. *Acta Sci. Pol., Technol. Aliment. 7(2) : 43-49*

Kurokawa, Y., Maekawa, A., Takahashi .and M. ,Hayasi, Y. (1990).Toxicity and Carcinogenicity of Potassium Bromate; A New Renal Carcinogen. *Environ. Health Persp*. 87:309-335.

Lazaridou, A., D. Duta, M. Papageogiou, N. Belc and G.C. Biliaderis. 2007. Effects of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulation. Jurnal of Food Engineering. 79: 1033-1047.

Leon, AE, Ribotta PD, Fernandez C, Lanada CA, Beltramo DM. 2000. Interactions of different carrageenan isoforms and flour components in breadmaking. J. Food Agric. Food Chem. Jul 48 (7): 2634-8.

Matz, S. A. (1992). Bakery Technology and Engineering, 3th edition. Van Nostrand Reinhold. Texas.

Mudjajanto, S.E. dan L.N.Yulianti. 2004. Membuat Aneka Roti. Penerbit Swadaya ; Jakarta.

Parsons, J.L. and Chipman, J.K.(2000).The role of glutathione in DNA damage by potassium bromate in vitro. *Mutagenesis*.15(4):311-316.

Peighambardoust, S. H., N. Ebrahimpour, A. O. Ghaffari and S. A. Damirchi. 2011. Effect of Pectin, Guar and Carageenan on Quality
Parameters and Staling of Gluten Free Pan Bread. Journal of Food Science and Engineering 1 : 226-236

Raychaudhuri, D.L. and R. Chakraborty. 2013. Role of hydrocolloids in improving the physical and textural characteristics of fennel bread. International Food Research Journal 20(5): 2253-2259.

Rodge, A.B, Sonkamble SM., Salve RV., and Syed Imran Hasmi. 2012. Effect of hydrocolloid (guar gum) Incorcopration on the Quality Characteristics of Bread. J Food Process Technol 3:2.

Rosell, C. M., Rojas, J. A. and Benedito, C. 2001. Influence of Hydrocolloids on Dough Rheology and Bread Quality. Food Hydrocol., 15: 75-81.

Salim-ur Rehman dan Ahmed, Mudassar. 2003. Effect of Carboxxy methyl Cellulose and Carrageenan gum on the Shelf life of Bread. Pak J. Food Science 13(1): 49-52

Sciarini, L. S. Ribotta, P. D. Leon, A. E. Perez G. T. 2012. Incorporation of Several Additives into Gluten Free Breads: Effect on Dough Properties and Bread Quality, *J.Food Eng.*, 111: 590–597.

Selomulyo, V. O. & Zhou, W. (2006). Frozen Bread Dough: Affects of Freezing Storage and Dough Improvers. Journal of Cereal Science. Elsevier Ltd.

September, Cindy. 2007. The Effect of enzymes, gums and proteins on the water absorption, loaf volume and shelf life of bread. Master Thesis. Univ. Johannesburg Oct

Suptijah. (2004). Rumput Laut: Prospek dan Tantangannya. http://tumoutou.net/702_04212/pipih_suptijah.htm

Syarbini, H., 2013. A-Z Bakery. Pt. Tiga Serangkai Pustaka Mandiri: Semarang

Tashiro, M, Nishizu, T, Hashizume, K. Sako, H, Goto, K.

Uchida, H. A., Sugiyama, H., Kanehisa, S., Harada, K., Fujiwara, K. and Ono, T.(2006). An elderly patient with severe acute renal failure due to sodium bromated intoxication. *Intern Med* 45: 151-154.

Venugopal, Vazhiyil 2008. Marine Products for Healthcare: Functional and Bioactive Nutraceutical Compounds from the Ocean. CRC Press. NW Boca Rato FL. 528 pgs.

Zlatica Kohajdová*, Jolana Karovičová, Štefan Schmidt. 2009. Significance of Emulsifiers and Hydrocolloids in Bakery Industry. Acta Chimica Slovaca, Vol.2, No.1, 2009, 46 – 6

The evaluation of physicochemical characteristics of royal icing sugar made from three different white eggs

Ellen Anggarini H.[1], Dr. Ir. B. Soedarini, MP[2], and Dr. V. Kristina Ananingsih ST, MSc.[2]
1) Students, Faculty of Food Agriculture, Soegijapranata Catholic University, Semarang 50234, Indonesia
2) Lecturer, Faculty of Food Agriculture, Soegijapranata Catholic University, Semarang 50234, Indonesia
ellen.anggarini@gmail.com

ABSTRACT

Royal icing sugar is usually used not only to decorate cakes or cupcakes, but also to add sweet taste. We use pasteurized chicken egg whites as foam maker to give texture to the royal icing sugar. Besides chicken egg, Indonesian people also consume duck and quail eggs. They are potentially substitute the use of chicken egg. This research was conducted to find out the physicochemical characteristics of duck, chicken and quail eggs and their influence to the final result of the royal icing sugar during storage. In this research, fresh chicken, duck and quail eggs were used in three times trials. The data were then processed by using Windows' SPSS. The result showed that water content and Aw of the products made from duck and quail eggs were lower than the one made from chicken egg. While crude protein and fat contents as well as TBA value of products made from duck and quail eggs were higher than the one made from chicken egg. The royal icing sugar made from duck egg showed the highest lightness degree while the one made from quail egg showed the lowest lightness degree. The royal icing sugar made from duck egg showed the best result because it had the highest lightness degree and lower TBA value than the one made from quail egg.

Keywords : royal icing sugar, white eggs

INTRODUCTION

Royal icing sugar is a sweet emultion made from whisked white egg, icing sugar and lemon juice which sets solid (BBC, 2015). It is usually used for decorating cakes or cupcakes and adding sweet taste. To make royal icing sugar, pasteurized white chicken egg is usually used as foam maker to give the texture of royal icing sugar. Besides chicken egg, Indonesian people also consume duck and quail eggs. They are potentially substitute the use of chicken egg. The quantity of nutrition contents of duck and quail eggs are higher than that of chicken egg (USDA, 2007). In Indonesia, the production quantity of duck and quail eggs are high. At 2015, the production of duck egg reached 277,827.5 ton, while the production of quail egg reached 20,651.105 ton (Directorate General of Animal Husbandry, 2015). Although duck and quail eggs have lower foam stability, the other characteristics are pretty much the same with chicken egg. Until now, white chicken egg is always used for making royal icing sugar. That is why the ability of duck and quail eggs to substitute chicken egg needs to be learned.

MATERIALS AND METHOD

The making of royal icing sugar

The egg whites were whisked in large bowl until they become frothy. After that, icing sugar, lemon juice and vanilla essence were added. The mixture was then beated until it was very stiff. Royal icing sugar was then stored in refrigerator.

Water content of royal icing sugar

Water content of royal icing sugar was determined using thermogravimetric analysis. First, empty porcelain cup was dried in the oven at 100-105°C for 18 hours and then the weight was measured. 5 grams of sample were put into the empty porcelain cup and dried in oven at 100-105°C for 18 hours. The porcelain cup of dry sample was then put into desiccator for 15 minutes before the weight measurement. The water content was then calculated.

Crude protein content analysis

The analysis of crude protein content was using Kjeldahl method. Digestion tube was cleaned using HCl and aquades. 0,5 grams of sample, 7 grams of K_2SO_4, 0,35 grams of HgO, and 15 ml of H_2SO_4 were put into digestion tube to be digested for 3 hours. After that, 70 ml of NaOH and $Na_2S_2O_3$ mixture was then added. The mixture was then destilated for 3 minutes (Erlenmeyer with 25 ml of 4% boric acid was used to collect destilate). 3 drops of methyl red blue were then added to the destilate for titration using HCl 0,1 N until the color changed to light purple. The protein content was then calculated.

Crude fat content analysis

The analysis of crude fat content was using Soxhlet method. 2 grams of sample were dried and put into filter paper. The filter paper containing sample was then put into extractor. Meanwhile, hexane was put into distillation flask. The fat extraction was done for 4 hours. The hexane containing fat from the extraction was put into empty porcelain cup and dried in the oven at 80°C for 12 hours. The porcelain cup was then put into desiccator for 15 minutes before the weight measurement. The crude fat content was then calculated.

Thiobarbituric acid analysis
0,5 grams sample was dissolved into 50 ml of aquades. The solution was put into destillation tube which contained 48,5 ml of aquades. 1,5 ml of HCl and 3 drops of antifoam were added into the solution. The destillation process was done until 25 ml of destilate was obtained. 5 ml of destilate was put into closed test tube and added with 2,5 ml of TBA reagent. The solution was then heated for 30 minutes. Absorbance was read at 528 nm.

Color intensity analysis
The color intensity of royal icing sugar was measured using Chromameter Minolta CR-400 at 3 different spots.

Foam stability of white eggs analysis
Foam stability was measured by whisking 50 ml of white eggs until stable foam was obtained in scale plastic cup. Then the volume and overrun were calculated. After 10 minutes, the volume and overrun were calculated again. Foam stability was then calculated.

Water activity analysis
The analysis was done by putting 35 grams of sample in transparent tube inside the Aw meter. After 10 minutes, the result was read at the Aw meter

Statistical analysis
The results of water content, crude protein, crued fat, TBA, water activity and color intensity analysis were analyzed using SPSS for Windows. This analysis was using One Way ANOVA. Significant differences between means were analyzed by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Water content of royal icing sugar

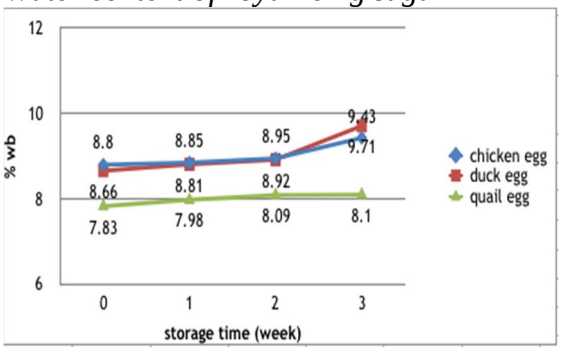


Figure 1. The water content of royal icing sugar during 4 weeks storage

The highest water content was obtained by royal icing sugar made from chicken egg whites while the lowest water content was obtained by royal icing sugar made from quail egg whites. The increase of water content during storage was caused by melting of foam as the result of the decrease of foam stability (Stadelman & Cotterill, 1995).

Crude protein content of royal icing sugar

Product	Protein content (%)
Chicken Egg Whites' Royal Icing Sugar	$1,74 \pm 0,36^{1}$
Duck Egg Whites' Royal Icing Sugar	$1,79 \pm 0,14^{1}$
Quail Egg Whites' Royal Icing Sugar	$2,05 \pm 0,16^{1}$

Figure 2. The crude protein content of royal icing sugar

The highest protein content was obtained by royal icing sugar made from quail egg whites while the lowest protein content was obtained by royal icing sugar made from chicken egg whites. The percentage of crude protein content of egg whites was higher than that of royal icing sugar because there are some addition of icing sugar and lemon juice in the royal icing sugar.

Crude fat content of royal icing sugar

Product	Fat Content (%)
Chicken Egg Whites' Royal Icing Sugar	$0,52 \pm 0,01^{1}$
Duck Egg Whites' Royal Icing Sugar	$0,73 \pm 0,01^{2}$
Quail Egg Whites' Royal Icing Sugar	$0,99 \pm 0,02^{3}$

Figure 3. The crude fat content of royal icing sugar

The highest fat content was obtained by royal icing sugar made from quail egg whites while the lowest fat content was obtained by royal icing sugar made from chicken egg whites. The percentage of crude fat content of egg whites was lower than that of royal icing sugar because there are some addition of lemon juice which also contained fat.

Thiobarbituric acid value of royal icing sugar

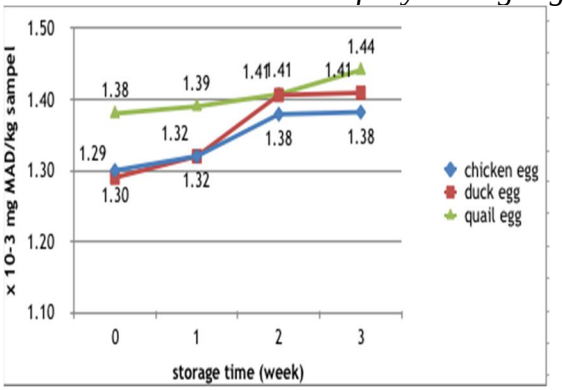


Figure 4. The thiobarbituric acid value of royal icing sugar during 4 weeks storage

The highest thiobarbituric acid value was obtained by royal icing sugar made from quail egg whites while the lowest thiobarbituric acid value was obtained by royal icing sugar made from chicken egg whites. The increase of thiobarbituric acid value was caused by product contact with oxygen and light which resulting in fat oxidation. The results are directly proportional with the crude fat content results.

Lightness degree of royal icing sugar

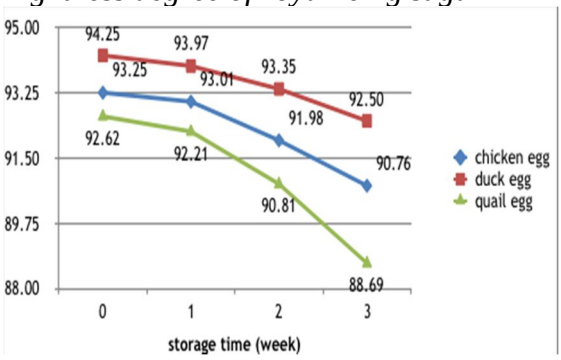


Figure 5. The lightness degree of royal icing sugar during 4 weeks storage

The highest lightness degree was obtained by royal icing sugar made from duck egg whites while the lowest lightness degree was obtained by royal icing sugar made from quail egg whites. The decrease of lightness degree during storage was in accordance to the theory of Cauvain & Young (2001) which said that royal icing sugar would undergo color changing during storage. Eskin (1990) added that the color changing was caused by browning of the amino content.

Foam stability of royal icing sugar

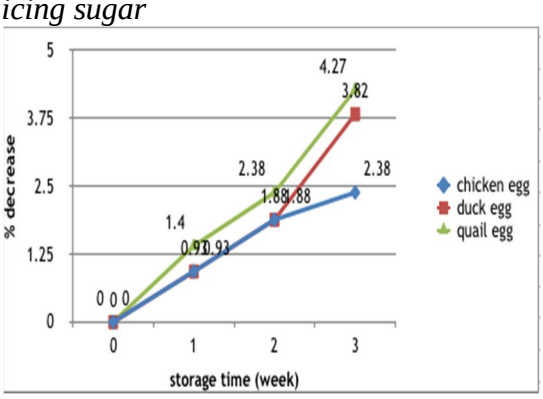


Figure 6. The foam stability of royal icing sugar during 4 weeks storage

Globulin content of duck and quail egg whites are lower than that of chicken egg whites. That is why the best foam stability was obtained by royal icing sugar made from chicken egg whites. Besides, the fat is also foam inhibitor. Product with higher fat content had lower foam stability. The foam stability had tight connection with the color intensity of product. Product with lower foam stability had lower product lightness.

Water activity of royal icing sugar

Product	Aw
Chicken Egg Whites' Royal Icing Sugar	0,86 ± 0,002[2]
Duck Egg Whites' Royal Icing Sugar	0,85 ± 0,001[2]
Quail Egg Whites' Royal Icing Sugar	0,85 ± 0,002[1]

Figure 7. The water activity of royal icing sugar

The result showed that water activity inversely proportional to product's water content. This was caused by icing sugar addition which has solid form and has the ability to create hydrogen bond which resulting in water content decrease (Kusnandar, 2010).

CONCLUSIONS

- The highest lightness was obtained by royal icing sugar made from duck egg whites while the lowest L value was obtained by royal icing sugar made from quail egg whites.
- Royal icing sugar made from duck and quail eggs had lower water content and foam stability than royal icing sugar made from chicken egg whites.
- Royal icing sugar made from duck and quail eggs had higher crude protein content, crude fat content and TBA value than royal icing sugar made from chicken egg whites.
- The royal icing sugar made from duck egg showed the best result because it had the highest lightness degree and lower TBA value than the one made from quail egg.

REFERENCES

Cauvain, Stanley & Linda Young. (2001). *Baking Problems Solved*. Woodhead Publishing Limited. Cambridge.

Direktorat Jenderal Peternakan. (2015). Produksi Telur Ayam Ras Petelur menurut Provinsi. Pertanian.go.id/ASEM2015-NAK/Prod_TelurAyamPetelur_Prop_2015.pdf

Direktorat Jenderal Peternakan. (2015). Produksi Telur Burung Puyuh. aplikasi.pertanian.go.id/bdsp/hasilKom.asp

Direktorat Jenderal Peternakan. (2015). Produksi Telur Itik menurut Provinsi. Pertanian.go.id/ASEM2015-NAK/Prod_TelurItik_Prop_2015.pdf

Eskin, N. (1990). Biochemistry of Food Second Edition. Academic Press. New York.

Kusnandar, Feri. (2010). Memahami Aktivitas Air dan Hubungannya dengan Keawetan Pangan. itp.fateta.ipb.ac.id/id/index.php?option=com_content&task=view&id=108&Itemid=94

SNI. (2009). Batas Maksimum Cemaran Mikroba dalam Pangan. SNI 7388 : 2009.

Stadelman, W. F. dan O. J. Cotterill. (1995). *Egg Science and Technology. 4th Edition.* Food Products Press. An Imprint of the Haworth Press, Inc. New York.

FOOD MICROBIOLOGY AND BIOTECHNOLOGY

The Development of *Centella asiatica* Extract-Loaded BSA Nanoparticles Production to Improve Bioavailability

Patchanee Yasurin[1*] and Kittiya Kesornbuakao[2]

[1]Food Biotechnology Program
[2]Food Technology Department
Biotechnology Faculty, Assumption University, Bangkok, 10240, Thailand

**Corresponding author. E-mail: patchaneeYsr@au.edu*

ABSTRACT

Centella asiatica (Bao-bog, Pennywort, Gotu kola) is used as a traditional drug widely in Asia. It was found that the chemical complexity of the herbal extracts seem to be important for the bioavailability. But, *C. asiatica* crude extracts showed excellent potential *in-vitro* but less or no *in-vivo* activity due to their poor lipid solubility or improper molecular size or both, resulting in poor absorption, poor dosing and poor bioavailability. The Bovine serum albumin (BSA) can attract macromolecular and carry wide variety of molecule. So this research was aimed to develop *C. asiatica* extract-loaded BSA nanoparticles (CBNP) to improve improve bioavailability. CBNP was prepared by the desolvation method using three different ratio *C. asiatica* crude chloroform extracts: BSA (1:2, 1:3, and 1.4.). The well agar diffusion method was used for evaluating antibacterial activity of CBNP with different concentration (100, 200, and 300 µl/ml) against five food borne pathogens (*Escherichia coli* ATCC25822, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), *S. enterica* 4,5,12:i:- (human) US clone, and *Bacillus cereus*). The results showed that the antibacterial activity of CBNP did not show significant different in different ratio of *C. asiatica* crude chloroform extracts: BSA and concentration in all food borne pathogens except *S. enterica* Enteritidis (human) ($P < 0.05$). The highest antibacterial active of CBNP was 1.07±0.46 cm against *S. enterica* Enteritidis (human) on ratio 1:4 of *C. asiatica* crude chloroform extracts: BSA using 200 µg/ml. The antibacterial activity of CBNP gave almost 2 times higher than free crude *C. asiatica* chloroform extracts. The modified Folin–Ciocalteu method was used for evaluating antioxidant activity. The highest antioxidant activity of CBNP was 21.11±6.60 µgGAE/mg using ratio 1:2 of *C. asiatica* crude chloroform extracts: BSA. The antioxidant activity of CBNP did not show significant different between on ratio 1:2 of *C. asiatica* crude chloroform extracts: BSA and others. The results indicated that CBNP showed the very promising to increase bioavailability of *C. asiatica*. The statistical analysis was done by ANOVA using SAS software version 9.3.

Keywords: *Centella asiatica*, Nanoparticles, Bioavailability, Antibacterial, Antioxidant

Introduction

C. asiatica (Bao-bog) is herbal in Asia. In Ayurvedic, an Indian system of medicine, *C. asiatica* is used for the treatment of leprosy, insanity, asthma, ulcers, eczema, skin and gastrointestinal disorder, arthritis, varicose vein and high blood pressure (Ariffin et al., 2011). The major biologically active compounds of *C. asiatica* extract are monoterpenes, sesquiterpene, and triterpenoids (Rattanakom, 2015). It was found that the chemical complexity of the herbal extracts seem to be important for the bioavailability. Even though, *C. asiatica* nanopowder gave higher yield of active compound (Asiatic acid) than natural *C. asiatica* powder (Borhan et al., 2013) there have been some limitations in *C. asiatica* crude extracts that it showed their extra ordinary potential in-vitro but less or no in-vivo activity due to their poor lipid solubility and improper molecular size, resulting in poor absorption, slow delivery, poor dosing and poor bioavailability. Especially, it is also difficult for water-soluble biological active compounds to enter through cell membrane of both human and pathogenic microorganism, which has hydrophobic characteristics. Albumin is a protein that can be obtained from a variety of sources, including egg white (ovalbumin), bovine serum albumin (BSA), and human serum albumin (HSA). Albumin is the major soluble protein of the circulating system and involved in the maintenance of osmotic pressure and binding and transport of nutrients to the cells. Albumin can soluble in water and diluted salt solution very well (Lohcharoenkal et al., 2014). The high solubility of albumin (up to 40% w/v). Albumin is stable in the pH range of 4 to 9 and can be heated at 60°C up to 10 hours without any deleterious effects and at pH 7.4 makes it an attractive macromolecular carrier capable of accommodating a wide variety of molecule (Kratz, 2008). The development of CBNP are the guild line to overcome these problems. Not only improve drug delivery system but the CBNP also reduce cost from purification steps, and can be applied to use in high valued industry products. Therefore, the objective of this research is to develop CBNP on antibacterial activity and antioxidant activity.

Materials and method

Preparation of sample

C. asiatica was purchased from local markets in Bangkok, Thailand. The aerial part of *C. asiatica* was used. Fresh *C. asiatica* were washed with tap water and cut into small pieces. Then it was air dried in oven (Memmert UM500) at 45°C. The dried samples were finely ground into powder. The powder were kept at 4°C before used (Rattanakom, 2015).

Preparation of C. asiatica crude choloroform extract

C. asiatica is extracted with chloroform using 1:10 ratio (g/ml). The mixtures are macerated at room temperature, 120 rpm, for 48 hours and then are filtered using whatman filter paper no.4. The crude extracts are concentrated using rotary evaporators at 45°C are kept at -20°C before use (Rattanakom, 2015). The *C. asiatica* chloroform crude extracts are further used for preparation of CBNP.

Preparation of C. asiatica extract-loaded BSA nanoparticles

CBNP are prepared by the desolvation method (Yu et al., 2014). The 100 mg of BSA is dissolved in 1 ml of sodium chloride solution (10 mM). Then, 8.0 ml of ethanol is added

dropwise into the BSA solution under magnetic stirring (400 rpm) at room temperature. Subsequently, the as-prepared BSA nanoparticles were cross-linked with 0.2% glutaraldehyde (GA). Then, *C. asiatica* crude extract is added into the solution for 24 hours at different ratio of *C. asiatica* to BSA (1:2, 1:3, and 1:4) in the preparation of CBNP. The particles are centrifuged and washed with distilled water. The centrifuged particles were resuspended and disperse in 2% mannitol, then freeze-dried for 24 hours. The dried nanopowder are further used for testing antimicrobial activity.

Antimicrobial activity

The modified agar well diffusion method (Rattanakom, 2015; Clark et al., 1981) is used. The 100 µl of bacteria (approximately 1.5×10^8 CFU/ml) is swab on Mueller-Hinton agar (MHA) plate. The *C. asiatica* crude extract and CBNP are used to test antibacterial activity at concentration 100, 200, and 300 µl/ml diluted with distilled water. The penicillin G is positive control. The inhibition zones were measured to determine the effectiveness of the *C. asiatica* crude extract and CBNP against each microorganism. The experiment was done in duplicate and three replication independently.

Antioxidant activity by Total phenolic content

The modified Folin–Ciocalteu method (Ragazzi and Veronese, 1973) was used for total phenolic content determination of *C. asiatica* crude extract and CBNP. The 20 µl of 10 mg/ml *C. asiatica* crude extract and CBNP was added to 1.58 ml distilled water and 100 µl Folin–Ciocalteu phenol reagent. The mixture was then allow to stand for 8 minutes 30 seconds and 300 µl saturated sodium carbonate solution was added to the mixture. Then the mixture was incubated without light at room temperature for 30 minutes and observed optical density (OD) at 765 nm. The experiment was done in triplicate and three replication independently.

Statistical analysis and Experimental design

All experiments were conducted in three replications and statistical analysis was accomplished using ANOVA with Tukey's multiple comparison and Dennett's test tests ($p < 0.05$) by SAS software version 9.3.

Results and Discussion

Antimicrobial activity

Table 1. Antibacterial activity as inhibition zone (cm) of CBNP and crude extract against different microorganism

	Inhibition zone of CBNP with different ratio between Crude:BSA and crude (cm.)								
	1:2			1:3			1:4		
Microorganisms	100µl/ml	200µl/ml	300µl/ml	100µl/ml	200µl/ml	300µl/ml	100µl/ml	200µl/ml	300µl/ml
Escherichai coli ATCC25822	$0.82\pm0.09^{AB,a}$	$0.85\pm0.04^{AB,a}$	$0.96\pm0.19^{A,a}$	$0.73\pm0.37^{AB,a}$	$0.66\pm0.33^{AB,a}$	$0.70\pm0.35^{AB,a}$	$0.96\pm0.25^{A,a}$	$0.58\pm0.45^{AB,a}$	$0.81\pm0.41^{AB,a}$
Salmonella enterica Thyphimurium U302 (DT104b)	$0.84\pm0.10^{A,a}$	$0.79\pm0.04^{A,a}$	$0.87\pm0.12^{A,a}$	$0.75\pm0.03^{A,a}$	$0.82\pm0.06^{A,a}$	$0.86\pm0.10^{A,a}$	$0.61\pm0.30^{AC,b}$	$0.72\pm0.37^{A,a}$	$0.68\pm0.34^{A,a}$
Salmonella enterica Enteritidis (human)	$0.86\pm0.04^{AB,a}$	$0.80\pm0.08^{ABC,a}$	$0.88\pm0.05^{AB,a}$	$0.78\pm0.05^{ABC,a}$	$0.88\pm0.08^{AB,a}$	$0.92\pm0.06^{AB,a}$	$0.84\pm0.12^{AB,b}$	$1.07\pm0.46^{A,a}$	$1.00\pm0.27^{A,a}$
Salmonella enterica 4,5,12:i: (human)US clone	$0.80\pm0.03^{ABC,a}$	$0.85\pm0.04^{ABC,a}$	$0.93\pm0.08^{A,a}$	$0.78\pm0.03^{BC,a}$	$0.88\pm0.08^{ABC,a}$	$0.85\pm0.09^{ABC,a}$	$0.74\pm0.05^{BC,b}$	$0.81\pm0.07^{ABC,a}$	$0.83\pm0.10^{ABC,a}$
Bacillus cereus	$0.75\pm0.07^{AB,a}$	$0.64\pm0.31^{AB,a}$	$0.89\pm0.10^{A,a}$	$0.77\pm0.05^{AB,a}$	$0.83\pm0.07^{AB,a}$	$0.88\pm0.06^{A,a}$	$0.82\pm0.08^{AB,b}$	$0.87\pm0.05^{A,a}$	$0.97\pm0.08^{A,a}$

Microorganisms	Crude 100µl/ml	200µl/ml	300µl/ml
Escherichai coli ATCC25822	0.45±0.38^{B,a}	0.54±0.30^{B,a}	0.52±0.32^{B,a}
Salmonella enterica Thyphimurium U302 (DT104b)	0.16±0.24^{B,a}	0.29±0.28^{BC,a}	0.27±0.34^{BC,a}
Salmonella enterica Enteritidis (human)	0.59±0.20^{ABC,a}	0.49±0.38^{ABC,a}	0.76±0.04^{ABC,a}
Salmonella enterica 4,5,12:i:- (human) US clone	0.42±0.38^{C,a}	0.62±0.31^{C,a}	0.67±0.22^{C,a}
Bacillus cereus	0.54±0.42^{B,a}	0.66±0.14^{B,a}	0.57±0.36^{B,a}

Different superscript within a row showed significant different at $p<0.05$
Different superscript within a column showed significant different at $p<0.05$

CBNP were prepare by a desolvation method (Yu et al., 2014). There are three different of the nanoparticles preparative. The different between these three nanoparticles were ratio of *C. asiatica* crude chloroform extracts to BSA 1:2, 1:3, and 1:4. The well agar diffusion method was used for evaluating antibacterial activity of CBNP and *C. asiatica* crude chloroform extracts with different concentration (100, 200, and 300 µl/ml) against five food borne pathogens (*Escherichia coli* ATCC25822, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), *S. enterica* 4,5,12:i:- (human) US clone, and *Bacillus cereus*). The results of CBNP and *C. asiatica* crude chloroform extracts antibacterial activity were interpreted by using Randomized Complete Block Design (RCBD) with Dennett's test in SAS program version9.3 as showed in table1. It was found that antibacterial activities trend of CBNP increased from *C. asiatica* crude chloroform extracts about 2-3 times significantly($p<0.05$). *C. asiatica* crude chloroform extracts have low antibacterial activity because there is a hydrophilic active compound so, it is difficult for water-soluble biological active compounds to permeate through cell membrane, which has hydrophobic characteristics. But, bovine serum albumin (BSA) of CBNP is the protein can attract macromolecular and carry variety of molecule of active compound (Kratz, 2008).Albumin nanoparticles are formed by linkage of amino acid by electrostatic and attraction and covalent linkage reagent (e.g. glutaraldehyde, genipin) and can be prepare to a size of between 100-200 nm (Weber et al., 2000). It also can readily bind and release small molecule (Kratz, 2008). All of these reasons made it can increase an efficiency of absorption to the cells. Furthermore, the results of CBNP antibacterial activity were interpreted by using Randomized Complete Block Design (RCBD) with Tukey's multiple comparison in SAS program version9.3. It was found that the different ratio of C. *asiatica* crude chloroform extracts: BSA and concentration showed no significant difference in antibacterial activity of CBNP against all pathogens except *S. enterica* 4,5,12:i:- (human) US clone($P < 0.05$). The highest antibacterial active was 1.07±0.46 cm using CBNP ration 1:4, 200 µg/ml against *S. enterica* Enteritidis (human). It can be conclude that CBNP at ratio 1:4 is most effective in an economical way.

Antioxidant activity by Total phenolic content

Table 2. Total phenolic content antioxidant potential of CBNP and crude extract

CBNP with different ratio between Crude:BSA and crude	Total phenolic content (µg GAE/mg)
1:2	21.11 ± 6.60^{A}
1:3	18.67 ± 3.98^{A}
1:4	20.52 ± 6.82^{A}
crude	21.51 ± 3.82^{A}

Different superscript within a column showed significant different at $p<0.05$

The antioxidant activity can be measured by the ability of the compound that can catch free radicals compounds by scavenging or trapping methods (Huang et al., 2005). Herb and spice are rich in phenolic compounds (Yanishlieva et al., 2006) which are an active compound that have antioxidant properties as protective agent against free radical compound (de Beer et al., 2002). Total phenolic content of CBNP was determined in comparison with standard garlic acid and the results were expressed in terms of µg GAE/mg as showed in table2The results

of CBNP antioxidant activity by total phenolic content were interpreted by using Randomized Complete Block Design (RCBD) with Tukey's multiple comparison in SAS program version9.3. According to the result, the crude extract had highest total phenolic content. Even though crude extract showed slightly higher total phenolic content than CBNP 1:2. While, CBNP 1:3 had lowest total phenolic content. Although, there are no significantly different between all of CBNP with different ratio of crude:BSA and crude extract. It can be conclude that CBNP at ratio 1:4 is most effective in an economical way because of using less crude extract.

Conclusions

The development of CBNP can improve in both antimicrobial activity and antioxidant activity. It also reduce using of crude extract but give higher or same properties. CBNP showed more antimicrobial efficiency than *C. asiatica* crude chloroform extracts at the same concentrations. Moreover, it shows no significantly different in total phenolic content. To conclude, preparation of CBNP at ratio 1:4 of crude:BSA is most effective in an economical way.

Acknowledgements

This research was supported by Assumption University, Research Grant P-58-388.

References

Ariffin F, Heong Chew s, Bhupinder K, Karim AA, Huda N.(2011), "Antioxidant capacity and phenolic composition of fermented *Centella asiatica* herbal teas", JSci Food Agric, Vol.91 no.15, pp.2731-9.

Clark AM, EI Feraly AS, Li WS. (1981), "Antimicrobial of phenolic constituents of Magnolia grandiflora L", J pharma sci, Vol. 70 no.8, pp.951-2.

D. de Beer, E. Joubert, W.C.A. Gelderblom, M. Manley. (2002), "Phenolic Compounds: A Review of Their Possible Role as In Vivo Antioxidants of Wine", Vol. 23 no.2.

Huang D., Ou B., Prior R.L. (2005), "The chemistry behind antioxidant capacity assays", J. Agric. Food Chem, Vol. 53, pp.1841-1856.

Kratz F. (2008), "Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles", PubMed, Vol.132 no.3, pp.171-83..

M. Z. Borhan, and T. Y. Nee. (2014), "Synthesis of TiO2 nanopowders from red gypsum using EDTA as complexing agent", Journal of Nanostructure in Chemistry.

Ragazzi E and Veronese G. (1973), "Quantitative analysis of phenolic compound after thin-layer chromatographic separation", J Chromatogr A, Vol 77 no.2, pp.369-75.

Rattanakom S, Yasurin P. (2015), "Antibacterial activity, antioxidant activity and chemical profiling of Centella asiatica under different extraction solvents", Orient. J. Chem, Vol.31 no.4, pp.2453-2459.

Warangkana Lohcharoenkal, Liying Wang, Yi Charlie Chen, and Yon Rojanasakul. (2014), "Protein Nanoparticles as Drug Delivery Carriers for Cancer Therapy", BioMed Research International.

Weber, C., et al. (2000), "Desolvation process and surface characterisation of protein nanoparticles", International Journal of Pharmaceutics, Vol.194, pp.91-102

Yanishlieva NV, Marinova E, Pokorny J. (2006), "Natural antioxidant from herb and spices", Eur J Lipid Sci Technol, Vol.108 no.9, pp.776-93.

Yu Z, Yu M, Zhang Z, Hong G, and Xiong Q. (2014), "Bovine serum albumin nanoparticles as controlled release carrier for local drug delivery to the inner ear", Nanoscale Research Letters, Vol.9 no.1, pp.343.

Assessment of Antimicrobial Activity of Concentrated Hypothiocyanite against *Escherichia coli* in Basic pH Environment

Ahmad Ni'matullah Al-Baarri[1]*, Anang Mohamad Legowo[1], Novia Tri Damayanti[2]

[1]*Food Technology Department, Faculty of Animal and Agricultural Sciences, Diponegoro University, Indonesia*

[2]*Animal Science Department, Faculty of Animal and Agricultural Sciences, Diponegoro University, Indonesia*

*Corresponding author: albari@undip.ac.id

ABSTRACT

The objective of this work was to analyze the concentrated hypothiocyanite against pathogenic bacteria of *Escherichia coli*. The antibacterial activities of 0.08–0.87 mM hypothiocyanite concentrations against *E. coli* were investigated in pH of 9.0 during 4 days. Hypothiocyanite was obtained from the enzymatic reaction of lactoperoxidase, hydrogen peroxide and thiocyanate. The obtained hypothiocyanite was stored in 36˚C for 4 h to analyze the decrease in concentration. The results showed that 0.87 mM hypothiocyanite was able to be produced and no remarkable decrease in its concentration during storage. The linear increase in antibacterial activity could also be seen along the increase of concentration of hypothiocyanite. All number of *E.* coli was able to be eliminated when 0.87 mM hypothiocyanite was employed in pH 9.0. This result may provide benefit to avoid contamination in food when it was stored in basic pH environment.

Keywords: hypothiocyanite, antibacterial activity, concentration, storage, pH range

Introduction

The mixture of three components, i.e. lactoperoxidase, hydrogen peroxide, and thiocyanate resulting in the production of hypothiocyanite (Al-Baarri, Hayashi, et al., 2011, Al-Baarri, Ogawa, et al., 2011, Seifu, Buys, et al., 2005). This compound has been widely understood to have a benefit for antibacterial and antifungal, and has been received its status as Generally Recognized As Safe (GRAS), thus it received many attentions from researcher to apply in various field including dairy technology (Nawangsari, Al-Baarri, et al., 2014), farming system (Nguyen, Ducamp, et al., 2005), and health sciences (Tenovuo, 2002).

Escherichia coli has been widely known as foodborne bacteria and it is a common knowledge that this microorganisms should be eliminated to zero at ready to eat's food. Traditionally, lactic acid, a weak-organic acid, has been widely used to control growth of pathogenic bacteria in foods for several decades. GRAS based preservative agent has received huge attention to reduce or destroy the presence of this bacteria in order to meet the requirement for health and safety. Several enzymes including lactoferrin and lysozyme successfully eliminated the foodborne bacteria. Application of these enzymes in order to inhibit pathogenic bacteria has commonly known to be existed in the same circumstances. It is understood that bacteria should be converged with the enzyme to realize the elimination reaction. Based on this mechanism, lactoperoxidase exhibit the unique mechanism to eliminate bacteria through the production of antibacterial agent of hypothiocyanite that was derived from the mixture of three compounds to build a system namely lactoperoxidase system. The enzyme-only is unable to generate the antimicrobial activity.

Organic acid has been widely applied to inhibit growth of pathogenic bacteria in foods (Ibrahim, Yang, et al., 2008, Touch, Hayakawa, et al., 2004), thus the fermentation should be an important application for avoiding foodborne. It is well understood that acid is associated with the ATP depletion resulting in the death of bacteria (Lansdowne, Beamer, et al., 2009). Strong basic pH is also generating the association to the swell and ultimate rupture of bacteria (Mendonca, Amoroso, et al., 1994). However fewer documents that was reported on mild basic condition in food. Moderate basic pH was reported unable to inhibit pathogenic bacteria, thus this research was done to analyze antibacterial activity of hypothiocyanite against E. coli in moderate basic pH.

Materials and Methods

Materials

Fresh bovine's milk was provided by campus farm at Faculty of Animal and Agricultural Sciences, Diponegoro University. Hydrogen peroxide, KSCN, 2,2-azino-bis(3-ethylbenzthia-zoline-6-sulfonic acid) (ABTS) and were purchased from Sigma. Rennet was purchased from Singapore. SP Sepharose Fast Flow was purchased from Amersham Pharmacia Biotech, Sweden. *E. coli* was purchased by Research Laboratory and Integrated Analysis, Gadjah Mada University. These strains were maintained on tryptic soy agar (TSA, Himedia, Mumbai, India) slants at 4 °C. Unless otherwise specified, all other chemicals were reagent grade.

Methods

Bacteria Preparation

The method for bacteria preparation was conducted as previous researcher with slight modification (Ibrahim, Yang, et al., 2008). Prior to use, *E. coli* were transferred from TSA to fresh tryptic soy broth (TSB, Himedia, Mumbai, India) and incubated for 24 h at 36˚C. The transfer into fresh TSB was conducted after 24 h incubation to achieve optimum growth of bacteria. The cultures were then centrifuged at 8000g for 15 min and the precipitate was resuspended (approximately 7 log CFU/ml) in 10 mM phosphate buffer pH 9.0. Serial dilutions using 10 mM phosphate buffer containing 0.88% NaCl were made to achieve an initial inoculum level of 5.0 log CFU/ml.

Lactoperoxidase Preparation

The method to obtain lactoperoxidase was conducted using the previous described method (Al-Baarri, Ogawa, et al., 2011). One liter of fresh bovine milk was defatted (by centrifugation at 8000g for 30min) and the collected skim milk was treated with 0.02% (w/v) rennet and 0.2% (v/v) lactic acid at 30 °C for 30 min. After the removal of curd through filtration using filter paper under vacuum, the whey was obtained. Whey was dialyzed against 10 mM sodium phosphate buffer (PB) (pH 6.8) at 4˚C for 48 h. The resulting whey solution was applied to a SP Sepharose Big Beads column. The preparation for using this column was performed using method of Hayashi *et al.* (2012). The eluate was collected using centrifuge tube (10 ml per tube). The purity of the LPO in the eluate was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). The tube that was highest in purity of protein was collected and filtered through a 0.22 µm filter (Millipore, MA, USA). Final LPO activity was determined using ABTS as a substrate (Pruitt, Kamau, et al., 1990). This research was successfully obtained 5 U/ml lactoperoxidase. The lactoperoxidase was stored at -20˚C prior to use.

Hypothiocyanite Preparation

The measurement hypothiocyanite was conducted using previous described method (Aune and Thomas, 1977) and the generation of hypothiocyanite was conducted using the method of Al-Baarri *et al.* (2010) with modification. The production of hypothiocyanite was performed by mixing H_2O_2, KSCN, and lactoperoxidase. Hypothiocyanite concentration was set by arranging the concentration of both substrates ranging from 0.1 to 1.5 mM. In order to set final hypothiocyanite in pH 9.0, all substrates was diluted to PB pH 9.0.

Antibacterial Activity Measurement

The antimicrobial activity of hypothiocyanite was determined using previous mentioned methods (Al-Baarri, Hayashi, et al., 2011) with slight modification. The mixture of 200 µl of 0,08~0.87 mM hypothiocyanite, 40 µL of *E. coli* (ca. 5 log CFU/ml), and 160 µL PB pH 9.0 were incubated for 4 h in a water bath at 37˚C. For bacteria enumeration, serial dilutions of the assay mixture were prepared with 0.88% NaCl solution. The final dilution was spread onto TSA and incubated at 37˚C for 24 h. The antimicrobial activity was calculated by log N0/Nt, where N0 is the CFU per milliliter of the mixture without hypothiocyanite and Nt is the CFU per milliliter of the mixture with hypothiocyanite.

Data Analysis
Data were presented as means±standard deviation of the mean. Obtained data were collected from three replications and descriptively explained to compare each treatment.

Result and Discussion
Hypothiocyanite production
It has been documented that the adequate substrates was required to achieve the high concentration of hypothiocyanite, however the excess of substrates resulted in the inactivation of lactoperoxidase (Boots and Floris, 2006, Seifu, Buys, et al., 2005), thus lessen the production of hypothiocyanite (Fonteh, Grandison, et al., 2005, Kussendrager and Hooijdonk, 2000). Therefore, this research was initiated with the analysis of the hypothiocyanite production from gradual increase of substrates (Figure 1). As can be seen, the applied substrates that were ranging from 0.1 to 1.0 mM resulted 0.08 to 0.87 mM hypothiocyanite.

The production of hypothiocyanite was understood to be linear to the applied substrates concentration (Björk, Rosén, et al., 1975), however the activity of enzyme was also appear to be important key to produce hypothiocyanite (Fonteh, Grandison, et al., 2005). Furthermore, the excess H_2O_2 exhibit the inactivation of lactoperoxidase enzyme through the formation of compound III that was associated with irreversible inactivation of lactoperoxidase enzyme (Seifu, Buys, et al., 2005) while the excess of KSCN resulted in the inhibition of catalytic activity of enzyme (Singh, Singh, et al., 2009). This reason explained non-linear graphic along the increase in substrates. The research was also continued by much higher concentration of substrates that was resulted in the not remarkable increase in hypothiocyanite concentration and the production was started to decrease when 2.0 mM substrates were applied (data not presented).

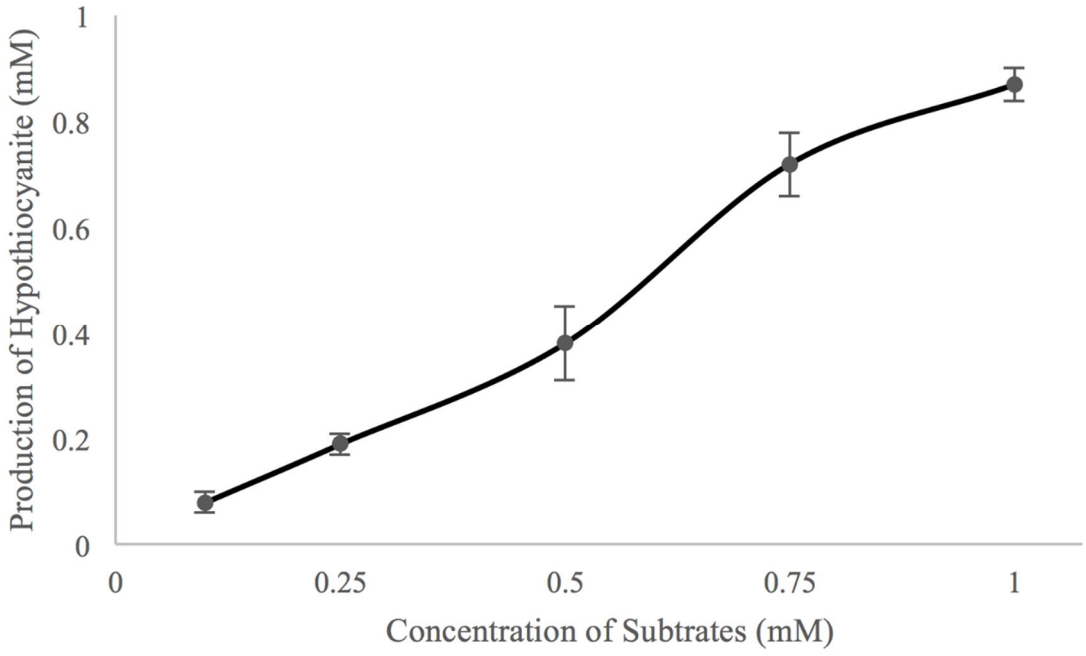


Figure 7. Concentration of hypothiocyanite after one hour in the reaction mixture containing two substrates (H_2O_2 and KSCN) in equal concentrations. Data represents average of three replicates while error bars represents standard deviation.

Remaining Hypothiocyanite during Storage

Figure 2 shows the remaining hypothiocyanite during storage in 37˚C for 4 hour and the result shows not remarkable decrease in the concentration of hypothiocyanite. There was a slight decrease about 3% from 100 to 97% in the concentration of hypothiocyanite, and interestingly, the storage until 4 hour in 4˚C was also decrease 2-3% indicating that the loss of hypothiocyanite concentration are not temperature independently.

Hypothiocyanite was produced from the reversible reaction between H^+ and $OSCN^-$ (Kussendrager and Hooijdonk, 2000, Zhou and Lim, 2009). Both HOSCN (hypothiocyanite) and $OSCN^-$ generate antibacterial activity (Seifu, Buys, et al., 2005). Since the reaction could be categorized as reversible reaction, the duration of storage didn't provide remarkable decrease in hypothiocyanite. Since 4 hour of storage exhibits negligible decrease in hypothiocyanite concentration, thus the research was continued to the antibacterial analysis for 4 h reaction time.

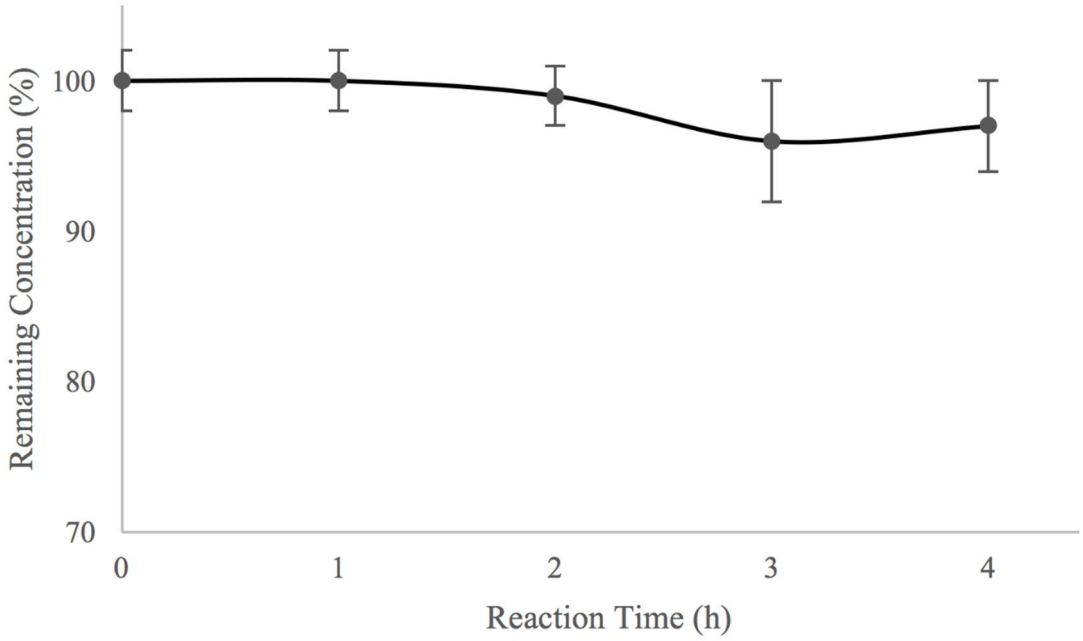


Figure 8. Concentration of remaining hypothiocyanite in the reaction mixture during 4 hour of storage. The solution containing high concentration of hypothiocyanite was stored at 37˚C up to 4 hour. Data represents average of three replicates while error bars represents standard deviation.

Antibacterial Activity against *E. coli*

Antibacterial activity of hypothiocyanite against *E. coli* is presented in Figure 3. Hypothiocyanite ranging from 0.08 to 0.87 mM was applied in the reaction solution containing *E. coli* at initial population of ca. 4 log CFU/ml. The results show that 0.38 mM

concentration of hypothiocyanite generated maximum elimination of bacterial population at two hours of reaction time and such antibacterial activity was also seen in the reaction mixture containing 0.72 and 0.87 at three hours of reaction time while the employed hypothiocyanite at the concentration less than 0.38 mM resulted in the antibacterial activity of 0.91 at maximum storage in this research.

It is generally recognized that the hypothiocyanite generates antibacterial activity, however the concentration was the limitation factor of antimicrobial activity (Hayashi, Naknukool, et al., 2012). The total concentration of 0.3 mM has been documented to have antibacterial activity and it was reported that concentration of 0.3 mM was able to inhibit growth of *Salmonella enteritidis* (Al-Baarri, Hayashi, et al., 2011, Touch, Hayakawa, et al., 2004). Optimum lactoperoxidase activity for working optimally has been explored at pH 6.4 (Kussendrager and Hooijdonk, 2000). This research used pH 9.0 since there was limitation work of antibacterial activity in basic pH. It was quite much documentation reporting antibacterial activity in acidic pH (Seifu, Buys, et al., 2005). This report shows that hypothiocyanite may able to work as antibacterial agent in the basic pH. This research may open the strategy for preserving the food in basic condition.

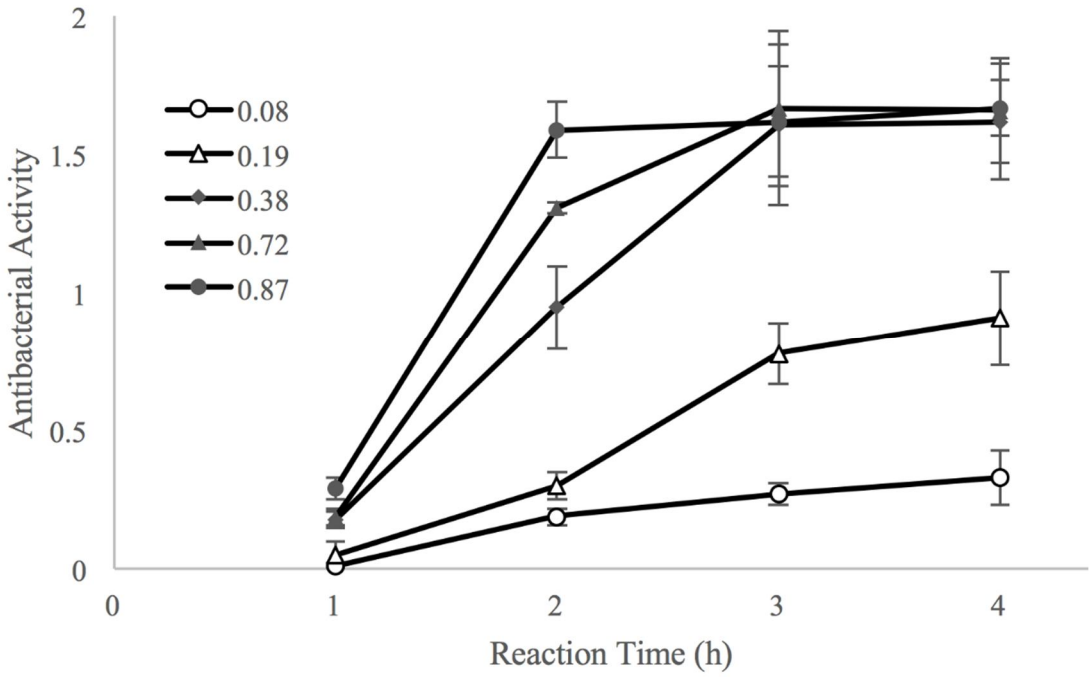


Figure 9. Antibacterial activity of hypothiocyanite against *E. coli* in the reaction mixture containing various concentrations of hypothiocyanite and *E. coli* (ca. 4 log CFU/ml) as initial load of population. The reaction mixture was set into pH 9.0. Data represents average of three replicates while error bars represents standard deviation**.**

Conclusion

The production of hypothiocyanite was substrates dependent and the concentration of 0.87 mM was successfully generated using the 1.5 mM of each substrates. The storage in 36˚C

hypothiocyanite for 4 hours exhibited no remarkable decrease in hypothiocyanite concentration. When basic pH was applied, the hypothiocyanite was still applicable to generate antibacterial activity against *E. coli*.

Acknowledgement
Authors would express our sincere thanks to Republic of Indonesia through financial support from research grants from Ministry of Research, Technology, and Higher Education.

Bibliography

Al-Baarri, A.N., M. Hayashi, M. Ogawa and S. Hayakawa. 2011. Effects of mono- and di-saccharides on the antimicrobial activity of bovine lactoperoxidase system. Journal of Food Protection 74: 134–139.

Al-Baarri, A.N., M. Ogawa and S. Hayakawa. 2010. Scale-up studies on immobilization of lactoperoxidase using milk whey for producing antimicrobial agent. Journal of the Indonesian Tropical Animal Agriculture 35: 185–191.

Al-Baarri, A.N., M. Ogawa and S. Hayakawa. 2011. Application of lactoperoxidase system using bovine whey and the effect of storage condition on lactoperoxidase activity. International Journal of Dairy Science 6: 72–78.

Aune, T.M. and E.L. Thomas. 1977. Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation od thiocyanate ion. European Journal of Biochemistry 80: 209-214.

Björk, L., C.-G. Rosén, V. Marshall and B. Reiter. 1975. Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other gram-negative bacteria. Applied Microbiology 30: 199–204.

Boots, J.-W. and R. Floris. 2006. Lactoperoxidase: from catalytic mechanism to practical applications. International Dairy Journal 16: 1272-1276.

Fonteh, F.A., A.S. Grandison and M.J. Lewis. 2005. Factor affecting lactoperoxidase activity. International Journal of Dairy Technology 58: 233-236.

Hayashi, M., S. Naknukool, S. Hayakawa, M. Ogawa and A.N.m. Al-Baarri. 2012. Enhancement of antimicrobial activity of a lactoperoxidase system by carrot extract and ß-carotene. Food Chemistry 130: 541–546.

Ibrahim, S.A., H. Yang and C.W. Seo. 2008. Antimicrobial activity of lactic acid and copper on growth of Salmonella and Escherichia coli O157:H7 in laboratory medium and carrot juice. Food Chemistry 109: 137–143.

Kussendrager, K.D. and A.C.M.v. Hooijdonk. 2000. Lactoperoxidase: physico-chemical properties, occurence mechanism of action and application. British Journal of Nutrition 84: S19-S25.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227: 680–685.

Lansdowne, L.R., S. Beamer, J. Jaczynski and K.E. Matak. 2009. Survival of Listeria innocua after Isoelectric Solubilization and Precipitation of Fish Protein. Journal of Food Science 74: 201–205.

Mendonca, A.F., T.L. Amoroso and S.J. Knabel. 1994. Destruction of gram-negative food-borne pathogens by high pH involves disruption of the cytoplasmic membrane. Applied Environmental Microbiology 60: 4009–4014.

Nawangsari, D.N., A.N. Al-Baarri, S. Mulyani, A.M. Legowo and V.P. Bintoro. 2014. Resistence of immobilized lactoperoxidase activity from bovine whey against storage solution. International Journal of Dairy Science 9: 56–62.

Nguyen, D.D.L., M.-N. Ducamp, M. Dornier, D. Montet and G. Loiseau. 2005. Effect of the Lactoperoxidase System against Three Major Causal Agents of Disease in Mangoes. Journal of Food Protection 68: 1497–1500(1494).

Pruitt, K.M., D.N. Kamau, K. Miller, B. Mansson-Rahemtulla and F. Rahemtulla. 1990. Quantitative, standardized assays for determining the concentrations of bovine lactoperoxide, human salivary peroxidase, and human myeloperoxidase. Analytical Biochemistry 191: 278-286.

Seifu, E., E.M. Buys and E.F. Donkin. 2005. Significance of the lactoperoxidase system in the dairy industry and its potential applications: a review. Trends in Food Science & Technology 16: 137-154.

Singh, A.K., N. Singh, S. Sharma, K. Shin, M. Takase, P. Kaur, et al. 2009. Inhibition of lactoperoxidase by its own catalytic product: crystal structure of the hypothiocyanate-inhibited bovine lactoperoxidase at 2.3-Å resolution. Biophysical Journal 96: 646-654.

Tenovuo, J. 2002. Clinical applications of antimicrobial host protein, lactoperoxidase, lysozyme, and lactoferrin in xerostomia: eficacy and safety. Oral Diseases 8: 23-29.

Touch, V., S. Hayakawa, S. Yamada and S. Kaneko. 2004. Effect of lactoperoxidase-thiocyanate-hydrogen peroxide system on Salmonella enteritidis in animal or vegetable foods. International Journal of Food Microbiology 93: 175-183.

Zhou, Y. and L.-T. Lim. 2009. Activation of Lactoperoxidase System in Milk by Glucose Oxidase Immobilized in Electrospun Polylactide Microfibers. Journal of Food Science 74: C170–C176.

Supplementation of Prebiotic Iles-Iles as a Source of Carbon in the MRS Media For Growth of *Lactobacillus casei*

Ngatirah[1], Meidi Syaflan[2]

[1]Faculty of Agricultural Technology, Stiper Agriculture University (ngatirah@yahoo.com)
[2]Faculty of Agricultural Technology, Stiper Agriculture University, (meidi Syaflan@yahoo..com)

ABSTRACT

This study aimed to study the effect of prebiotic supplementation fraction of iles-iles as a carbon source to support the growth of Lactobacillus casei. The experimental design used was a randomized complete block desgn (RCBD) 1 factor is the type of carbon source and consists of three levels ie MRS medium + glucose (A), Media MRS + glucomannan (B) and Media MRS + hydrolyzate glukoman (C), each treatment was repeated 3 times restating. Analysis was conducted on the analysis of the amount L.casei, pH analysis and analysis of volatile fatty acids (acetic acid, propionate acid and butyric acids). The results showed that the fraction of prebiotic supplementation iles-iles that glucomannan and glucomannan hydrolyzate does not affect the number of bacteria and pH media growth. supplementation of glucomannan hydrolysates on MRS media produce the amount of acetic acid and butyric acid which is higher than supplementation with glucomannan and glucose (control).

Keywords : Iles-iles, Prebiotic, Supplementation, *L. casei*

Introduction

Attention to healthy food tends to increase with the increasing of degenerative diseases as a result of not eating well. One type of healthy food nowadays is functional food. According Grajek *et al*, (2005), functional food include: (i) food that is naturally contain bioactive compounds (eg dietary fiber), (ii) food enriched with bioactive compounds (eg probiotics, antioxidants), and (iii) substituted foodstuffs in food traditional (eg prebiotics). Nowadays, the concept of functional food began to lead to sinbiotic concept is a combination of probiotics and prebiotics.

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Sander, 2008). From various studies, the benefits of probiotics on health has been widely studied. *Lactobacillus casei* is one type of probiotic that is widely applied in the dairy industry. Prebiotics are defined as food that is not digested that benefit the host by selectively stimulating and or activity of specific bacteria in the colon host (Gibson and Roberfroid, 1995) .Some kind of material that has potential as prebiotics can be extracted / isolated from various plants. One type of local plant as a potential source of prebiotics is iles-iles (*Amorphophalus onchophyllus*) (Grajeck *et al*, 2005; Tuhoy *et.al.*, 2005, and Gibson and Ratsall, 2006). Iles-iles flour containing glucomannan 41.14%, 24.09% starch, crude fiber 11.26%, 4.96% protein, 6.1% ash, 0.08% fat and 6.24% calcium oxalate. High levels of glucomannan, starch and fiber in the flour iles-iles has potential as a prebiotic. From some research, glucomannan on iles-iles flour can reduce cholesterol and control the glycemic index (Alvin and Bodin, 1995; Vuksan et.al, 2000; Yoshida et.al, 2005).

Iles-iles is one of the local plant species with the potential to be developed as a source of prebiotic, but has not been many studies evaluating this potential. Iles-iles contain some fraction of which can serve as a prebiotic that is starch, fiber, glucomannan and mannooligosaccharides. Iles-iles containing of 91.79% carbohydrates, 10,61% crude fiber and 23,52% glucomannan. The high carbohydrate, fiber and glucomannan potential as a source of prebiotics. Prebiotics are food ingredients that can not be digested by the human gut, but can serve as a source of food (substrate) for the beneficial bacteria so as to reduce the amount of harmful bacteria. Prebiotic components of the most widely used is the oligosaccharide groups such as those found in bananas (inulin), apples, corn, potato (Solanum tuberosum) and tubers (oligosaccharides) including iles-iles. On iles-iles contained oligosaccharides which can be broken down into acids by probiotic bacteria eg Lactic Acid Bacteria (LAB) (Silalahi and Netty, 2003).

Iles-iles potential as a source of prebiotic can be in various fractions such as flour, starch, glucomannan extract and glucomannan hydrolysates. Iles-iles flour is produced iles-iles that still has such a mixture of starch, fiber, carbohydrates and glukoman. Starch is the separation of the components of iles-iles flour which still contains amylose and amylopectin. Glucomannan extract resulting from the process of separation of components of iles-iles flour that contains the form of oligosaccharides. Glucomannan hydrolyzate is yielded from hydrolysis of iles-iles flour with α-amylase enzyme, so it's contains of simple sugars (glucose). That fractions has different potentially as a prebiotic so this paper need to learn fractions that can contribute the most good in supporting the growth of lactic acid bacteria and capable to fermentation prebiotic into short chain fatty acids such as acetic acid,

propionate acid and butyric acid. Short chain fatty acids produced by lactic acid bacteria are able to balance the pH in the intestine making it suitable for the life of the microflora in the gut. The purpose is to study the effect of supplementation with glucomannan and glucomannan hydrolysates as a carbon source for growth of L. casei and short chain fatty acids produced.

Materials and method

Glucomannan and hydrolyzate glucomannan preparation

Preparation of glucomannan based on Ashadi and Mardiah (2006) methods were modified. Iles-iles flour mixed with distilled water (1:30), then heated at 75 ^{o}C for 1 hour while stirring / homogenized with a speed of 560 rpm to form a gel, then cooled at room temperature. After that, the addition of 96% alcohol in the ratio 1: 2, while stirring until homogeneous ± 5 minutes. then filtering, the filtrate is discarded and the solid are taken and dried in the oven at 50 ^{o}C for 48 hours. After that the solid are milled and sieved of 100 mesh to obtained glucomannan flour and then stored in a sealed jar.

Preparation of glucomannan hydrolyzate made with reference to the method of Nurjanah (2010) are modified. Iles-iles flour mixed with distilled water with a ratio of 1:30 g / ml then heated for 2 hours on a hot plate at a temperature of 75 ^{o}C until gelatinized. Then do the process of hydrolysis with α-amylase enzyme bb 0.15% while stirring at a speed of 560 rpm at 75 ^{o}C for 2 hours. Then left overnight and form two phases, filtrat taken and the sediment was removed. The filtrate is subsequently extracted using 95% alcohol in the ratio (1: 2) for 5 minutes and filtered and then dried in an oven at a temperature of 45 ^{o}C for 48 hours, then the hydrolyzate glucomannan obtained wrapped in aluminum foil and stored in a glass bottle which contains silica gel.

Studies of glucomannan and glucomannan hydrolyzate as a carbon source

Prepared materials to make MRS broth medium, the carbon source was added, namely MRS broth with 1% glucose as carbon source (control), MRS broth with 1% glucomannas iles-iles asc carbon source and MRS broth with 1% hydrolyzate glucomannan as carbon source. Each subsequent media were sterilized at 121 ° C for 15 minutes. Then inoculated with 1 ose *L. casei* FNCC 0090 (which obtained from UGM) and incubated for 18 hours at a temperature of 35 °C. After incubation calculating the number of bacteria *L. casei* (with plate count method), pH media with a pH meter and total volatile fatty acids (acetic acid, propionate acid and butyric acid) with Chromatography Gas.

Statistical analysis

The randomized complete blok design used as experimental design. The treatment was repeated 3 times replications as blocks. Data were analyzed diversity, then if there is a significant difference continued with Duncan test level 5%

Results and Discussion

The amount of L. casei

The amount of *L. casei* that growth in the MRS broth with various carbon source can be seen in Table 1.

Table 1. the amount of lactic acid bacteria L. casei (CFU / ml)

Media	Amount of cell (CFU/ml)
MRS + Carbon source glucose	$4{,}13 \times 10^6$
MRS + Carbon source glucomannan	$1{,}18 \times 10^7$
MRS + Carbon source glucomannan hydrolisates	$1{,}19 \times 10^7$

Table 1 shows that the carbon source utilization of glucomannan and glucomannan hydrolyzates on MRS broth media is able to provide growth of *L. casei* higher about 1 log compared to control (glucose carbon source). Maybe it is because *L. casei* capable of using glucomannan as a carbon source for cell growth

pH Medium

pH is one indicator of the formation of lactic acid. The average of pH medium in MRS broth with various carbon source can be seen in Table 2.

Table 2. The average pH medium

Media	pH medium
MRS + Carbon source glucose	4.55
MRS + Carbon source glucomannan	5.87
MRS + Carbon source glucomannan hydrolisates	6.95

Table 2 shows that the media MRS broth with carbon source of glucomannan and glucomannan hydrolyzate having a pH higher than the growth in MRS media with glucose carbon source. Maybe It was caused by the growth of existing BAL more slowly so the acids produced less so the higher pH although not statistically significantly different.

Analysis of volatile fatty acids (acetic acid, propionate acid and butyric acid)

The amount of acetic acid, proponat acid and butyric acid produced by *L. casei* during growth on MRS media with various carbon source can be seen in Figure 1.

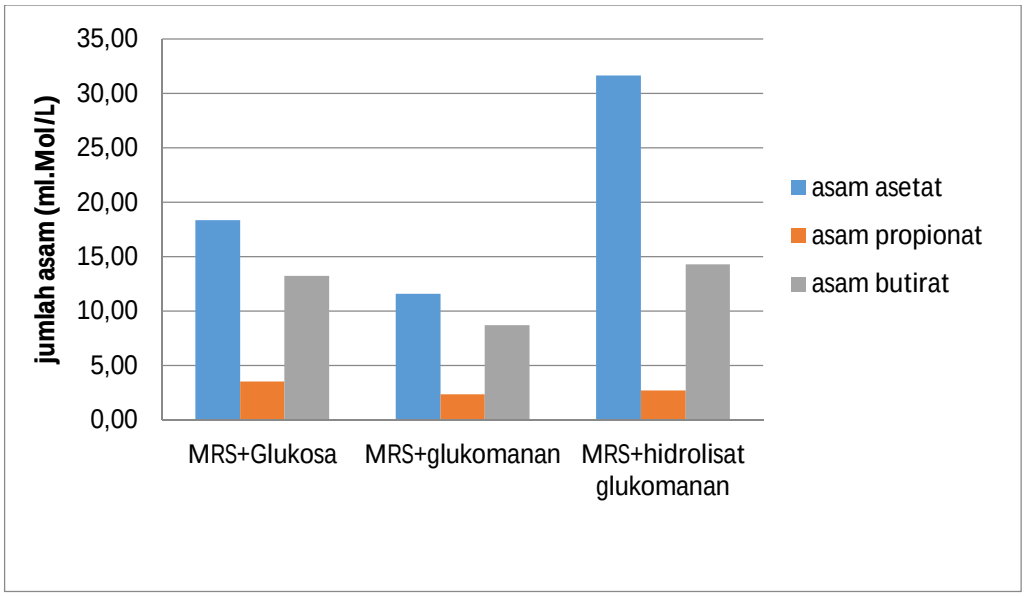


Figure 1. Number of acetic acid, propionate acid and butyric acid during the growth of L. casei on MRS media with different carbon sources

In Figure 1 shows that the type of volatile fatty acid produced by *L. casei* dominant during the growth of acetic acid followed by butyric acid and propionate. *L. casei* ditumbuhan in MRS medium with glucomannan hydrolysates carbon source capable of generating volatile fatty acids that most high though not significan, maybe it cause the number of *L. casei* produced the most.

Conclusions

The use of carbon sources of glucomannan and glucomannan hydrolyzates on MRS broth medium is able to provide growth of L. casei higher about 1 log compared to control (glucose carbon source). The addition of glucomannan hydrolyzate as a carbon source in a MRS broth medium is able to produce the amount of acetic acid, butyric acid and propionate acid higher than control or glucomannam

Acknowledgements

The author would like to thank the Dirjen Dikti that has provided research funding through a competitive grant (Hibah Bersaing) in 2015-2016.

References

Ashadi, R.W., dan Mardiah, 2006. *Utilizion of Amorphophallus Oncophyllus For Decreasing Blood Sugar On Hyperglycaemic Rat.* Faculty of Agribussines and Food Technology. Djuanda University. Bogor

Gibson and Roberfroid, 1995. Dietary modulation of the human colonic microbiota introducing the concept of prebiotic,. Journal of nutrition, 125:1401-1412

Grajek W., Anna Olejnik and Anna Sip, 2005.Probiotic, Prebiotics and antioxidants as funtional foods. Acta Biochimia Polonica, Vol. 52 No. 3: 665-671
Hermansyah 1985. Berbagai Macam Penggunaan Temulawak dalam Makanan dan Minuman. Simposium Nasional temulawak UNPAD Bandung

Johnson, 2007. Konjac-An Introduction. http://www.konjac. info. Di akses 24 April 2012

Nurjanah, S., 2010. *Kajian Proses Pemurnian Tepung Glukomanan dari Umbi Iles-Iles (Amorphophallus oncophyllus) dengan Menggunakan Enzim α-Amilase* [skripsi]. Bogor: Fakultas Teknologi Pertanian Institut Pertanian Bogor.

Safii (1981). Percobaan Pembuatan Tepung Mannan dari Umbi Iles-Iles (Amorphophallus variabilis BI) [skripsi]. Bogor: Fakultas Teknologi Pertanian Institut Pertanian Bogor

Sander, ME. 2008. Probiotics: Definition, Sources, Selection, and Uses. Clinical Infectious Journal, Volume 46 issue supplemen 2, pp558-561

Silalahi dan Netty, 2003. Manfaat dan khasiat probiotik untuk mencegah Penyakit. www.kompas. com/kcm/produk.htm. Diakses 12 Feberuari 2012, Yogyakarta.

Tuohy, K.M., G.C.M. Rouzaud, W.M. Bruck and G.R. Gibson. 2005. Modulation of the human gut microflora towards improved health using prebiotics-assesment of efficacy. Current Pharmaceutical Design, 2005, 11

Tjokroadikoesoemo, 1986. Tjokroadikoesoemo P.Soebijanto, HFS dan Industri Ubi Kayu Lainnya, PT Gramedia, Jakarta

Vuksan V, Jenkins DJ, Spadafora P, Sievenpiper JL, Owen R, Vidgen E,Brighenti F, Josse R, Leiter LA, Bruce-Thompson C. 1999. Konjac-mannan (glucomannan) improves glycemia and other associated risk factors for coronary heart disease in type 2 diabetes. A randomized controlled metabolic trial. Diabetes Care. 1999 Jun;22(6):913-9.

Lactic Acid Bacteria Isolated From Fermented Foods Made of Indonesian Local Sources: Potential and Application

Amelia Juwana [1], Cynthia Christine [2], Tesyara Danesh [2] Cindy Kusuma [2], Lindayani [3], Laksmi Hartayanie [3]

[1]Student of Master Program on Food Technology, Faculty of Agricultural Technology, Soegijapranata Catholic University
[2] Graduate Student of Food Technology, Faculty of Agricultural Technology, Soegijapranata Catholic University
[3] Lecturer of Master Program on Food Technology, Faculty of Agricultural Technology, Soegijapranata Catholic University

e-mail: amelia.juwana@gmail.com

ABSTRACT

Lactic acid bacteria (LAB) still become the most beneficial microorganisms. Besides promoting health for human due to their probiotic potential, metabolites of LAB are potentially used for natural food preservatives. Indonesian local sources have been found to be potential habitats of LAB. The aim of this study is to evaluate probiotic potential of lactic acid bacteria (LAB) which screened from *mandai* (fermented *cempedak* (*Artocarpus champeden* Spreng) *dami*), fermented *sawi pahit* (*Brassica juncea* (L.)), *betung* bamboo shoot (*Dendrocalamus asper*) pickle, fermented cacao (*Theobroma cacao* L.) seed and viability of LAB isolates in food products during storage. The results showed selected isolates isolated from all fermented foods were proven to have probiotic potential (acid tolerance test (pH 3 & 7), bile salt tolerance test (0.3% & 0.5%), and antimicrobial test against indicator pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella typhimurium*)). The selected isolates were identified as *Lactobacillus sp.* and *Lactobacillus plantarum*. Isolates from fermented *sawi pahit* (*Brassica juncea* (L.)), *betung* bamboo shoot (*Dendrocalamus asper*) pickle, fermented cacao (*Theobroma cacao* L.) seed were applied subsequently to food products (pineapple juice, minced beef, and sweet potato concentrate) to test the viability (quantitative) during cool storage (4° C) for 4 days. Based on viability test results, all applied isolates were able to grow during storage (day 1 until 4). The ability of LAB to grow described the resistance of LAB when applied in food products and thus indicating potential use as natural preservatives.

KeyWords: Lactic Acid Bacteria, Indonesian local sources, probiotic, viability

Introduction

To date, lactic acid bacteria (LAB) still become the most beneficial microorganisms due to their probiotic potential. Some LAB as probiotics have proven the numerous health promoting effects by clinical evidence (FAO/WHO, 2010 in Ogueke *et al.*, 2010). Beside potential of probiotics, LAB also have inhibitory activities such as antimicrobial production which able to inhibit the growth of spoilage microorganisms and pathogenic bacteria, thus can maintain the hygienic and quality of the products or host health (Kormin, *et al.*, 2001). Furthermore, utilization of LAB in food processing have been proven to prolong the shelf life of the products. Therefore, LAB are potential to be used as preservatives (Nagalakshmi *et al.*, 2013).

LAB have complex nutritional requirements because of their limited biosynthetic capabilities. Essential components (*i.e.* carbohydrates, amino acids, peptides, fatty acid esters and vitamins) must be obtained by LAB from their habitats. Indonesia has plenty local sources that contain protein and sugar units, which, therefore should be suitable environments for LAB. Many fermented products have been made from Indonesian local sources. Indonesian fermented foods such as *tempoyak* (fermented durian), *tape ketan* (fermented glutinous rice), *sayur asin* (fermented *sawi pahit* (mustard leaf)), *mandai* (fermented *cempedak dami*) and bamboo shoot pickle have been consumed for centuries. However, there are little investigations have been conducted to assess the diversity of LAB in Indonesian fermented foods (Mustopa & Fatimah, 2014). This study has been conducted to evaluate probiotic potential of LAB which already screened from *mandai* (fermented *cempedak* (*Artocarpus champeden* Spreng) *dami*), fermented *sawi pahit* (*Brassica juncea* (L.)), *betung* bamboo shoot (*Dendrocalamus asper*) pickle, fermented cacao (*Theobroma cacao* L.) seed and viability of LAB isolates in food products during storage.

Materials and Method

Equipment

Equipments used in this study *i.e.* glass jars, reaction tubes, petridish, dilution flask, Erlenmeyer, bekker glass, micropipette, bluetips, yellowtips, microtubes, ose, hot plate, vortex, Memmert incubator, Binder incubator, Hirayama autoclave, All America autoclave, volume pipette, McFarland tubes, 0.45 μm (milipore) filter, Sartorius vacuum filter, centrifuge, autoclavable centrifuge tubes, digital scale, pH meter, Olympus trinocular microscope, UV-spectrofotometer, API 50 CHL medium.

Materials

Materials and chemicals used *i.e. mandai cempedak* (*Artocarpus champeden* Spreng.), *betung* bamboo shoot (*Dendrocalamus asper*) pickle, *sayur asin* (from *sawi pahit* (*Brassica juncea* (L.)), fermented *Lindak* cocoa seed (*Theobroma cacao* L.), salt, water, saline water, *Lactobacillus* broth acc. to De Man, Rogosa and Sharpe (MRS Broth), *Lactobacillus* agar acc. to De Man, Rogosa and Sharpe (MRS Agar), aquadestillata, sodium hydroxide (NaOH), hydrogen chloride (HCl), nutrient agar (NA), nutrient broth (NB), bile salt (Fluka, Sigma-Aldrich), pathogenic indicator bacteria (*Staphylococcus aureus* FNCC 0047, *Escherichia coli* FNCC 0091, *Listeria monocytogenes* FNCC 0156, *Salmonella typhimurium* FNCC 0050) purchased from *Pusat Antar Universitas*, Gadjah Mada University, Yogyakarta), pineapple juice, minced beef, purple sweet potato concentrate.

Method

Source of Lactic Acid Bacteria Cultures

Aside from *mandai*, the LAB cultures from *betung* bamboo shoot pickle, *sayur asin* and fermented *Lindak* cocoa seeds were obtained from previous research. Detections of antimicrobial activity that have been conducted in previous research were used to select five isolates from each fermented food in this study. After verifications (re-screening) of all isolates were done, screenings probiotic potential were conducted subsequently. Detections of antimicrobial activity were repeated for five selected isolates from each fermented food.

Maintenance of Cultures

Lactic acid bacteria (LAB) cultures which isolated from *mandai cempedak* (*Artocarpus champeden* Spreng.), *betung* bamboo shoot (*Dendrocalamus asper*) pickle, *sayur asin* (from *sawi pahit* (*Brassica juncea* (L.)), fermented *Lindak* cocoa seed (*Theobroma cacao* L.) were identified as *Lactobacillus* genus (based on Bergey's Manual of Systematic Bacteriology). All cultures were preserved in MRS broth added with 15% (vv^{-1}) glycerol and stored at −20°C prior to use (Nanasombat *et al*., 2012; Tamang *et al*., 2007). The working cultures were prepared by transferring 0.5-1 ml of the frozen stock culture to 9-10 ml of MRS broth and incubated for 16-48 hours at 30-37º C (Ishola & Tayo, 2012; Santoso, 2008). LAB cultures which have been identified to genus were further screened to probiotic potential followed by antimicrobial detection.

Screening Probiotic Properties

Acid Tolerance

Overnight cultures were inoculated into MRS broth medium that has pH of 7 and 3. Adjusting pH of MRS using HCl 1 M or NaOH 0.5 M. Bacterial growth were observed with enumerated viable colony at the 0, 1.5, and 3 hours with pour plate method. Incubations were done at 37º C for 48 hours (Asraf *et al*., 2009 modified and Yavuzdurmaz, 2007 modified).

Bile Salt Tolerance

Overnight cultures were inoculated into MRS broth medium containing bile salt concentration of 0.3%. Bacterial growth were observed with enumerated viable colony at the 0, 2 and 4 hours with pour plate method. Incubations were done at 37º C for 48 hours (Asraf *et al*., 2009 modified and Yavuzdurmaz, 2007 modified).

Detection of Antimicrobial Activity by Agar Well Diffusion Method

Preparation of Culture

Overnight cultures which already inoculated into MRS broth medium were centrifuged at 6000 rpm for 10 minutes to separate the supernatant and cells. The culture cells (pellets) were added into 0.85% NaCl. The solution then adjusted with McFarland tube number 5 (Iñiguez-Palomares *et al*., 2007). One ml solution which contain each culture were added into 100 ml MRS broth media then incubated for 24 hours at 37º C to obtain culture suspension (Khunajakr, 2008 modified). Cell-free supernatants were obtained by centrifugation (6000 rpm for 10 minutes) of culture suspensions (Kormin *et al*., 2001).

Detection of Antimicrobial Activity

Overnight cultures of pathogenic (purchased from *Pusat Antar Universitas,* Gadjah Mada University, Yogyakarta) including *Staphylococcus aureus* FNCC 0047, *Escherichia coli* FNCC 0091, *Listeria monocytogenes* FNCC 0156, *Salmonella typhimurium* FNCC 0050, grown in NA medium at 37 °C diluted with 0.85% NaCl solution until the turbidity equal with McFarland tube number 3. 10 µl each of pathogenic bacteria then mixed with 10 ml NA. Three holes of 7 mm of diameter at similar distance were punched and filled with 50 µl culture suspension and 20 µl cell-free supernatant. The petri dishes stored at 4 °C for 3 hours to allow the diffusion of culture suspension and cell-free supernatant on the medium. After that, the incubation will be carried out for 24 hours at 37 ºC. The measurement of the clear zone will be performed using a calliper. Positive results then recorded when the zone of inhibition of at least 1 mm around the well (Khunajakr, 2008).

Identification of Lactic Acid Bacteria to Species

Based on antimicrobial detection result, isolate with the widest inhibition spectrum was selected and identified to species. Two methods were used to identify the species of LAB: molecular technique and analytical profile index (API) reading. Molecular identification were conducted using DNA fingerprinting by polymerase chain reaction (PCR), sequencing and phylogenetic construction, while API reading was conducted using API 50 CHL test kit from BioMerieux, France.

Viability Test of Lactic Acid Bacteria on Food Product During Storage

In this study, viability tests were done to selected isolates from *betung* bamboo shoot pickle, *sayur asin* and fermented *Lindak* cocoa seed, whereas isolates from *mandai* were not subjected to viability test.

Preparation of Food for Starter Culture

Making of Purple Sweet Potato Concentrate

Purple sweet potato that have been boiled, peeled and cut, was put into a blender with addition of water (1:3). The juice then separated from the residue, and filtered using cheesecloth. 100 ml concentrate were filled into 5 sterile bottles to be further used as LAB starter culture substrate (Suhartini, 2009 modified; Siregar *et al*., 2014).

Making of Pineapple Juice

Pineapple that has been peeled and cut was put into blender with addition of water (1:1). The juice then separated from the residue by filtering with cheesecloth. The juice filled into sterile bottles to be further used as LAB starter culture substrate.

Preparation of Starter Culture

Lactic acid bacteria cultures were inoculated to MRS broth and incubated at 37°C for 4 days prior to use. Substrates (100 ml purple sweet potato concentrate, 500 ml pineapple juice, water) which added with 4% skim milk and 5% sugar were boiled to 90° C and stirred for 5 minutes. The substrates then pasteurized at 95° C for 5 minutes. After the temperature of all substrates reached ± 40° C, 2-3% active LAB cultures were inoculated and incubated at 37°C for 24 hours. The starter cultures then stored in refrigerator. To ensure that the amount of LAB

colonies have reached 10^8 -10^9 CFU/ml in order to meet minimum amount of bacteria for probiotic food or beverage, examination was done after incubation by surface platting at dilution of 10^{-5} -10^{-8}. Total colonies were counted using following equation:

Colony-Forming Unit/ml (CFU/ml) = $\text{number of colony} \times \frac{1}{\text{dilution factor}}$

Preparation of Probiotic Food

Purple Sweet Potato Concentrate Probiotic Drink

Twelve bottles were filled with 100 ml purple sweet potato concentrate added with 2% sugar and 4% skim milk which have been boiled to 90° C and stirred for 5 minutes, then pasteurized at 95° C for 5 minutes. After the temperature of purple sweet potato concentrates reached ± 40° C, following treatments were applied:

a. Negative Control :Purple Sweet Potato Concentrate without LAB and sodium benzoate
b. Positive Control :Purple Sweet Potato Concentrate +0.05% sodium benzoate
c. Purple Sweet Potato Concentrate + 3% starter culture
d. Purple Sweet Potato Concentrate + 3% starter culture + 0.05% sodium benzoate

All purple sweet potato concentrates were incubated at 37° C for 16-24 hours before stored at 4° C (refrigerator) for 4 days.

Pineapple Juice Probiotic Drink

Twelve bottles were filled with 100 ml pineapple juice added with 2% sugar and 4% skim milk which have been boiled to 90° C and stirred for 5 minutes, then pasteurized at 95° C for 5 minutes. After the temperature of pineapple juice reached ± 40° C, following treatments were applied:

a. Negative Control : Pineapple juice without LAB and sodium benzoate
b. Positive Control : Pineapple juice +0.1% sodium benzoate
c. Pineapple juice + 3% starter culture
d. Pineapple juice + 3% starter culture + 0.1% sodium benzoate

All pineapple juice were incubated at 37° C for 16-24 hours before stored at 4° C (refrigerator) for 4 days.

Probiotic Minced Beef

Fresh meats were boiled until done and minced with blender. Each 10 g of minced beef were treated as following:

a. Control : Minced beef without LAB and nitrite
b. Minced beef + 2% starter culture
c. Minced beef + 2% starter culture + 2% nitrite

Addition of 2% nitrite was done before boiling the meat. After addition with nitrite, the meats were stored in refrigerator for 24 hours. After that, meats were boiled at 80° C for 40 minutes. Addition with 2% starter culture was done after the meats were cooler. The minced beef then incubated at 37° C for 24 hours before stored at 4° C (refrigerator) for 4 days (Erkkila, 2001 and Arief, 2000 modified).

Viability Test of Lactic Acid Bacteria During Storage

Viability test of LAB during storage at 4° C storage was done from day 1 until day 4. Surface platting method was used for testing at 10^{-5}-10^{-8} (for purple sweet potato concentrate and pineapple juice) and 10^{-4}-10^{-7} (for minced beef) dilution. The observation was conducted

qualitatively, with positive (+) and negative (–) results. Positive result indicated that LAB were able to grow (resistant) in probiotic food while negative result indicated that LAB were not able to grow (not resistant) in probiotic food (Wireko-Manu *et al*., 2010 and Supavititpatana *et al.,* 2009 modified).

Result and Discussion

Screening Probiotic Potential

Acid Tolerance

Table 1. The Ability of Lactic Acid Bacteria to Survive at pH 3 and 7

Source	Isolate Code	Acid Tolerance					
		pH 3			pH 7		
		0h	1.5h	3h	0h	1.5h	3h
Mandai	A2	+	+	+	+	+	+
	A14	+	+	+	+	+	+
	B30	+	+	+	+	+	+
	E52	+	+	+	+	+	+
	F71	+	+	+	+	+	+
Sayur Asin	43J	+	+	+	+	+	+
	22J	+	+	+	+	+	+
	52J	+	+	+	+	+	+
	42J	+	+	+	+	+	+
	71J	+	+	+	+	+	+
Betung Bamboo Shoot Pickle	1.1.S	+	+	+	+	+	+
	2.1.S	+	+	+	+	+	+
	4.1.S	+	+	+	+	+	+
	5.1.S	+	+	+	+	+	+
	6.2.S	+	+	+	+	+	+
Fermented *Lindak* Cocoa Seed	23	+	+	+	+	+	+
	32	+	+	+	+	+	+
	33	+	+	+	+	+	+
	35	+	+	+	+	+	+
	47	+	+	+	+	+	+

Key :

- "+"= bacteria grow
- "–" = bacteria not grow

Based on result, all LAB isolates were able to survive at low pH (pH 3) for 3 hours. According to Casiano-Colon & Marquis (1998), lactic acid bacteria are able to survive from acidic damage due to the presence of histidine decarboxylase and arginine deiminase enzyme. LAB have higher tolerance in acidic condition is attributed to the ability to maintain constant gradient between extracellular and cytoplasmic (have internal pH regulatory mechanism) (Hutkins & Nannen, 1993). Cotter & Hill (2003) added that gram positive bacteria use F_0F_1-ATPase mechanism *via* proton expulsion and amino acid decarboxylase for protection against acidic conditions.

Bile Salt Tolerance

Table 2. The Ability of Lactic Acid Bacteria to Survive at 0.3% Bile Salt

Source	Isolate Code	Bile Salt Tolerance 0.3%		
		0h	2h	4h
Mandai	A2	+	+	+
	A14	+	+	+
	B30	+	+	+
	E52	+	+	+
	F71	+	+	+
Sayur Asin	43J	+	+	+
	22J	+	+	+
	52J	+	+	+
	42J	+	+	+
	71J	+	+	+
Betung Bamboo Shoot Pickle	1.1.S	+	+	+
	2.1.S	+	+	+
	4.1.S	+	+	+
	5.1.S	+	+	+
	6.2.S	+	+	+
Fermented *Lindak* Cocoa Seed	23	+	+	+
	32	+	+	+
	33	+	+	+
	35	+	+	+
	47	+	+	+

Key :

- "+"= bacteria grow
- "–" = bacteria not grow

Based on result, all LAB isolates were able to survive at 0.3% bile salt for 4 hours. According to Smet *et al.* (1995), some *Lactobacillus* have bile-salt hydrolases (BSHs) which has been proposed to confer protection in bile salt environment through bile salt deconjugation. BSHs changes the physical and chemical characteristic of bile salt into non-toxic substance which not harmful for LAB. However, the ability to survive in bille condtion was different for every isolate. Ngatirah *et al*. (2000) explained that isolates which obtained from the same source have different survival ability to bile salt or it can be certain that survival ability to bile salt is strain dependent.

Detection of Antimicrobial Activity of Lactic Acid Bacteria

Table 3. Spectrum of Inhibition of Lactic Acid Bacteria

Source	Isolate	Inhibition Zone (ZOI) (mm)			
		Escherichia coli	*Staphylococcus aureus*	*Listeria monocytogenes*	*Salmonella typhimurium*
Mandai	A2	12.03	N/D	13.37	2.73
	A14	8.87	N/D	9.40	5.03
	B30	9.70	N/D	10.43	9.00
	E52	7.37	N/D	10.33	6.03
	F71	10.73	N/D	13.73	6.40
Sayur Asin	43J	6.2-9.2	10.8-11.6	N/D	N/D
	22J	10.0-10.9	7.7-14.7	N/D	N/D
	52J	9.2-10.7	4.8-10.4	N/D	N/D
	42J	6.2-8.7	9.9-13.4	N/D	N/D
	71J	4.6-6.9	9.8-14.5	N/D	N/D
Betung Bamboo Shoot Pickle	1.1.S	10.6-11.8	11.6-12.9	N/D	N/D
	2.1.S	9.5-10.3	10.9-12.7	N/D	N/D
	4.1.S	7.7-8.8	8.5-14.7	N/D	N/D
	5.1.S	9.2-12.5	11.0-11.8	N/D	N/D
	6.2.S	8.4-11.9	10.8-14.0	N/D	N/D
Fermented *Lindak* Cocoa Seed	23	8.60-8.80	9.75-11.60	N/D	N/D
	32	7.60-12.20	7.50-13.00	N/D	N/D
	33	6.70-12.25	11.00-16.00	N/D	N/D
	35	8.60-11.35	6.00-6.50	N/D	N/D
	47	9.90-12.90	7.00-10.40	N/D	N/D

Key :

- N/D = No Data (no antimicrobial test against specific pathogenic bacteria)

From the result of antimicrobial detection, it was found that in general, inhibition zones of *Lactoobacillus* isolates to *Listeria monocytogenes* and *Staphylococcus aureus* were bigger than to *Escherichia coli* and *Salmonella typhimurium*. According to Naufalin (2013), inhibition zone formations vary between pathogens and are related to outer membrane composition. The outer membrane of Gram-positive *Staphylococcus aureus* and *Listeria monocytogenes* consist of 90% peptidoglycan and thin layers of teichoic acid and teichuronic acid. The outer membrane of Gram-negative *Escherichia coli* and *Salmonella typhimurium* mostly contains (>90%) glycolipid lipopolysaccharides (LPS) on its surface with 5-20% peptidoglycan. LPS is generally thought to be a protective wall with two potential barriers, one is hydrophilic (provided by densely packed oligosaccharide core), and the other is hydrophobic hydrocarbon chain region, which contains six fatty acidsper molecule (Papo & Shai, 2005). According to Rattanachaikunsopon & Phumkhachorn (2010), LPS weaken the activity of antimicrobial produced by LAB to Gram-negative compared to Gram-positive. In accordance with Lebeer *et al*. (2008), lactic acid can be considered to be a key of antimicrobial compound produced by *Lactobacillus*. Besides exerting its activity through lowering the pH and through its undissociated form, lactic acid is also known as permeabilizer of Gram-negative bacterial outer membrane, allowing other compound to act synergistically with lactic acid.

Identification of Lactic Acid Bacteria

Based on antimicrobial detection result, one isolate from each fermented foods (*sayur asin*, *betung* bamboo shoot pickle, and fermented *Lindak* cocoa seed) which has the widest spectrum of inhibition was selected. Isolate 22J was selected from *sayur asin*, isolate 33 was selected from fermented *Lindak* cocoa seed, isolate 6.2.S was selected from *betung* bamboo shoot pickle. Based on API reading, isolate 22J was identified as *Lactobacillus plantarum* 1 (sig. 96.6%), isolate 33 was identified as *Lactobacillus plantarum* (sig. 99.9%), isolate 6.2.S was identified as *Lactobacillus plantarum* (sig. 97.7%). Identification of selected isolates from *mandai* which using molecular technique (DNA fingerprinting) is still being conducted, and there are no specific species have been reported.

Viability of Lactic Acid Bacteria During Storage

Table 4. Viability of Lactic Acid Bacteria During Storage at Refrigeration Temperature

Source	Product	Isolate	Treatment	Storage at 4° C (Day)			
				1	2	3	4
Sayur Asin	PSPC	43J	PSPC + 43J	+	+	+	+
		22J	PSPC + 22J	+	+	+	+
		52J	PSPC + 52J	+	+	+	+
		42J	PSPC + 42J	+	+	+	+
		71J	PSPC + 71J	+	+	+	+
		43J	PSPC + Sodium Benzoate + 43J	+	+	+	+
		22J	PSPC + Sodium Benzoate + 43J	+	+	+	+
		52J	PSPC + Sodium Benzoate + 52J	+	+	+	+
		42J	PSPC + Sodium Benzoate + 42J	+	+	+	+
		71J	PSPC + Sodium Benzoate + 71J	+	+	+	+
Betung	MB	1.1.S	MB + 1.1.S	+	+	+	+
Bamboo		2.1.S	MB + 2.1.S	+	+	+	+

Shoot Pickle		4.1.S	MB + 4.1.S	+	+	+	+
		5.1.S	MB + 5.1.S	+	+	+	+
		6.2.S	MB + 6.2.S	+	+	+	+
		1.1.S	MB + Nitrite + 1.1.S	+	+	+	+
		2.1.S	MB + Nitrite + 2.1.S	+	+	+	+
		4.1.S	MB + Nitrite + 4.1.S	+	+	+	+
		5.1.S	MB + Nitrite + 5.1.S	+	+	+	+
		6.2.S	MB + Nitrite + 6.2.S	+	+	+	+
Fermented *Lindak* Cocoa Seed	PJ	23	PJ + 23	+	+	+	+
		32	PJ + 32	+	+	+	+
		33	PJ + 33	+	+	+	+
		35	PJ + 35	+	+	+	+
		47	PJ + 47	+	+	+	+
		23	PJ + Sodium Benzoate + 23	+	+	+	+
		32	PJ + Sodium Benzoate + 32	+	+	+	+
		33	PJ + Sodium Benzoate + 33	+	+	+	+
		35	PJ + Sodium Benzoate + 35	+	+	+	+
		47	PJ + Sodium Benzoate + 47	+	+	+	+

Key :

- PSPC : Purple Sweet Potato Concentrate
- MB : Minced Beef
- PJ : Pineapple Juice
- \+ : Bacteria able to grow (resistant)

Based on result, from day 1 to day 4, all isolates (both in PSPC and PJ) were able to grow under refrigeration temperature (4°C) which also means resistant at 4°C for 4 days. Suhartini (2009) explained that during storage at refrigerator, probiotic drinks have shelf life up to approximately 1 month. From the result, PSPC and PJ with addition of sodium benzoate as food preservatives resulting LAB in greater amount qualitatively compared to PSPC and PJ without addition of sodium benzoate. The presence of sodium benzoate as preservatives gives no antagonistic effect to the growth of LAB, instead, increasing the resistance of LAB in PSPC and PJ. This might due to the ability of sodium benzoate to inhibit yeast and mold as well as spoilage bacteria *i.e. Streptococcus* and *Pseudomonas,* thus exert a selective effect on the microorganisms present on PSPC and PJ (Nebedum & Obiakor, 2007; Frazier & Westhoff, 1988). Contrary, addition of preservatives (nitrite) in MB decreasing the viability of LAB qualitatively during 4 days storage compared to MB without nitrite addition. This probably because some LAB are not resistant against nitrite and die.

Conclusion

- All genus *Lactobacillus* which isolated from *mandai*, *betung* bamboo shoot pickle, *sayur asin*, and fermented *Lindak* cocoa seed have probiotic potential which demonstrated by ability to survive in stress condition (acid and bile), and ability to

produce antimicrobial substance that can inhibit the growth of pathogenic bacteria *in vitro*.

- Isolate 22J (from *sayur asin*) was identified as *Lactobacillus plantarum* 1 (sig. 96.6%), isolate 33 (from fermented *Lindak* cocoa seed) was identified as *Lactobacillus plantarum* (sig. 99.9%), isolate 6.2.S (from *betung* bamboo shoot pickle) was identified as *Lactobacillus plantarum* (sig. 97.7%).
- All probiotic LAB in purple sweet potato concentrate and pineapple juice were able to grow under 4°C storage for 4 days with or without addition of sodium benzoate.
- All probiotic LAB in minced beef were decreased in number during storage at 4°C storage for 4 days with nitrite addition.

Refferences

Arief, I.I. **(**2000). *Pengaruh Aplikasi Kultur Kering dengan Beberapa Kombinasi Mikroba Terhadap Kualitas Fisiko-kimia dan Mikrobiologi Sosis fermentasi*. Program Pasca Sarjana IPB, Bogor. (Thesis).

Ashraf, M., Arshad, M., M. Siddique, and G. Muhammad. (2009). In Vitro Screening of Locally Isolated Lactobacillus Species for Probiotic Properties. *Pakistan Veterinary Journal* 29(4): 186-190.

Casiano-Colón A, and R.E. Marquis. (1998). Role of The Arginine Deiminase System In Protecting Oral Bacteria and An Enzymatic Basis for Acid Tolerance. *Journal of Applied and Environmental Microbiology*. 54(6): 1318-1324.

Cotter, P. D., and C. Hill. (2003). Surviving The Acid Test: Responses of Gram-Positive Bacteria to Low pH. *Microbiology and Molecular Biology Reiews* 67: 429-453.

Erkkila, S. (2001). *Bioprotective and Probiotic Meat Starter Cultures for The Fermentation of Dry Sausages.* Department of Food Technology. University of Helsinki. (Disertation).

Frazier, W. C. and D. C. Westhoff. (1988). *Food Microbiology, Fourth Edition*. MCGraw-Hill Book Co. Singapore.

Hutkins, R.W. and Nannen N.L. (1993). pH Homeostatis in Lactic Acid Bacteria. *Journal of Dairy Science* 76: 2354-2365.

Iñiguez-Palomares C., Pérez-Morales R., Acedo-Félix E. (2007). Evaluation of Probiotic Properties in Lactobacillus Isolated from Small Intestine of Piglets. *Revista Latino Americana de Microbiologia* 49(3-4):46-54.

Ishola, R.O.and B.C.A-Tayo. (2012). Screening of Lactic Acid Bacteria Isolated from Fermented Food for Bio-molecules Production. *Assumption University Journal of Technnology* 15(4):205-217.

Khunajakr, N., A.Wongwicharn, D. Moonmangmee, S. Tantipaiboonvut. (2008). Screening and Identification of Lactic Acid Bacteria Producing Antimicrobial Compounds From Pig Gastrointestinal Tracts. *KMITL Science and Technology Journal* 8(1):8-17.

Kormin, S., G.Rusul, Son Radu, Foo Hooi Ling. (2001).Bacteriocin-Producing Lactic Acid Bacteria Isolated from Traditional Fermented Food. *Malaysian Journal of Medical Sciences* 8(1): 63-68.

Lebeer, S., J. Vanderleyden, S.C.J. De Keersmaecker. (2008). Genes and Molecules of Lactobacilli Supporting Probiotic Action. *Journal of Microbiology and Molecular Biology Reviews* 72(4): 728-764 DOI: 10.1128/MMBR.00017-08.

Mustopa, A.Z. and Fatimah. (2014). Diversity of Lactic Acid Bacteria Isolated from Indonesian Traditional Fermented Food. *Journal of Microbiology Indonesia* 8(2): 48-57.

Nagalakshmi, P.K., R. Sumathi., K. Kanimozhi., and T. Sivakumar. (2013). Isolation of Bacteriocin Nisin Producing *Lactococcus lactis* from Dairy Products. *Journal of Academia and Industrial Research* 1(10): 627-630.

Nanasombat, S., S. Phunpruch, T. Jaichalad. (2012). Screening and Identification of Lactic Acid Bacteria from Raw Seafoods and Thai Fermented Seafood Products for Their Potential Use as Starter Cultures. *Songklanakarin Journal of Science and Technology* 34(3): 255-262.

Naufalin, R. (2013). *Aktivitas Antimikroba Formula Kulit Buah Kecombrang* (*Nicolaia speciosa* Horan) *sebagai Pengawet Alami Pangan.* Seminar Nasional PATPI.

Nebedum, J.O. and T. Obiakor. (2007). The Effects of Different Preservation Methods on the Quality of Nunu, a Locally Fermented Nigerian Dairy Product. *African Journal of Biotechnology* 6(4): 454-458.

Ngatirah, A., Harmayanti E.S. dan T. Utami. (2000). Seleksi Bakteri Asam Laktat sebagai Agensia Probiotik yang Berpotensi Menurunkan Kolesterol. *Prosiding Seminar Nasional Industri Pangan.* PATPI (II): 63-70.

Ogueke, C.C., C.I.Owuamanam, N.C.Ihediohanma, J.O.Iwouno. (2010). Probiotics and Prebiotics: Unfolding Prospect for Better Human Health. *Pakistan Journal of Nutrition* 9(9): 833-843.

Papo, N. and Y. Shai. (2005). A Molecular Mechanism for Lipopolysaccharide Protection of Gram-Negative Bacteria from Antimicrobial Peptides. *The Journal of Biological Chemistry* 280(11): 10378-10387 DOI: 10.1074/jbc.M412865200.

Rattanachaikunsopon, P. and P. Phumkachorn. (2010). Lactic Acid Bacteria: Their Antimicrobial Compounds and Their Uses in Food Production. *Annals of Biological Research* 1(4): 218-228.

Santoso, E. (2008). *Bakteri Asam Laktat (BAL) pada Cumi-Cumi Kering Asin dan Aktivitas Penghambatannya Terhadap Bakteri Patogen dan Bakteri Pembusuk. Jurnal Agroteksos.* 18(1-3): 46-53.

Siregar, W.F., S. Ginting, dan L.N. Limbong. (2014). *Pengaruh Perbandingan Ubi Jalar Ungu dengan Air dan Konsentrasi Starter terhadap Mutu Minuman Probiotik Sari Ubi Jalar Ungu. Jurnal Rekayasa Pangan dan Pertanian* 2(3): 22-29.

Smet, I.D., L. van Hoorde, M.V. Woestyne, H. Christiaens and W. Verstraete. (1995). Significance of Bile Salt Hydrolytic Activities of *Lactobacilli. Journal of Applied Bacteriology* 79: 292-301.

Suhartini. (2009). *Prospek Ubijalar sebagai Bahan Baku Minuman Probiotik*. http://pangan.litbang.pertanian.go.id/files/05-suhartini.pdf. Diakses 3 Februari 2015.

Supavititpatana, P; T.I. Wirjantoro; and P. Raviyan. (2009). Effect of Sodium Caseinate and Whey Protein Isolate Fortification on the Physical Properties and Microstructure of Corn Milk Yoghurt. *CMU Journal National Science* 8(2): 247-263.

Tamang, J.P., S. Dewan, B. Tamang, A. Rai, U. Schillinger, W.H. Holzapfel. (2007). Lactic Acid Bacteria in Hamei and Marcha of North East India. *Indian Journal of Microbiology* 47: 119-125.

Wireko-Manu, F.D., W.O. Ellis, and I. Oduro. (2010). Production of a Non-alcoholic Beverages from Sweet Potato (*Ipomoea batatas* L.). *African Journal of Food Science* 4(4): 180-183.

Yavuzdurmaz, H. (2007). Isolation, Characterization, Determination Of Probiotic Properties Of Lactic Acid Bacteria From Human Milk. Izmir Institute of Technology. (Thesis).

APPENDIX

Appendix 1. Probiotic Pineapple Juice

Pineapple juice with addition of LAB starter culture

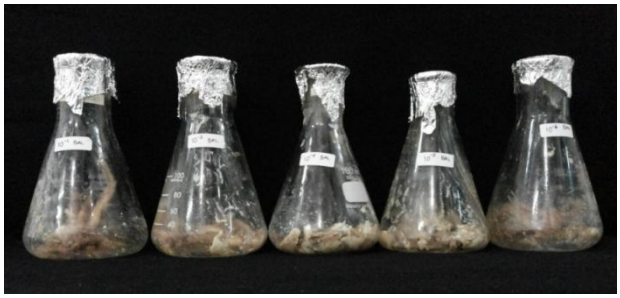
Pineapple juice with addition of LAB starter culture and sodium benzoate

Appendix 2. Probiotic Purple Sweet Potato Concentrate

Purple sweet potato concentrate with addition of LAB starter culture

Purple sweet potato concentrate with addition of LAB starter culture and sodium benzoate

Appendix 3. Probiotic Minced Beef



Minced beef with addition of LAB starter culture

Minced beef with addition of LAB starter culture and nitrite

The antibacterial and Antioxidant Activity of *Zingiber Officinale* Rhizomes (Ginger) in Thailand

Fiorency Santoso[1] and Patchanee Yasurin[2]

[1]Food Technology Department, Faculty of Agricultural Technology, Soegijapranata Catholic University, Semarang, 50234, Indonesia
(Email: fiorency_santoso@yahoo.co.id)
[2]Food Technology Department, Biotechnology Faculty, Assumption University, Bangkok, 10240, Thailand
(E-mail: patchaneeYsr@au.edu)

ABSTRACT

Nowadays, the use of herbs is dramatically increasing over all the world. *Zingiber officinale* (ginger) has been used as a medicinal plant all over Asia. *Z. officinale*, a member of the Zingiberaceae family, is a household spice used in the daily in many Asian countries. This research was aimed to investigate the bioavailability potentials of *Z. officinale* as natural antibacterial and antioxidant agents. The well agar diffusion method was used for evaluating antibacterial activity of crude *Z. officinale* ethanol and hexane extracts against pathogenic bacteria; *Bacillus cereus* and *Salmonella enterica* Typhimurium U302 (DT104b). The antibacterial activity results showed that the concentration of extracts did not showed significant different in both solvent. The highest antibacterial activity was crude Z. *officinale* ethanol extracts against *B. cereus* (2.26 ±0.715 cm.). The solvent showed the significant different effect on the antibacterial activity on both bacteria. Both crude *Z. officinale* ethanol and hexane extracts showed antibacterial activity against *B. cereus* better than *S. enterica* Typhimurium U302 (DT104b). This indicated that the *Z. officinale* crude extracts affected on gram positive bacteria better than gram negative bacteria. The modified Folin–Ciocalteu method was used for evaluating antioxidant activity. The results showed that crude *Z. officinale* hexane extracts showed 2 times higher antioxidant activity than crude *Z. officinale* ethanol extracts. So crude *Z. officinale* extracts shows the promising as natural antibacterial and antioxidant agents.

Keywords: *Zingiber officinale*, Antibacterial, Antioxidant

Introduction

Foodborne disease is a pervasive problem caused by consumption of contaminated food and drink. Foodborne diseases caused by pathogenic bacteria are still a major threat to public health. To prevent foodborne illness, it is a necessity to use the chemical preservatives to prevent the growth of food spoiling microbes in the food industry. Furthermore, many food industries used preservatives (synthetic antioxidant) in food processing to lengthen product's shelf life and to inhibit off-flavor. Nowadays, many people concerned about the safety of food containing preservative as synthetic chemicals, therefore, there is a growing interest to use natural antibacterial and antioxidant compounds. Plants have secondary metabolites that can be responsible for the natural resistance, perhaps for this reason a lot of the plants used for the treatment of infections and research to discover compounds as potential antimicrobial properties (Hashim *et al.*, 2010). One of them is *Zingiber officinale* rhizomes (ginger). *Z. officinale*, a member of the Zingiberaceae family, is a household spice used in the daily in many Asian countries. Ginger contains of secondary metabolites, especially the flavonoid, phenol, terpenoids and essential oils. Those secondary metabolites produced can inhibit the growth of pathogenic microorganisms. The phenol compound content in ginger, such as gingerol and shogaol affects the aroma and pungent taste of ginger, yet it is also potentially as an antioxidant (Nursal *et al.*, 2006). Instead of that, ginger usually used as herbal medicine for headache, cold, treat rheumatism, cholera, diphtheria, and can be used to increase appetite. Due to the benefits of *Z. officinale*, this research had been done to evaluate the effects of solvent extractions of *Z. officinale* on antibacterial activity against some foodborne pathogens, and antioxidant activity.

Materials and method

Preparation of Sample and Crude Extracts

Z. officinale rhizomes (ginger) were obtained from local fresh market in Bangkok, Thailand. For making dry crude extract, herb was peeled and cut into small pieces. The 1:5 wv^{-1} of solvent (95% ETOH and hexane) was added to 50 g of the cut ginger and soaked for 2 days at room temperature in a closed bottle. After 2 days, the crude extract was separated with filter paper to collected the liquid part, then was concentrated in rotary evaporator at 45^0C until become very concentrate slurry then dissolved with DMSO to make concentration 1 mg $ml^{-}1$, 10 mg ml^{-1}, 20 mg ml^{-1}, and 30 mg ml^{-1}. Crude extract was kept in freezer at -20^0C until use.

Antibacterial Activity Determination

The modified agar well diffusion method was used in this experiment. The 100µl of bacteria was swabbed on the MHA (Mueller-Hinton Agar) plate. The 50µl of 10mg/ml, 20mg/ml, and 30mg/ml crude *Z. officinale* ethanol and hexane extracts were used to test the antibacterial activity against *Bacillus cereus* and *Salmonella enteric typhimurium U302* (DT104b). The 20µl of 50mg/ml penicillin G were used as positive control for comparison of the antibacterial activity of samples. All plates were incubated at 37^0C for 24 hours. Clear zone result was measured to determine the effectiveness of crude *Z. officinale* against each bacterium .All experiment in the laminar air flow hood under aseptic condition and was performed in duplicate and three replications independently.

Antioxidant activity

The modified Folin-Ciocalteu method was used for total phenolic content determination in crude *Z. officinale* ethanol and hexane extracts. The 20µl of 1mg/ml crude *Z. officinale* ethanol and hexane extracts was added to 1.58 ml distillated water and 100µl Folin-Ciocalteu reagent. The mixture was then allowed to stand for 8 minutes and 30 seconds. After that 300µl saturated sodium carbonate was added to the mixture and the mixture was incubated at room temperature and without light for 30 minutes. Then, the mixture was observed optical density (OD) at 765 nm. Gallic acid were used as the standard curve. The result were expressed as microgram gallic acid equivalent (GAE/ml). The experiment was done in triplicate and three replications independently.

Results and Discussion

Antibacterial Activity

Table 1. Mean and SD of inhibition zone of *Z. officinale* hexane and ethanol extract in 3 different concentration against *Bacillus cereus* and *S.enterica* Thyphimurium U302 (DT104b)

Microorganisms	Concentration (mg/ml)	Inhibition zone (cm)	
		Hexane	Ethanol
Bacillus cereus	10	$1.73 \pm 0.502^{BC,a}$	$2.21 \pm 0.658^{B,bc}$
	20	$1.77 \pm 0.228^{C,ab}$	$1.98 \pm 0.772^{B,abc}$
	30	$1.65 \pm 0.326^{BC,a}$	$2.26 \pm 0.715^{B,c}$
S. enterica Typhimurium U302(DT104b)	10	$1.16 \pm 0.331^{A,ab}$	$1.05 \pm 0.250^{A,a}$
	20	$1.17 \pm 0.354^{A,ab}$	$1.13 \pm 0.305^{A,ab}$
	30	$1.43 \pm 0.325^{AB,b}$	$1.22 \pm 0.537^{A,ab}$

*A = different superscript within a column , compare the microorganisms and the concentrations

*a = different superscript within a row, compare the solvent and the concentration

Same symbol indicate no statistically significant difference ($p>0.05$) and different symbol indicate significant difference ($p<0.05$) among same column.

Two different solvents and three different concentrations were used in extracting antibacterial compounds from *Z. officinale*. From table 1 we can see that there is no significant different concentration of extracts in both solvent, but different microorganisms show significant different in both solvent. Table 1 showed that extraction with hexane 10 mg/ml and ethanol 10 mg/ml resulted significant different against to *Bacillus cereus*, but not significant different against *S. enterica Typhimurium U302*. This is the same results with hexane 30 mg/ml and ethanol 30 mg/ml extraction. Hexane 20 mg/ml and ethanol 20 mg/ml extraction was not significant different against both bacteria.[1]

The highest antibacterial activity was crude Z. *officinale* ethanol extracts against *B. cereus* with inhibition zone of 2.26 ± 0.715 cm. However ethanol extracts against *S. enterica* Typhimurium U302 show the lowest antibacterial activity with inhibition zone of 1.05 ± 0.250 cm. This results show that both crude *Z. officinale* ethanol and hexane extracts showed antibacterial activity against *B. cereus* better than *S. enterica* Typhimurium U302 (DT104b). [1] This indicated that the *Z. officinale* crude extracts affected on gram positive bacteria better than gram negative bacteria. The result also can be seen also in Figure 1. [2]

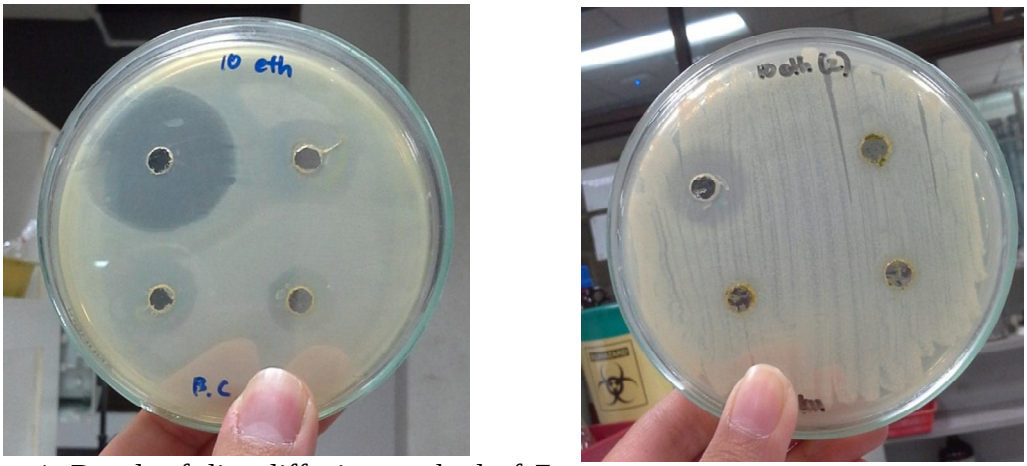


Figure 1. Result of disc diffusion method of *Z. officinale* with extraction solvent of 10mg/ml ethanol against: (a) *B. cereus* and (b) *S. enterica* Typhimurium

This result similar to the work of Sivasothy *et al*., (2011) that essential oil in ginger showed better result in prevent gram positive bacteria (*B. cereus*). Essential oils in ginger are generally more effective against gram positive bacteria compared to gram negative. According to Silhavy (2010), there are three principal layers in the gram negative envelope; the outer membrane, the peptidoglycan cell wall and the cytoplasmic or inner membrane. The outer membrane contains lipopolysaccharide (LPS) and acts as a protective barrier. This organelle is essential because it prevents the entry of compounds that are not needed by cells, including bacteriocins, enzymes and another compound that are hydrophobic (Davidson *et al*., 2005). To prevent the bacteria, antimicrobial compounds should penetrate the lipopolysaccharide (LPS) from the cell wall. Gram positive bacteria do not have the LPS, so there is no barrier function (Ousallah *et al*., 2006). It can be concluded that gram negative bacteria are more resistant than gram positive.

This result also similar to the research by Rosevicka *et al*., (2007) that ginger oleoresin extraction with ethanol can produce more than oleoresin extraction with hexane. Polarity of the solvent effects the extraction. Some compound in ginger can be dispersed in water easily. Water is polar, then the ginger more easily extracted by solvent which are polar as well. Sudarmadji and Haryono (1996) added that chemical compounds will be soluble in the solvent that has the same polarity. As ethanol and oleoresin in ginger has smaller different in polarity so therefore compound in ginger more easily extracted in ethanol and it make the antibacterial activity also higher.

The content of secondary metabolites found in the ginger, especially the flavonoid, phenol, terpenoids and essential oils can inhibit the growth of pathogenic microorganisms. Based on research that being conducted by Nursal *et al*., (2006), it is said that the inhibition of the bacteria caused by the damage in the structural component of the bacterial cell membrane. Terpenoids compounds can bind proteins and lipids in cell membranes so it is caused cell lysis. Damage to the cell membrane makes the transport of nutrients (compounds and ions) through the cell membrane disrupted so that the bacterial cells lack of nutrients and then it will be dead.

Antioxidant activity

Most of life that use oxygen to obtain energy will make the body produces free radicals. Free radical compounds can come from outside the body (exogenous), such as environmental pollution, smoke, UV light, etc, and inside the body (endogenous) from the result of metabolism. Free radical compounds will produce hydroperoxides, and lipid peroxides that have damaging effects. It can damage the structure and function of cells and caused cancer to our body (Winarsi, 2007). To control these compounds, antioxidants are needed. Phenol compounds in plants can help to inhibit lipid oxidation by donating a hydrogen atom to the free radical. Active compounds non-volatile phenols in ginger such as gingerol, shogaol and zingeron, proven ability as an antioxidant. Gingerol and shogaol act as primary antioxidants against lipid radicals. Gingerol and shogaol have antioxidant activity because it contains a benzene ring and a hydroxyl group (Ibrahim *et al*., 2015).

Table 2. Mean and SD of total phenolic content of *Z. officinale* hexane and ethanol extract in 1mg/ml concentration

Solvent	Total phenolic content (microgramGAE/mg)
Hexane	15.75 ± 0.474^{a}
Ethanol	7.18 ± 0.265^{b}

Same symbol indicate no statistically significant difference ($p>0.05$) and different symbol indicate significant difference ($p<0.05$)

Two different solvents were used; hexane and 95% ethanol with the concentration of 1 mg/ml *Z. officinale* extracts. From the Table 2, we can see that both solvent were not significant different in antioxidant activity. Crude *Z. officinale* hexane extracts showed 2 times higher antioxidant activity than crude *Z. officinale* ethanol extracts.[3]

Several other studies have shown that ginger compounds effect on antioxidant activity. Based on research by Ravindran (2005) found that shogaol components and zingiberene shows strong antioxidant activity. Ravindran (2005) also concluded that the antioxidant activity depends on the structure of the side chain and the structure the benzene ring. Furthermore, another study by Tsushida *et al*., (1994), found 12 components on ginger has a higher antioxidant activity than ∝-tocopherol. From those 12 components, gingerol and hexahydrocurcumen are mainly influenced with antioxidant activity. Tsushida *et al*., (1994) also proved that one of the components of ginger phenolic antioxidants, is shogaol has high antioxidant activity. According to Koswara (1995), it is said that bioactive compounds such as gingerol and shogaol are non-polar. The chemical compounds will be soluble in the

solvent that has the same polarity. Hexane is non-polar. So that's why hexane extraction has higher antioxidant activity.

Conclusions

Secondary metabolites in *Zingiber officinale* rhizomes (ginger), such as flavonoid, phenol, terpenoids and essential oils can inhibit the growth of pathogenic microorganisms. The active compounds non-volatile phenols such as gingerol, shogaol and zingeron, proven ability as an antioxidant. Ginger extracts show antibacterial properties against gram positive bacteria better than gram negative bacteria. The best solvent to produce higher antibacterial activity is ethanol. Meanwhile, crude *Z. officinale* hexane extracts showed 2 times higher antioxidant activity than ethanol extracts.

References

Clinical and Laboratory Standards Institute. (2006). *Performance standards for antimicrobial disk susceptibility tests.* Approved standard M2-A9. CLSI, Wayne, PA.

Davidson, P.M., Sofos, J.N., & Brannen, A.L. (2005). *Antimicrobial in Food : 3rd edition.* CRC Press : Taylor and Francis Group. London.

Hashim, H., Kamali, EL., & Mohammed, Y. (2010). "Antibacterial activity and phytochemical screening of ethanolic extract obtained from selected Sudanese medicinal plants", *Journal of Biological Science*, Vol. 2 No. 2, pp.143-146.

Ibrahim, Agus M., Yunianta, & Sriherfyna, F.H. (2015). "Pengaruh Suhu dan Lama Waktu Ekstraksi Terhadap Sifat Kimia dan Fisik Pada Pembuatan Minuman Sari Jahe Merah (*Zingiber officinale var. Rubrum*) Dengan Kombinasi Penambahan Madu Sebagai Pemanis". *Jurnal Pangan dan Agroindustri.* Vol. 3 No. 2, pp. 530-541

Koswara, S. (1995). *Jahe dan Hasil Olahannya*. Pusat Sinar Harapan, Jakarta, Indonesia.

Nursal, S., Wulandari, & Juwita, S.W. (2006). "Bioaktifitas Ekstrak Jahe (*Zingiber officinale Roxb*) dalam Menghambat Pertumbuhan Koloni Bakteri *Escherichia coli* dan *Bacillus subtilis*", *Jurnal Biogenesis*, Vol. 2 No. 2, pp. 64-66.

Oussalah, M., Cailletm, S., Saucier, L., & Lacroix, M. (2006). "Antimicrobial effects of selected plant essential oils on the growth of a *Pseudomonas putida* strain isolated from meat", *Journal of Meat Science*, Vol. 73, pp. 236-244.

Ravindran, P.N. (2005). *Ginger: The genus of Zingiber*. CRC Press. USA.

Rosevicka D.O., Aylianawati, Y., & Sudaryanto. (2007). "Ekstraksi Oleoresin dari Jahe", *Jurnal Widya Teknik*, Vol. 6 No. 2, pp.131-141.

Silhavy, T.J., Daniel, K., & Suzanne, W. (2010). "The Bacterial Cell Envelope", available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2857177/ (accessed March 4th, 2016).

Sivasothy, Y., Chong, W.K., Hamid, A., Eldeen, I.M., Sulaiman, S.F., & Awang, K. (2011). "Essential oils of *Zingiber officinale var. rubrum theilade* and their antibacterial activities", *Journal of Food Chemistry*, Vol. 124, pp.514-517.

Sudarmadji, S., Haryono, B. (1996). *Analisa Bahan Makanan dan Pertanian*. Liberty. Yogyakarta, Indonesia.

Tsushida, T., Suzuki, M., & Kurogi, M. (1994). "Evaluation of antioxidant activity of vegetable extracts and determination of some active compunds". *Journal of Japan Soc. Food Sci Tech*, Vol. 41 No. 9, pp. 611-618.

Winarsi, H. (2007). *Antioksidan Alami dan Radikal Bebas*. Kanisius. Yogyakarta, Indonesia.

FOOD PROCESSING AND ENGINEERING

The effect of dry cell *Haematococcus pluvialis* equivalent to Astaxanthin extracted for color enhancing in goldfish

Suchawadee Wiratthikowit[1*] and Wipa Niranartlumpong[1],

[1]Assumption University, Huamark, Bangkok, Thailand,10240

**Corresponding author. E-mail: pannuun@hotmail.com*

ABSTRACT

This research aim to study the effect of astaxanthin for feeding application in form of dry whole cells. The extraction of astaxanthin from *Haematococcus pluvialis* was also done to evaluate the amount of astaxanthin produce from the studied cultivation method for comparing with other feeding formula. The yield of astaxanthin was 1.76 mg/g dry weight of *Haematococcus pluvialis*. The dry whole cell of *Haematococcus pluvialis* was then mixed with commercial fish feed at varies amount (2.5-7.5 mg/ 1 kg) using commercial feed only and commercial feed with testosterone as control. The change in color of fish was observed the data using Munsell book in RGB color scale. Moreover, the data was collected at the first day of the study and continue checked every week for four weeks. For treatment 1st noticed that during four weeks of feeding the control commercial fish feed, the color of all ten gold fish did not change too much. Following the treatment 2nd showed that color of some fish had been changed which following this it noticed that the assumption of genetic factor affect to ornamental aquatic's color is not true. The feed mixed with hormone could enhance color of gold fish but not well. Next, treatment 3rd, 4th and 5th, feeding with commercial fish feed mixed with dry cell of *Haematococcus pluvialis* 2.5 mg, 5.0 mg and 7.5 mg were performed respectively. According to the results treatment 5th showed the highest change in color in which the fish's color was redder than fish in treatment 4th and 3rd. Furthermore, there was also separately RGB color value into three color zones following the grade of gold fish (due to color of market demand). It's showed that controlled treatment (1st) was represented color from zone B and Y (Brown and Yellow) into zone R (Red) only 10%, while treatment 3rd , 4th and 5th were represented equal to 40%, 80% and 80% respectively on the last week. Finally, the highly amount of dry cell *Haematococcus pluvialis* added had effected to the fish color enhancing. To be able to use astaxanthin effectively, one must consider the relationships between the amounts of astaxanthin used its price, the appropriate level of the red color of fish, and the price of fish that could be sold was selected underlying economic cause.

Keywords: Astaxanthin, *Haematococcus pluvialis,* Whole cell, Munsell book, RGB color scale

Introduction

Nowadays, Astaxanthin has become important in aquaculture feed such as salmonids, crustraceans to increase the flesh color to be red appetizing and increasing price. Astaxanthin is a red pigment occurring naturally in a wide variety of living organisms, including shrimp, crawfish, crabs and lobster, are pigmented red by accumulated Astaxanthin. The coloration of fish is often due to astaxanthin; the pink flesh of a healthy wild salmon is a conspicuous example. In commercial fish and crustacean farms, astaxanthin is commonly added to feeds in order to make up for the lack of a natural dietary source of the pigment. Astaxanthin will be used worldwide in the price of 455 million US Dollar. The 5-10% concentration of astaxanthin can increase price to 2,500-5,000 US Dollar per kg (Johnson E.A. *et. al.,* 1995) Moreover, it can be used as food additive or nourishment for human as carotenoid, source of pro-vitamin A, and resist free radical.(Palozza, P. and Krinsky, N. I., 1992). In the commercial ornamental fish, astaxanthin also plays the important role by adding in feed to benefit in color enhancement and reduce stress that can result in increasing the growth and survival rate. (Jyonouchi, H. *et. al.*, 1994)

In natural, there are many microorganisms which can produce astaxanthin such as bacteria *Mycobacterium lacticola* and *Brevibacterium* can produce 30 μg per grams dry weight (Jyonouchi, H. *et. al.,* 1995a), *Halobacterium salinarium* produce 265 μg per grams dry weight (Jyonouchi, H. *et. al.*, 1995b), yeast *Phaffia rhodozyma* produce 2,700 μg per grams dry weight (Jyonouchi, H. *et. al.*, 1996) and algae *Chlorella fusa, Chlorella zofingiensis* (Jyonouchi, H. *et. al.*, 2000). However, the amount of astaxanthin production from them is quite low when compared with *Haematococcus* sp. especially *Haematococcus pluvialis* which can produce to 55,000 μg per grams dry weight. This microalgae can produce astaxanthin in highest amount and fortunately, the new strain of *Haematococcus* sp. Was isolated from Huay Kha Khaeng Wildlife Sanctuary, Uthaithani Province, identified as *H. lacustris* subsp. *Siamensis*, was able to produce 30 milligram/liter of astaxanthin. (Sudsaichon H., 1998)

Consequently, this research had concentrated on studying the effect of dry cell *Haematococcus pluvialis* microalgae equivalent to Astaxanthin extracted and to observe the color enhancing in gold fish. Finally, it was to compare the color results between gold fish feeding with hormone testosterone and feeding commercial fish feed mixed with dry cell *Haematococcus pluvialis* microalgae in various amounts. The aim of this study were performed to study the effect of of dry cell *Haematococcus pluvialis* microalgae equivalent to Astaxanthin extracted. Besides, to observe and compare the color enhancing of gold fish by the way of gold fish feeding with hormone testosterone and feeding commercial fish feed mixed with dry cell *Haematococcus pluvialis* microalgae in various amounts.

Materials and methods

This study was an experimental research to study the effect of dry cell *Haematococcus pluvialis* microalgae equivalent to Astaxanthin extracted and to observe the color enhancing of gold fish and to compare the color results between gold fish feeding with hormone testosterone and feeding commercial fish feed mixed with extracted astaxanthin in various amounts which *Haematococcus pluvialis* stock culture (from Thailand Institute of Scientific and Technological Research (TISTR), Klong 5, Amphoe Klong Luang Pathumthani province), Hormone testosterone from TP drug laboratory company, Bangkok, Thailand, Gold fish (from Sunday fish market, Bangkok) and Commercial fish feed from CP company

The production of Astaxanthin extraction

The method of cultivate microalgae *Haematococcus* sp. was followed by production of astaxanthin from *Haematococcus* sp. (Sudsaichon H., 1998) Microalgae, *Haematococcus* sp., were taken from Thailand Institute of Scientific and Technological Research (TISTR). One milliliter of sample was cultured into 100 mL prepared MCM medium. Then the sample was incubated at 27°C under fluorescence light for 16 hours and without light for 8 hours in each day. Microalgae were left cultivated until the red color of suspension change to green. Enumeration of these microalgae was performed for another generation by adding microalgae in proportion 1:10 (Cell suspension: media). Study phase change of microalgae from green to red by press stress condition was conducted by; increasing light density from UV fluorescence white light to UV fluorescence violet light, increasing temperature from 27°C into 32°C, and diluting nutrients 1 times. The experiment was done in three parts; first press stress condition to microalgae only one condition change, second combined two factors changed and last press all stress condition together then observed and collected as result.

Astaxanthin was extracted by solvent extraction method. First, centrifuged 10 ml of cell culture at 30,000 rpm for 5 min. then separated cell out. Added 5 ml of 90% acetone into tube which contain filtrated cell. Covered tube with aluminum foil and kept in dark and low temperature place about one night for preventing the deterioration by light and heat. Grinded cell in test tube until cell color was faded. Observed the color of cell precipitate that changed to light orange. Then, filtrated the cell solution by no. 1 Whatman's filter paper. Then dried the extracted solution in desiccators for 4-5 hours, checking that it completely dry and kept in the refrigerator at 5-10°C for further study.

For measuring the amount of astaxanthin yield, extracted filtrate part after filtrated cell solution with petroleum ether by separatory funnel, the upper part is carotenoid or astaxanthin that dissolved in acetone and petroleum ether, the lower part is water. Released the water out and kept the upper solution to analyze amount of astaxanthin. Adjusted the solution into 100 ml by petroleum ether then measured the photo absorption at 470 nm and absorption coefficient A1% equal to 2,500. Then it was calculated with below formula; Total Astaxanthin mg/ml = $\frac{\text{OD 470 x 100(amount of petroleum ether) x } 10^3}{2{,}500 \text{ x 100 x ml of sample}}$

The efficiency of dry cell *Haematococcus pluvialis* microalgae equivalent to Astaxanthin extracted for color enhancing of gold fish

The observation of the color enhancing of gold fish by comparing the color results from five difference treatments. The control treatment (T1) was only commercial fish feed, T2 was commercial fish feed mixed with hormone testosterone and T3, T4 and T5 were commercial fish feed mixed with dry cell *Haematococcus pluvialis* microalgae equivalent to Astaxanthin extracted in various amounts. Then, fed the mixed diets 2 times a day for 30 days, the amount of diet was equal to 5 % of fish's weight in each tank. Finally, observed the color and expression changing of fish. Compared the color with Munsell book then collect the data as RGB color.

Data Analysis

After collected the data from Munsell book, all data were arranged by Munsell value and chroma. Value increases from the bottom of the page to the top in increments of 1, while chroma increases outward from left to right I increments of 2. The standard way to write the description of a color using Munsell notations is to write the alpha-numeric designation for the Munsell hue (H) and the numeric designations for value (V), and chroma (C) in the form of HV/C. The data were compared between each treatment and within treatments themselves. There were five treatments, in treatments, there were ten fishes as repeat RGB color was the qualitative or discriminated data because it was not able to calculate. They showed only the difference and preference of the color. The shade from Munsell book of color was selected only pages of gold fish's color and it was separated into three zones following the grade of gold fish due to color of market demand. Brown zone present as low grade or under standard gold fish (dark brown color). Yellow zone present as medium grade or the standard gold fish (light/fade color). Red zone represent the brighten color of gold fish or high standard gold fish (dark red/orange color)

Result and discussion

Preparing the micro algae named *Haematococcus pluvialis* Inoculation of stock culture

In the result by preliminary study, it showed that the color of *Haematococcus pluvialis* stock culture had changed from red to green within 7 days which indicated that under laboratory condition, selected media and method, this stock culture could grow normally (Nultthawee T., 2000). The total incubation time was 14 days since the organisms turned from red to green and back again to red which showed the success of regeneration of these microorganisms.

Phase Change Observation

Under microscopic observation, the organisms was put under stress such as reduction of food, increase of light intensity and temperature increase from 27 to 32^{o}C, cells number increased, however, shrunk and moved faster. Until reach day 14th, cells turned red and number of cells decreased, however, bigger and disappearance of flagella was found (John K., 2002). When increased light intensity by dividing green algae to expose two different light sources, white lights and purple lights, there was not changing in color of algae. The algae were also incubated at 32°C with light absence for 7 days. All the algae were found as dead phase which indicated light had the strong influence due to lack of possibility for photosynthesis. In the case of variation in nutrition, media was diluted by adding double more water during algae's growth. The results showed that there was not development of red

color in algae. When algae ran out of nutrition, they could not enter seed phase (red cells, indicating production of astaxanthin).

However, when combined more than one factors together, for example, light and temperature, algae showed some changes. In this case, algae turned red but only in a little amount . When light intensity was varied with nutrient reduction, algae turned red under higher light intensity. In contrast, when nutrients were limited with under varied temperature, there was not change. This indicated that light was the most important factor for algae to produce astaxanthin. Finally, when combined all three factors, it concluded that increasing in light intensity and temperature and reduction of nutrients were the best condition for production of astaxanthin. Because of algae cells were put under stress, but not enough to cause death, so they entered the seed phrase and produce astaxanthin. Therefore, this experiment followed the first hypothesis but not all of it.

Extraction of Astaxanthin

The production of dry cell *Haematococcus pluvialis* microalgae equivalent to Astaxanthin extracted

After inoculated the algae until the red cells had spread whole the flask. The five ml of cells were exactly sampling Astaxanthin by centrifuged it then separated cell out, kept separated red cell in desiccators about one night to use as dried cell. The percent moisture content was 96.05 percent. Astaxanthin was quite less amount to extract from the algae, thus the dry cells were directly mixed with fish's feed. The yield of astaxantin from this study was 0.07 mg/mL. It was quite good compared to other researches, for instance, 0.024 mg/mL (W. Majewski, *et. al*. 1999), 0.123 mg/mL (A. Domínguez, 2005) and 0.07 mg/mL (Storebakken, T., *et. al.* 1987). The yield of astaxantin extracted differential amount might be from two factors. Firstly, the extracted solvent (Ethanol) would extract all carotenoid included astaxanthin, therefore, some unwanted solute (other carotenoid) would be extracted even through this algae consisted of 90 – 99 % of astaxanthin. Secondly, it might be not suitable for extracting astaxanthin in case of only one method was conducted which was solvent extraction (Co-solvent were Ethanol and Ether). For commercial, the industrial scale should be conducted. The obtained product (dried cell *Haematococcus pluvialis* equivalence as Astaxanthin follow by calculated amount) was used further as raw materials for study the effect of astaxanthin on goldfish in the next steps.

The efficiency of Astaxanthin extracted for color enhancing of gold fish

The powder of dry cells which received from filtration and drying in desiccators was then mixed with the commercial fish's feed followed formula. The fish were fed by the five difference formulas of mixture for one month. There were ten fish in each tank. The colors of fish were differentiated by RGB color from the Munsell's book. From that results, they showed the color of ten experiment's gold fish in each condition as RGB color from Munsell book. The data was collected at the first day of the study and continue checked every week for four weeks. The results were collected in RGB value that showed together with their real color band of the above and easily compared. Moreover, all figures also showed the cluster chart for the no. of fish in each tank, display three bar as three color zone of gold fish following the grade of gold fish due to color of market demand. Brown zone present as low grade or under standard gold fish (dark brown color). Yellow zone present as medium grade

or the standard gold fish (light/fade color). Red zone represent the brighten color of gold fish or high standard gold fish (dark red/orange color).

For the 1st tank, they noticed that during four weeks of feeding the control commercial fish feed; the color of all ten gold fish did not change too much. There were just a little bit changing in week 3rd and 4th, which might cause of the commercial fish feed CP brand which was the lowest grade had contained some color pigment. (Monthol S., 2004). As see tank no.1, there was showed that 1 fish enhance its color to red zone. For tank no. 2, feeding by commercial fish feed mixed with hormone testosterone, by the fact that genetic factor could affect to ornamental aquatic's color, the appearance of aquatic animal related with age, species, gender, disease, and hormone. As the Thailand farmer gold fish believed that feeding men hormone (testosterone) would enhance the color of gold fish. This condition was emphasized under the assumption that "without the color pigment, only feeding with hormone testosterone cannot enhance the gold fish color". Following the result in tank no. 2, they showed that color of some fish had been changed, dark brown color changed to light orange brown color, light orange color changed to dark orange color. But also noticed that five out of ten fish did not change in color, which following this it noticed that the assumption is not true, only the feed mixed with hormone could enhance color of gold fish but not well. (Torrissen, O. J. and Christiansen, R. 1995)

Next, tank 3rd , 4th and 5th , feeding with commercial fish feed mixed with dry cell of *Haematococcus pluvialis* 0.1 g, 0.2 g and 0.3 g which equal to amount of astaxanthin 2.5 mg, 5.0 mg and 7.5 mg respectively. Most fish in these three tanks changed their color to be brighter since week one. However tank no. five showed the highest change in color in which the fish's color was redder than fish in tank no. four, followed by fish in tank no. three, respectively. Therefore, the highly amount of astaxanthin added to the feed, the changed in the fish color able to be redder. (Torrissen, O. J. and Christiansen, R. 1995) The most economical amount of astaxanthin added into the feed that could increase the color of the gold fish to the desirable level should be studied in the next future. To be able to use astaxanthin effectively, one must consider the relationships between the amounts of astaxanthin used its price, the appropriate level of the red color of fish, and the price of fish that could be sold.

It was noted that the white-colored fish (fish no. 10), showed no observable change in their colors in every conditions used. Although the fish was feed with the hormone (tank no. 2), the color of fish was still unchanged. This was reasonable since the white-colored fish, possibly the 'albino' fish, contains no color pigment in its body. (Monthol S., 2004.) Since the fish has no color pigment, any addition to the feed for the increasing of the color would be impossible. The results were divided into five individual tanks in order to show the comparison of color change from the beginning week. In the first week, the color of gold fish was observed to be quite equally light brown. However, when looked closely, tank 1st or control tank showed the lowest rate of color changed from week 1st to 4th. During week 2nd, the table showed the color had changed already in tank 5th which contained the highest amount of astaxanthin. In the last week, the color changed in tank 4th and 5th were quite the same. Therefore, it is more recommended that the addition of astaxanthin could be optimized at 5 mg/1000 g commercial fish feed since the results were not very much different

(Torrissen, O. J. and Christiansen, R. 1995.). Consequently, as results of color changed in each treatment noticed that the color changed in tank 4th and 5th were quite the same. Therefore, Treatment 4 or 5 mg/1000 g was selected underlying economic cause.

CONCLUSION

As the study had accomplished, the result and discussion from this study had led to this conclusion as follow ; Microalgae *Haematococcus* was alternate growth to stress state to produce color pigment astaxanthin by increasing light intensity, temperature and reduce nutrition. After inoculated *Haematococcus pluvialis* sample which in red state, it turned to green (reproduction and grow) within 7 days and turned to red (produce astaxanthin) after pressed it to stress state by 7 days approximately. So, the period of growth *Haematococcus pluvialis* from red to red (one session) was 14 days follow this experiment. Under the microscope, when *Haematococcus pluvialis* became stress state and form red cell, they noticed that cell had not flagella and no motion as in normal green state. An average yield (amount of astaxanthin) in sample five ml was equal to 0.07 mg/ml. Various amounts of dry cell *Haematococcus pluvialis* microalgae equivalent to Astaxanthin extracted in feed affected the color enhancing of gold fish. Without cooperate dosage with color enhancing feed (asthaxanthin), hormone testosterone was affected the color enhancing of gold fish. The highly the amount of dry cell *Haematococcus pluvialis* equivalent to Astaxanthin extracted added to the feed had effected to the fish color changing. The addition of astaxanthin could be optimized at 5 mg/1000 g commercial fish feed was selected underlying economic cause.

References

A. Domínguez, M. Ferreira, P. Coutinho, J. Fábregas and A. Otero. 2005. Delivery of astaxanthin from *Haematocuccus pluvialis* to the aquaculture food chain., Laboratorio de Microbiología, Facultad de Farmacia, Universidad de Santiago, 15782 Santiago, Spain

Jyonouchi, H., Zhang, L., Gross, M. and Tomita, Y. 1994. Immunomodulating actions of carotenoids: enhancement of in vivo and in vitro antibody production to T-dependent antigens. Nutr. Cancer 21: 47–58.

Jyonouchi H., S. Sun, and M.D. Gross. 1995a. Astaxanthin, a carotenoid without vitamin a activity, augments antibody responses in cultures including T-helper cell clones and suboptimal doses of antigen. J. Nutr. 125(10): 2483-2492.

Jyonouchi H, S. Sun, and G. Myron. 1995b. Effect of carotenoids on in vitro immunoglobulin production by human peripheral blood mononuclear cells: Astaxanthin, a carotenoid without vitamin A activity, enhances in vitro immunoglobulin production in response to a T-dependent stimulant and antigen. Nutr. Cancer. 23(2):171-183.

Jyonouchi H, Sun S, Mizokami M, Gross M D. 1996. Effects of various carotenoids on cloned, effector-stage T-helper cell activity. Nutr Cancer. 26: 313–324.

Jyonouchi H., S. Sun, K. Iijima and M. Gross. 2000. Antitumor activity of astaxanthin and its mode of action. Nutrition and Cancer. 36(1): 59-65.

Monthol Siripanchai. 2004. The alternate Gold fish, D.K. Publishing,: 325

Nultthawee Thamjedsada. 2000. Enhanced production of astaxanthin in a microalga Haematococcus pluvialis NIES-144 Thesis (M.Sc.)--Chulalongkorn University

Palozza, P. and Krinsky, N. I. 1992. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. Arch. Biochem Biophys 297: 291-295.

Storebakken, T., P. Foss, K. Schiedt, E. Austreng, S. Liaaen-Jensen, and U. Manz. 1987. Carotenoids in diets for salmonids IV. Pigmentation of Atlantic salmon with astaxanthin, astaxanthin dipalmitate and canthaxanthin. *Aquaculture*, 65:279-292.

Sudsaichon Homthong. 1998. Production of Astaxanthin from *Haematococcus* sp. thesis, Kasetsart University.

Torrissen, O. J. and Christiansen, R. 1995. Requirements for carotenoids in fish diets. *J. Appl. Ichthyol.*, 11:225-230.

W. Majewski, M. Perrut and J. O. Valderrama1. 1999. Extraction of Astaxanthin from *Haematococcus Pluvialis* by Supercritical Carbon dioxide Univ. de La Serena, Fac. de Ingeniería, Casilla 554, La Serena – Chile

Designing Pulper as Efforts to Increase Production Quality of Black mulberry (Morus nigra) Juice

Yusman Taufik, Yudi Garnida, Yelliantty
Program Studi Teknologi Pangan-Fakultas Teknik, Universitas Pasundan
Jl. Setiabudhi 193 Bandung, West Java, Indonesia 40153
Correspondence e-mail: yusman_taufik@yahoo.com

ABSTRACT

Market demand for the product Black Mulberry fruit is very high, especially in the form of processed juice. In the process of making juice needed a tool that is used to separate the juice with pulp, the pulper. In designing pulper should be optimized and adapted to the product to be processed, so that it can produce fruit juice effectively and efficiently. The purpose of this study was to design and build a pulper which can be used in juice processing Black Mulberry effectively and efficiently to improve the quality and quantity of products. The methods used in this study is testing and optimization factor rotation speed, frictional forces, and perforation of the filtering devices on pulper, as well as an assessment of the quality and quantity of Black Mulberry juice products. The results of this research prototype pulper created with the following specifications: Dimensions: 0.35 x 0.75 meters; Construction: SS304 and Steel Profile; Electric Power: 1.5 HP; Operating Systems: Continuous. The trial results showed that the juice produced in accordance with the organoleptic response expected, and had good raw material efficiency. In addition, the specifications and the model, the resulting pulp can be immediately separated and the time required producing the juice to be faster than the process of crushing / pressing usual. From this study we concluded that the pulper built were optimal in producing juice Black Mulberry with both quantity and quality, and has the potential to be applied as processing technology in small and medium enterprises.

Keywords: black mulberry, *Morus nigra*, pulper

INTRODUCTION

The current market demand for the product Black Mulberry fruit is very good, other than in the form of fresh fruit also in processed form (which is a very attractive market) such as syrup, juice, jam, juice, dodol, puree, and tea leaves for a replacement. The juice is defined as a liquid extortion by pressure or other mechanical means to the edible part of the fruit, the liquid can be cloudy or clear fluid depending on the type of fruit used (Hulme, 1971 in Padma 1981). Meanwhile, according to Indonesian Industrial Standard (1979), is defined as a liquid juice obtained by extortion fruit, filtered or not, and intended for direct drinking fresh drinks (Peter, 1983). In the process of making juice to separate the juice needed a tool pulper to separate the juice with pulp.

The factors that affect the process of spending juice by using the tool pupler among others:
- Rotation speed of the tool
- Friction happens
- Perforation of filtering devices

The function of this tool pulper, which is to improve the process of spending the juice in a relatively short time, so that the processed juice employers no longer feel the loss with their production. And besides, for an SME the development of this technology, it would be obviously very favorable for the production generated by small businesses is increasing, wages are relatively cheap and the quality is guaranteed. To motivate farmers to grow and develop black mulberries that can lift the economy and to improve the resilience of farmers to save, usefulness and economic value of the black mulberry fruit. Therefore, a need to manufacture pulper tool that will give effect to the quality of black mulberry juice is produced as functional beverages produced.

METHODS

Calculation of dimensions

Calculating the dimensions of the tool is an activity to determine the size of the tools and modeling tools based on the results of the calculation of the size of the tool.
Framework designed using brackets with dimensions in accordance with the capacity and size of the desired device is based on medium-scale industries.
Using peeler tank ST 304 material and designed to be able to accommodate a capacity of 5 kg black mulberry with the cylindrical shape of the container, ie the calculation:
$V = \pi.r2.t$

Preparation of Basic Design

The initial step of the design process of this engine is started with the initial drawing engine design.

Determination Instrumentation

Components of the tools used in the design and manufacture of machinery is the key instrument on - off and speed control. Button instrument is adapted to a commercially available goods.

Detail Design
After going through the steps above then the machine design process is then performed a more thorough along with the specification tool.

Revised Pictures
Reviewing the re-design machines that have been drawn to further simplify the process of designing the next machine.

Overall Design Process
After designing the components - the main component and then do the process of designing the overall machine by assembling the components - the main component into a machine planned.

Animation
Creating animation tools before the assembly as a whole.

Assembling Tools
After designing a whole, then performed the assembly by assembling the components - the main component into a machine planned.

Equipment Test
Once the engine is assembled, then tested the machine in order to determine whether the machine that made it feasible or not feasible for use with the parameter: tank capacity, speed and pressing results.

RESULT
Results of design tools can be seen in Figure 1 and 2. The design of the tool is based on several considerations can be acquired when doing literature review.

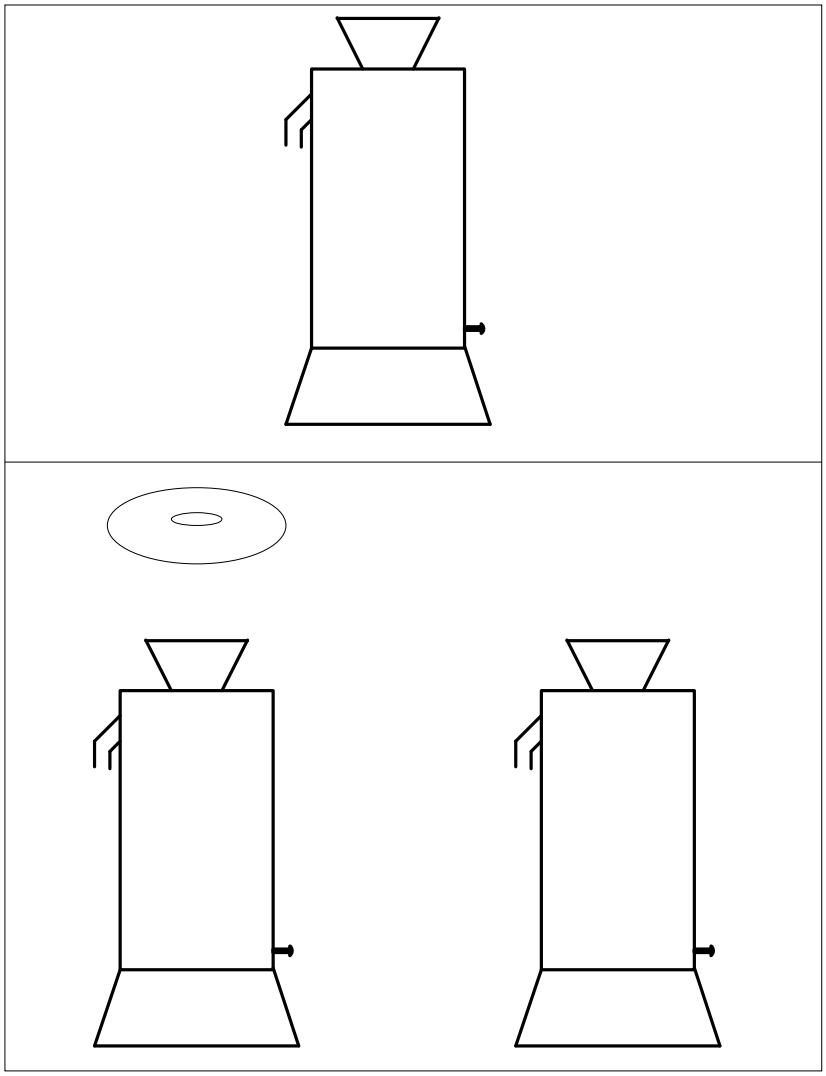


Figure 1. Design Results of Pulper

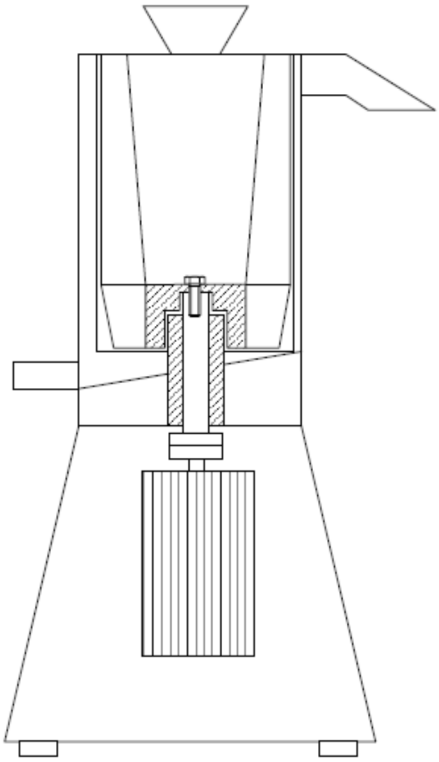


Figure 2. Inside of Pulper

Once the design is done, the next step is to manufacture pulper tool based on the design. The result of tool-making can be seen in Figure 3.

Figure 3. Results of design tools pulper

Table 1. Specifications of Pulper

Specifications	
Name	Fruits Pulper
Dimensions	350 x 750 mm
Construction	SS304 and steel profiles
Power	1.5 HP
Operation	Continue

Tool design results were then tested using the black mulberry fruit. Tests conducted in the laboratory instruments Machinery and Equipment Food Industry of Pasundan University. Results of testing tools can be seen in Figure 4.

Figure 4. Results of testing tools pulper

The test results indicate that the tool tool design results can be used to produce the black mulberry juice. Characteristics of the black mulberry juice obtained in the form of fruit juice does not have pulp but still have fruit pulp. Results juice produced as expected. The existence of fruit pulp obtained in processing results are expected to increase the fiber content of fruit so that it can improve the nutritional value may also reduce the need for food additives, such as stabilizers.

The next stages of research that will be done is to research the manufacture of beverages ready-made from Black Mulberry juice as a drink with health functions. Results were then expected to become the basis of making instant beverage products made from Black Mulberry Juice can be applied to both small-scale industries, medium, even large industry.

CONCLUSIONS

In this study it can be concluded that the results pulper design tool can be used to produce refined black mulberry juice in accordance with the expected character.

ACKNOWLEDGMENTS

Thanks to the BP3IPTEK

REFERENCES

Anonim.2009. Robinia Pseudacacia. - Leguminosae Black Mulberry *(Morus nigra)*

Anonim. 2009. Murbei,http://www.herbal-obatalami.com.

AOAC. 1995. Official Methods of Analysis of International. 16th Edition, Association of Official Analytical Chemists, Inc., Washington, DC.

Apriyantono, (1988), Petunjuk Laboratorium Analisis Pangan, Penerbit PAU Pangan dan Gizi IPB, Bogor.

Astutik, S.,. 2009. Murbei, Tanaman Berhasiat dan Bermanfaat, sehat.wordpress.com.

Baedhowie, M. and S. Pranggonawati, B.Sc., .1983. Petunjuk Praktek Pengawasan Mutu Hasil Pertanian I, Penerbit Departemen Pendidikan dan Kebudayaan, Direktorat Pendidikan Menengah Kejuruan.

Buckle, A. K., R.A. Edwards., G. H. Fleet., and M. Wooton..1987. Ilmu Pangan, Penerbit Universitas Indonesia (UI-PRESS), Jakarta.

Cruess, W. V., .1985. Commercial Fruit and Vegetable Product, Mcgraw-Hill Book Co., Inc. New York.

DeMan, J., .1997. Kimia Makanan, Edisi Kedua, Penerbit ITB, Bandung.

Endriany, R., .1998. Mempelajari Pengaruh Penambahan Pektin dan Gula Terhadap Mutu Jam dari Pulp Jeruk Siam, Skripsi, Universitas Pasundan, Bandung.

Garsari .1999. Pengaruh Konsentrasi Sukrosa dan Konsentrasi CMC Terhadap Karakteristik Konsentrat Bubur Buah Jeruk Fremont, Skripsi, Universitas Pasundan, Bandung.

Gaspersz, V., .1995. Teknik Analisis dalam Penelitian Percobaan, Cetakan Kedua, Penerbit Tarsito, bandung.

Glicksman, M., .1969. Gum Technology in Food Industry, Academic Press, New York, San Francico, London.

Goel, R. K.,. 1980. Food Industrial manual Technology of Food Product (Food Processing Industries), Small Business Publication, Roop Negar, Delhi.

Evaluation of Ginger *(Zingiber officinale* Roscoe) as Antioxidative Agent in Protein Fraction Characteristics of Sweet Bread

Katharina Ardanareswari, Wahyu Putri Sari, Kristina Ananingsih

Faculty of Agricultural Technology, Soegijapranata Catholic University, Semarang, Indonesia
(e-mail : kristina@unika.ac.id)

Abstract

Addition of antioxidant is well-accepted in wide range of product to enhance nutritional properties and product qualities. Ginger (*Zingiber officinale* Roscoe) is known with its natural antioxidant potential. Supplementation of antioxidant in sweet bread may affect its physical properties, due to the potential disrupt in the formation of disulphide bond in gluten development. The purpose of this research is to understand effect of ginger *(Zingiber officinale* Roscoe) powder as antioxidant in protein fraction characteristics of sweet bread. Various concentration of ginger powder (0, 1.5, 3%) was added to flour and water mix, and analyzed for its hardness. The extraction of gliadin, glutenin, albumin, and globulin was done, and the glutenin fraction was assayed using electrophoresis technique. The result showed that higher concentration of ginger powder resulted in higher dough hardness. Different molecular weight of protein subunits was detected in each sample.

Keywords : ginger, sweet bread, antioxidant, electrophoresis

Introduction

Antioxidant is compounds being able to inhibit or delay oxidation reaction. The positive benefits range from longer shelf life of product due to the slower rate of rancidity to health benefit for consumer. Ginger (*Zingiber officinale* Roscoe) is well known for its antioxidant potential (Balachandran et al., 2006). It is well known that gingerol, shogaol, zingerone, and another flavonoid content in ginger show antioxidant, anti-inflammation, analgesic, and anti-cacinogenic effect. Dried and powdered ginger shows more stable antioxidant activity (Vankar et al.,2006).

However, the addition of antioxidative ingredients in bread may affect its physical properties. It may acts as reducing agents which interrupt the gluten formation. Gluten plays important roles in the development of bread structure. During mixing, the precursors of gluten i.e. gliadin and glutenin is hydrated, developing elastic mass which is able to retain gas produced during yeast fermentation (Hoseney, 1994). Redox reaction plays important roles in determining physical characteristic of bread. During mixing, oxidation will be naturally take place in gluten formation. During the gluten formation, oxidation of sulfhydryl group (S-H) from cystein residue occurs and forms interchain disulphide bonds (S-S), which results in higher dough strength.

Materials and method

Processing of Ginger Powder

Ginger rhizome was obtained from local market, cleaned and dried. The ginger rhizome was peeled, washed, sliced (about 0.5 - 1 mm height) and dried in cabinet drying for approx. 5 h to reach 9% moisture content. The dried ginger was crushed and shieved in 625 mesh. The ginger powder was stored in ambient temperature in tight container with dried silica gel.

Ginger-supplemented dough Hardness

The dough was made of water (32.25 g) (Aqua, Danone, Indonesia), high protein flour (50 g) (Bogasari, Indonesia) and 3 level of ginger powder concentration, i.e. 0, 1.5, and 3 % (0, 0.75, and 1.5 g, respectively). This ingredients was kneaded in high scale for approx. 2 minutes until it formed a stiff and elastic mass. The molecular protein fraction and the hardness of the dough was analyzed.

Hardness analysis

The hardness of the dough was analyzed by Texture Profile Analysis (ILYOD *texture analyzer*) with ball probe. The condition of the test is 5 mm/s speed, trigger of 10 gf, sample compress of 50%.

Molecular protein fraction analysis

Extraction of protein fraction

The protein of the dough was fractioned with Osborn method which consisted of 4 steps, i.e. albumin extraction, globulin extraction, gliadin extraction, and glutenin extraction described by Lookhart & Bean (1995). Albumin is the water-soluble protein fraction. The amount of

100 mg sample was immersed in 500 µl ddH_2O for 30 min. In every 10 min intervals, the solution was vigorously shook with vortex for 1 min. After 30 min, the sample was centrifuged in 2000 rpm, for 5 min. The supernatant obtained was albumin. This procedure was repeated twice by immersing the solid residue in 400 µl ddH_2O for 5 min followed by centrifugation. The obtained albumin was combined.
Globulin is the NaCl-soluble protein fraction. The solid residue left from albumin extraction was used for globulin extraction which followed the described method above, with NaCl 0.5 N as immersion solution. The supernatant obtained was globulin.
For gliadin extraction, the left solid residue should be rinsed first. It is immersed in 400 µl ddH_2O and vigorously shook with vortex for 1 min, left aside for 5 min, and centrifuged in 2000 rpm for 5 min. The left solid residue was used for gliadin extraction with the method described above, with ethanol 70% for the solvent. After three times centrifugation which results in gliadin fraction, the left solid residue was immersed in 400 µl mixed solution of 50% 1-propanol + 1% DTT for 30 min. In every 10 min intervals, the solution was vigorously shook with vortex for 1 min. After 30 min, the sample was centrifuged in 2000 rpm for 5 min. The obtained supernatan was glutenin.

Electrophoresis

The electrophoresis procedures was followed Bollag & Stuart (1991) with modification). For SDS-Page, 12.5% Separating Gel and 4% Stacking Gel was used. Glutenin extract was added with 2x Sample Buffer with 1:1 ratio and heated in waterbath for 5 min in 90° C. An amount of 1 µl coloring agent (1g Bromophenol Blue + 10 ml ddH_2O) was added. This colourized sample (20 µl) was added to the well. SDS-PAGE was operated in 300V, 50A for 135 min. After the gel was taken, the gel was colorized with Comassive Blue. The gel was agitated in Comassive Gel Stain for 45 min, rinsed with ddH2O, and agitated in Comassive Gel Destain overnight. The gel was scanned afterwards and the molecular weight of the fractionated protein was calculated according to low molecular weight BSA.

RESULTS AND DISCUSSION

Ginger-supplemented dough Hardness

During dough mixing, the air is incorporated during mixing and contributes in increasing oxygen contact to the sulfhydryl residue. The presence of oxygen triggers oxidation reaction of disulfhydryl residue and results in the formation of interchain disulphide bond. The formation of disulphide bonds results in higher elasticity yet lower hardness. However, the addition of antioxidant compound reverse this process. Antioxidant inhibits the oxidation reaction and reduces disulphide bond produced to its initial form (Ananingsih & Zhou, 2011). This chemical reaction also affects physical characteristic of the dough.
In this research, antioxidant was added in the form of ginger powder. Addition of 1.5 and 3% ginger in the dough affected the hardness of the dough significantly. Dough with higher ginger concentration showed higher hardness (Table 1).

Table 1. Hardness of ginger-supplemented dough

Ginger Concentration (%)	*Hardness* (gf)
0	28,63 ± 1,74[1]
1.5	41,68 ± 1,22[2]
3	48,91 ± 1,37[3]

[a] Each value represents the means ± standard deviation of three replicates.
[b] Different superscript in the same column shows significant difference ($p<0.05$) according to Duncan's Multiple Range Test

Molecular protein fraction analysis

SDS-PAGE analysis is intended to separate protein into fractions based on its molecular weight (Wasinger *et al.*, 1995). Flour contains two protein type, gluten and non-gluten. Albumin and globulin are classified as non-gluten protein, while gliadin and glutenin are classified as gluten protein. Physical characteristic of dough is highly affected by the gluten protein. In this research, glutenin showed higher portion than gliadin (Fig. 1). Glutenin is the major protein fraction of flour which consists about 45% of flour total protein (Zilic *et al.*, 2011). Glutenin is composed of polypeptide chain connecting by interchain disulphide bond (Zilic *et al.*, 2011), therefore it plays a major role in determining dough elasticity.
Based on that reason, electrophoresis analysis was conducted for the glutenin fraction only. Glutenin fraction of each sample consists of different amount of protein sub unit chain.

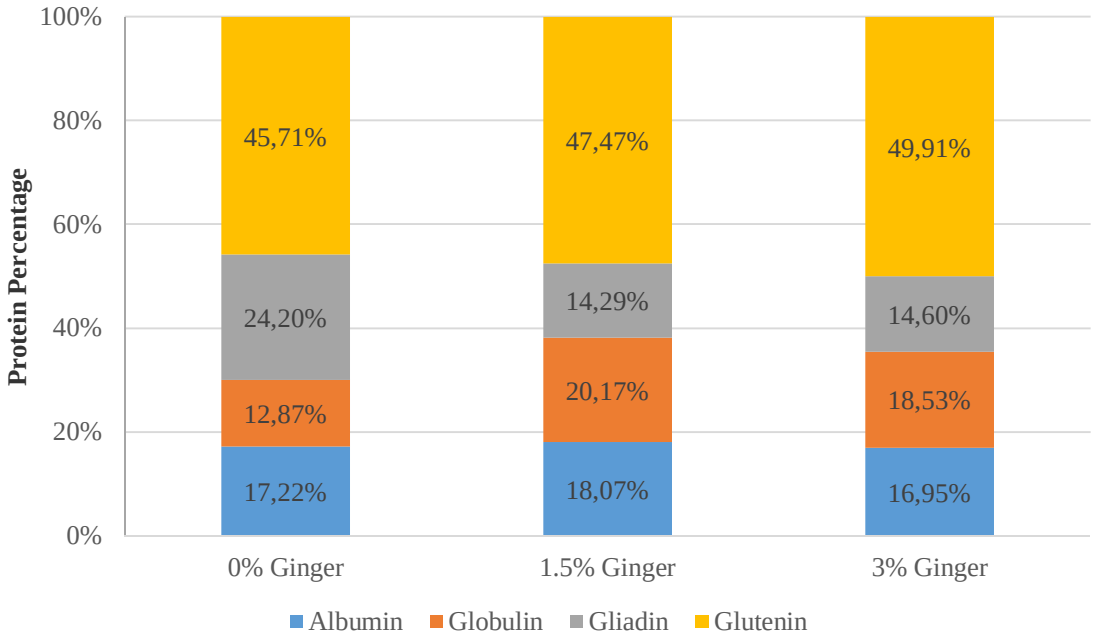


Fig. 1. Protein fraction percentage of ginger-supplemented dough

Addition of antioxidant compound may dissociate the disulphide bond and increase the amount of free sulfhydryl group (Ananingsih & Zhou, 2011). Ginger addition in various concentration resulted in different amount of protein subunit in each concentration. The control sample showed 7 protein subunit, i.e. 63.73 kDa; 57.20 kDa; 52.82 kDa; 46.08 kDa; 42.68 kDa; 39.86 kDa and 31.30 kDa. Meanwhile, sample with 1.5% ginger powder contained 5 protein subunit i.e. 63.01 kDa; 55.13 kDa; 52.52 kDa; 46.88 kDa; and 41.01 kDa. Sample with 3% ginger powder contained 5 subunit, i.e. 61.94 kDa; 56.55 kDa; 51.20 kDa; 43.41 kDa and 41.72 kDa (Fig. 5). Oxidation and free radical formation during mixing may cause a breakdown of disulphide bond to form sulfhydryl group, therefore it may change the molecular weight of gluten protein.

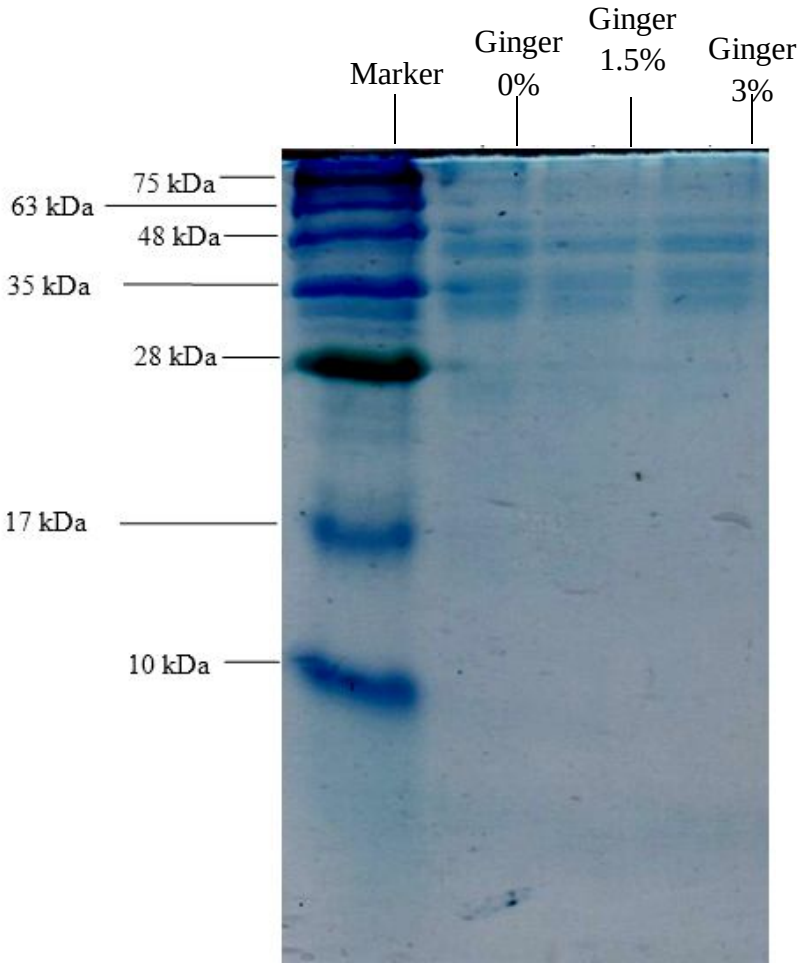


Fig. 2. Glutenin protein fraction profile of ginger-supplemented dough

Conclusion

Addition of ginger powder increased dough hardness. Higher ginger powder concentration resulted in higher dough hardness. Glutenin is the major protein of ginger-supplemented dough. Addition of ginger powder triggered change of molecular weight of glutenin protein fraction. Different amount of protein subunit with different molecular weight in each fraction was found.

References

Ananingsih, V. K., and Zhou, W. (2011). Effect of Green Tea Extract on Large Deformation Rheological Properties of Steamed Bread Dough and Some Quality Attributes of Steamed Bread. In *11th International Congres on Engineering and Food*, (pp. 647 – 648). Athens, Greece.

Balachandran, S., S. E. Kentish and R. Mawson. (2006). The Effect of Both Preparation Method and Season on the Supercritical Extraction of Ginger. Sep. Purif. Technol. 48 (2) : 94-105.

Bollag, D.M., and Stuart, J.E. (1991). Protein Methods. Department of Biochemistry. University of Geneva. Geneva. Switzerland.

Hoseney, R. C. (1994). Principles of Cereal Science and Technology 2nd Edition. American Association of Cereal Chemist, Inc. USA.

Lookhart, G. and S. Bean. (1995). Separation and Characterization of Wheat Protein Fractions by High-Performance Capillary Electrophoresis. American Association of Cereal Chemist, Inc. USA.

Vankar, P. S., Shanker, R., Srivastava, J., and Tiwari, V. (2006). Change in Antioxidant Activity of Spices Tumeric and Ginger on Heat Treatment. Di dalam Purnomo, H.,

Jaya, F., and Widjanarko, S. B. (2010). The Effects of Type and Time of Thermal Processing on Ginger (*Zingiber officinale* Roscoe) Rhizome Antioxidant Compounds and its Quality. International Food Research Journal. 17: 335-347.

Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K. L. and Humphery-Smith, I. (1995). Progress with Gene-product Mapping of the Mollicutes: Mycoplasma Genitalium. Electrophoresis. 16:1090-4.

Zilic, S., Miroljub B., Mirjana P., Dejan D., and Dragana I. M. (2011). Characterization of Proteins from Grain of Different Bread and Durum Wheat Genotypes. International Journal of Molecular Sciences 12, 5878-5894.

Examination of Antioxidant Activity and Color Characteristic of Herbal Infusion From Soursop Leaves

Albertus Adrian Sutanto*[1], Stefany P. A. Budhy[1], V. Kristina Ananingsih[1]
[1]Department of Food Technology, Faculty of Agricultural Technology, Soegijapranata Catholic University
*Corresponding author: adrian@unika.ac.id

ABSTRACT

Soursop (*Annona muricata* Linn.), which is known as *sirsak* in Indonesia, is one of the plants that contains bioactive compounds. However, many people only consume its fruits rather than its leaves. Previous studies showed that soursop leaves contain various antioxidant compounds, such as flavonoid, triterpenoid, phenolic (tannin), cardiac glycosides, and reducing sugar. Soursop leaves can be prepared as a herbal infusion. This study reports the effect of infusion time and temperature on antioxidant activity and color characteristic of herbal infusion from soursop leaves. The antioxidant activity of the infused solution was increasing both for infusion at 80°C and 100°C during 3 minutes of observation. The highest antioxidant activity of 82.53 ± 0.36 was obtained from the 3-minutes infusion of soursop leaves at 100°C. The color changes of the infused solution from red-yellowish color to dark brown color were observed. The color changes of the infused solution were due to the oxidation of the extracted tannin.

Keywords: soursop, leaves, infusion, antioxidant activity, color characteristic

INTRODUCTION

One of plants that has a potential as an antioxidant source is Soursop (*Annona muricata* Linn.), which is also known as *sirsak* in Indonesia. However, most people in Indonesia only consume its fruit. It has been shown in many researches that soursop leaves contain various antioxidant compounds, such as flavonoid, triterpenoid, phenolic (tannin), cardiac glycosides, and reducing sugar (Trevor, 1995; Artini *et al.*, 2012; Gajalakshmi *et al.*, 2012; George *et al.*, 2012). Moreover, soursop leaves also contain acetogenins, annocatacin, annocatalin, annohexocin, annoanacin, annomuricin, and annonol (Dewi and Hermawati, 2013). Those compounds are known to be able to boost body immunity and also to be used as a remedy for certain diseases (Dewi and Hermawati, 2013).

Soursop leaves can be prepared as either fresh leaves or dry leaves. Fresh soursop leaves are usually prepared by boiling them directly in the water, while dry soursop leaves are often served as an herbal tea by infusing them in hot water. Serving soursop leaves by infusion of dry soursop leaves offer some advantages, such as longer shelf-life and easiness of serving. Both of drying and infusion parameters play an important role to the bioactive compounds content and also the physical characteristics of the herbal infusion from soursop leaves (Horžić *et al.*, 2009; Zuhud, 2012).

The dry soursop leaves can be packed inside tea bag or served as loose tea. In general, consumers prefer tea bag to loose tea due to its serving simplicity. During the production process of tea bags, pulp is bleached with chlorine (Modric, 2015). In a study conducted by Putri (2014), it was suggested to not consume tea brewed from tea bag infusion more than two times a day and to infuse the tea bag no more than 5 minutes, because it was found that the chlorine level in tea was increasing as the infusion time increased. Thus, infusion time will be a limiting parameter in serving herbal infusion from soursop leaves.

Basically, the infusion of tea and herbal beverages is an extraction process by using water as the solvent. Horžić *et al.* (2009) have investigated that the effective temperature for tea and herbal beverages was 100°C. Since dry soursop leaves contain many bioactive compounds which are sensitive to heat, the infusion parameters such as temperature and time will affect the content of bioactive compounds extracted to the infused water. These parameters also affect the color characteristic of herbal infusion from soursop leaves (Goh *et al.*, 2003).

As far as our knowledge, there is no report about the antioxidant activity and color characteristic of herbal infusion from soursop leaves depending on various infusion conditions. Therefore, this study was aimed to evaluate the effect of infusion time and temperature towards the antioxidant activity and color characteristic of herbal infusion from soursop leaves.

MATERIALS AND METHODS

Materials

Fresh soursop leaves were obtained from a yard located in Bukit Kencana Jaya, Semarang. Calcium chloride was used as a drying agent. DPPH (2,2-diphenyl-1-picrylhydrazyl) and methanol 99.98% were used for antioxidant activity determination. Teabags were used to pack the dry soursop leaves.

Preparation of Dry Soursop Leaves
Fresh soursop leaves were washed with clean water. Prior to drying, the leaves were pre-treated by immersing them in 0.5% calcium chloride solution, and then the leaves were steam-blanched at 70°C for 4 minutes followed by cooling at room temperature for 5 minutes. Dry soursop leaves were prepared by drying them using solar tunnel dryer. The drying process was carried out until water content of the sample was below 10%.

Soursop Leaves Infusion
Dry soursop leaves were ground into powder and packed inside tea bags. Each tea bag contained 2 grams of dry soursop leaves. Infusion experiments were conducted by pouring 200 mL of hot water (80°C and 100°C). Samples were taken at intervals of 30 seconds within 3 minutes for further analyses.

Antioxidant Activity Assay
The determination of antioxidant activity was based on DPPH (2,2-diphenyl-1-picrylhydrazyl) method developed by Molyneux (2004). 0.1 mL of sample was added to 3.9 mL of 0.029 mg/mL DPPH solution. The mixture was left for 30 minutes in the dark to facilitate the reaction between DPPH and the antioxidant compounds. Blank solution was prepared by adding 0.1 mL methanol to 3.9 mL of 0.029 mg/mL DPPH solution. The absorbance of the sample and blank solution then were measured at 515 nm. The antioxidant activity was expressed as the reduction of DPPH (Q) and calculated using the following equation:
$Q = 100\ (A_0 - A_c)/A_0$
where A_0 is the absorbance of blank solution and A_c is the absorbance of the sample.

Color Analyses
Color analyses of the infused soursop leaves tea were carried out using chromameter (Konica Minolta, CR–400). The instrument was standardized against a white plate before each measurement. Color was expressed in L*, a*, and b* parameter. L value expressed lightness. +a* value expressed redness and –a* greenness. +b* expressed yellowness and –b* blueness (MacDougall, 2002).

Statistical Analyses
All measurements were performed in triplicate and reported as mean ± standard deviation. One-way analyses of variance (one-way ANOVA) with confidence level of 95% were conducted and followed by post-hoc Duncan test using SPSS software.

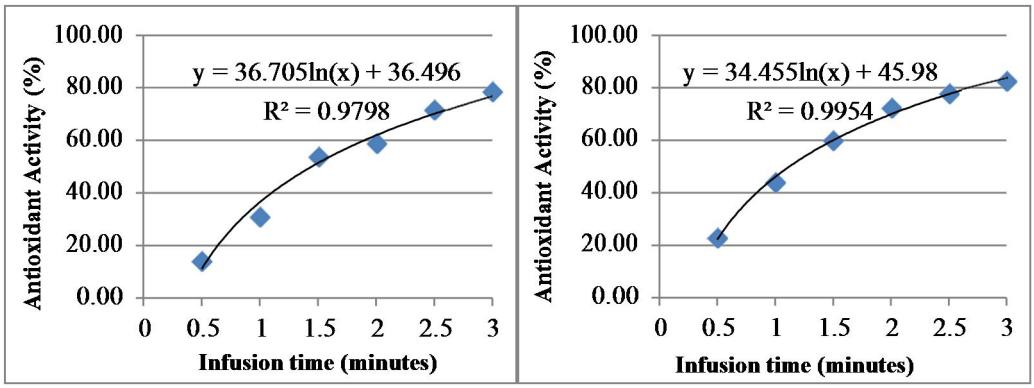
RESULTS AND DISCUSSION
Dry soursop leaves were infused with water at 80°C and 100°C. The antioxidant activity of soursop leaves infusion was measured every 30 seconds for 3 minutes. Table 4 showed the antioxidant activity of soursop leaves infusion on various infusion conditions.

Table 4 Antioxidant activity of soursop leaves infusion on various infusion conditions

Infusion Time (minutes)	Antioxidant Activity (%)	
	Temperature = 80°C	Temperature = 100°C
0.5	14.10 ± 1.40^{a}	22.76 ± 1.79^{b}
1.0	30.91 ± 2.03^{c}	44.01 ± 1.02^{d}
1.5	53.77 ± 0.86^{e}	59.87 ± 2.04^{f}
2.0	58.85 ± 0.78^{f}	72.30 ± 2.06^{g}
2.5	71.64 ± 1.96^{g}	77.81 ± 1.71^{h}
3.0	78.55 ± 3.95^{h}	82.53 ± 0.36^{i}

Note: Values followed by the different superscripts showed significant differences by the Duncan test

The highest antioxidant activity of 82.53 ± 0.36 was obtained from the soursop leaves infused at 100°C for 3 minutes. The phenomena observed in this study was in agreement with the previous study of Horžić *et al.* (2009), where the bioactive compounds were effectively extracted in water at 100°C because of the higher extraction rate. At high temperature, the diffusion rate of the bioactive compounds increases due to reduction of solvent viscosity and higher mass transfer occurs from the plant cells (in this case soursop leaves) to the solvent (Hadiyanto *et al.*, 2014).



(a) (b)

Figure 10 Profiles of the antioxidant activity versus infusion time at (a) 80°C and (b) 100°C

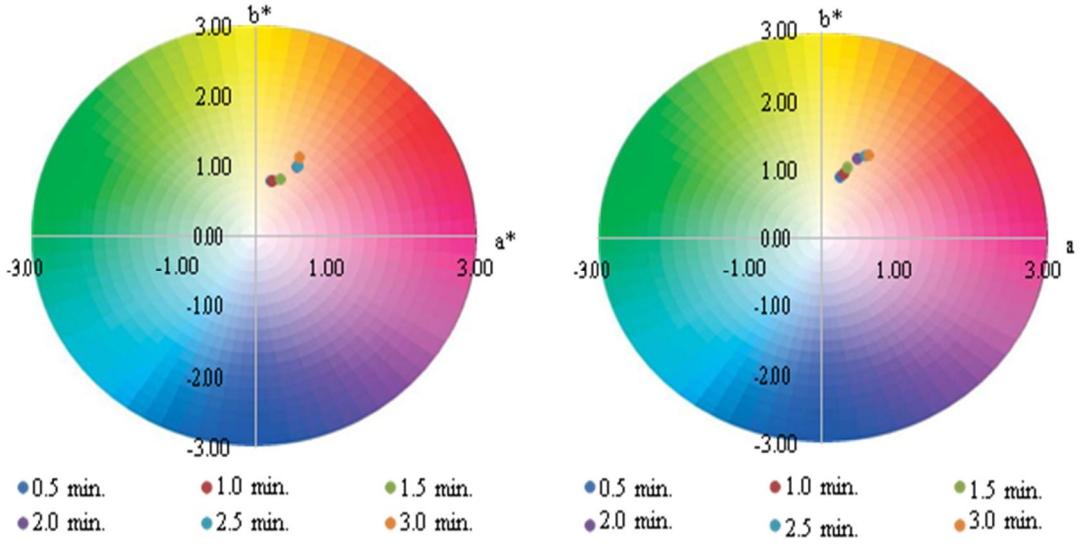
Profiles of the antioxidant activity versus infusion time at different temperature were plotted on Figure 10. Mathematical models were obtained from the curves fitting. The antioxidant activity was increasing from time to time for infusion both at 80°C and 100°C. The longer infusion time gives the longer contacting time between solvent and the antioxidant compounds, thus more antioxidant compound extracted from the samples (Hadiyanto *et al.*, 2014).

Table 5 Color intensity of soursop leaves infusion on various infusion conditions

Infusion Time (minutes)	Temperature = 80°C			Temperature = 100°C		
	L* value	a* value	b* value	L* value	a* value	b* value
0.5	26.04±0.14^{c}	0.23±0.02^{a}	0.77±0.16^{a}	25.45±0.46^{bc}	0.25±0.04^{a}	0.90±0.15^{a}
1.0	25.34±0.53^{b}	0.25±0.12^{a}	0.78±0.06^{a}	25.29±0.34^{b}	0.30±0.10^{a}	0.95±0.38^{a}
1.5	25.23±0.48^{b}	0.35±0.09^{a}	0.81±0.43^{a}	25.15±0.61^{b}	0.35±0.16^{a}	1.04±0.31^{a}
2.0	24.47±0.09^{a}	0.56±0.32^{a}	0.98±0.09^{a}	24.25±0.62^{a}	0.48±0.14^{a}	1.17±0.41^{a}
2.5	24.17±0.28^{a}	0.57±0.36^{a}	0.99±0.54^{a}	24.12±0.09^{a}	0.58±0.49^{a}	1.21±0.28^{a}
3.0	24.07±0.14^{a}	0.59±0.04^{a}	1.11±0.49^{a}	23.96±0.16^{a}	0.63±0.35^{a}	1.22±0.41^{a}

Note: Values followed by the different superscripts showed significant differences by the Duncan test

Lightness values of the infusion of soursop leaves were tabulated on Table 5. It was observed both for infusion at 80°C and 100°C that the longer infusion time, the lower lightness value was. The lower lightness value indicated that the solution became darker. Slight difference in the lightness value was observed between infused solution at 80°C and 100°C. The solution infused at 100°C was slightly darker, and it did not show a significant difference compared to the solution infused at 80°C.



(a) (b)

Figure 11 a*b* color space plot

For all infusion parameters, both a* and b* values were positive. The positive a* and b* values were showing red and yellow color, respectively. The a* and b* values were plotted on color space plot shown on Figure 11. The infused solutions' color changed

from red-yellowish color at the beginning of infusion to dark brown color at the end of infusion (Figure 12). The final color of the solution was in agreement with Indonesian National Standard SNI 3753:2014 about black tea bag (BSN, 2014). The color changes of the infused solution were due to the extraction of tannin from soursop leaves. Tannin was originally a colorless compound. During the infusion of soursop leaves, tannin was oxidized into phlobaphenes which contributed to reddish color of the solution (Trevor, 1995).

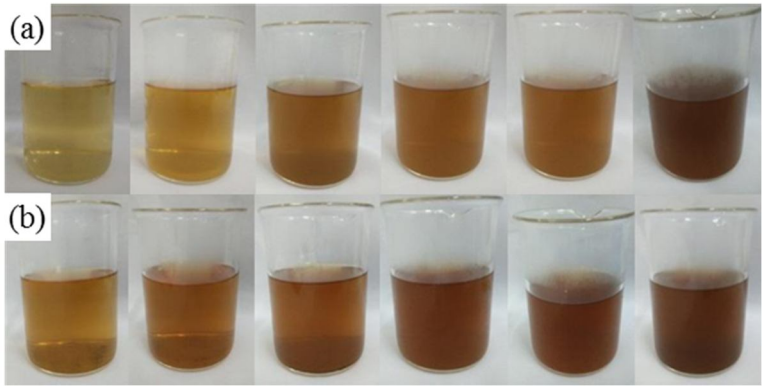


Figure 12 Physical appearance of soursop leaves infusion every 30 seconds interval for 3 minutes at (a) 80°C and (b) 100°C

CONCLUSION

The results from this study showed the effect of various infusion condition (time and temperature) on antioxidant activity and color characteristic. The antioxidant activity of the infused solution was increasing both for infusion at 80°C and 100°C during 3 minutes of observation. The highest antioxidant activity of 82.53 ± 0.36 was obtained from the 3-minutes infusion of soursop leaves at 100°C. The color changes of the infused solution from red-yellowish color to dark brown color were observed. These changes were well supported by L*, a*, and b* values of the solution. The color changes of the infused solution were due to the oxidation of the extracted tannin.

REFERENCES

Artini, N. P. R., Wahjuni, S. and Sulihingtyas, W. D. (2012) 'Ekstrak Daun Sirsak (*Annona muricata* L.) sebagai Antioksidan pada Penurunan Kadar Asam Urat Tikus Wistar', *Jurnal Kimia*, 6(2), pp. 127-137.

BSN (2014) *SNI 3753:2014 : Teh Hitam Celup*. National Standardization Agency of Indonesia (BSN).

Dewi, H. A. C. and Hermawati, R. (2013) *Khasiat Ajaib Daun Sirsak*. Malang: Padi.

Gajalakshmi, S., Vijayalakshmi, S. and Devi Rajeswari, V. (2012) 'Phytochemical and Pharmacological Properties of *Annona muricata* : A Review', *International Journal of Pharmacy & Pharmaceutical Sciences*, 4(2), pp. 3-6.

George, V. C., Kumar, D. R. N., Rajkumar, V., Suresh, P. K. and Kumar, R. A. (2012) 'Quantitative Assessment of the Relative Antineoplastic Potential of the n-butanolic Leaf Extract of *Annona muricata* Linn. in Normal and immortalized Human Cell Lines', *Asian Pacific Journal of Cancer Prevention*, 13(2), pp. 699-704.

Goh, L. M., Barlow, P. J. and Yong, C. S. (2003) 'Examination of antioxidant activity of *Ginkgo biloba* leaf infusions', *Food Chemistry*, 82(2), pp. 275-282.

Hadiyanto, H., Sutanto, A. A. and Suharto, Y. (2014) 'Ultrasound assisted extraction of antioxidant from *Coleus tuberosus* peels', *Carpathian Journal of Food Science and Technology*, 6(1), pp. 58-65.

Horžić, D., Komes, D., Belščak, A., Ganić, K. K., Iveković, D. and Karlović, D. (2009) 'The composition of polyphenols and methylxanthines in teas and herbal infusions', *Food Chemistry*, 115(2), pp. 441-448.

MacDougall, D. B. (2002) *Colour in Food : Improving Quality*. Cambridge: Woodhead Publishing Limited.

Modric, J. (2015) *Bleach in Tea Bags - Helath Risk*. Available at: http://www.healthhype.com (Accessed: 23 March 2015).

Molyneux, P. (2004) 'The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity', *Songklanakarin J. Sci. Technol.*, 26(2), pp. 211-219.

Putri, H. D. W. (2014) *Pengaruh Lama Variasi Perendaman terhadap Kadar Tanin Acid dan Chlorine pada Teh Celup*. Bachelor thesis. University of Jember.

Trevor, R. (1995) *Kandungan Organik Tumbuhan Tinggi*. Translated by Padmawinata, K. Bandung: Penerbit ITB.

Zuhud, E. A. M. (2012) *Kanker Lenyap Berkat Sirsak*. Jakarta: Agromedia Pustaka.

Potency of High Protein Oyek for Decreasing Cholesterol of Diabetic Rats

Bayu Kanetro1, Sri Luwihana2, Dwiyati Pujimulyani3
1Food Technology Department Mercu Buana Yogyakarta University (bayu_kanetro@yahoo.co.id)
2Food Technology Department Mercu Buana Yogyakarta University (sri_luwihana@yahoo.co.id)
3Food Technology Department Mercu Buana Yogyakarta University (dwiyati2002@yahoo.com)

ABSTRACT

High protein oyek is traditional food from Kulon Progo Special Region of Yogykarta that was made of cassava and developed by addition of cowpeas sprout for increasing the protein of oyek This research was aimed to determine the potency of hypocholesterolemic of oyek through in vivo bioassay by using Sprague Dawley male rats. There were two treatments of the research, the first treatment were normal rats and diabetic rats which was induced by aloxan injection, and the second treatment were standard feed and oyek feed. The blood triglyceride, cholesterol total, High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL) cholesterol were analysed on 3^{th}, and 18^{th} days for the treatment and before the treatment as control (0^{th}). The result of this research showed that the total cholesterol of normal and diabetic rats increased during the feed treatment but the increase of cholesterol of diabetic rats were higher than normal rats. The potency of hypocholesterolemic were shown by decreasing of blood triglyceride, cholesterol total, LDL, and increasing of blood HDL in diabetics rats with oyek feed treatment.
Keywords: oyek, protein, cassava, cowpeas, hypocholesterolemic

INTRODUCTION

Oyek is traditional food from Kulonprogo Yogyakarta Indonesia. Oyek is staple food providing energy especially in dried climate condition that is high price of rice. Oyek is produced by spontaneous fermentation of cassava in water for 5 days, and then the fermented cassava are pressed to remove water, formed, steamed, and dried (Kanetro and Luwihana, 2015). The same product as oyek in the other country is *gari* or *rale* that is fermented cassava using lactic acid bacteria (Eduardo et al., 2013). Oyek had been developed into artificial rice that its glycemic index was lower than original rice, and the artificial rice was more preferably than oyek according to the sensory testing by Kalirejo community (Kanetro et al., 2015)

The protein of oyek was lower than rice, so the addition of legumes flour into oyek is important to increase protein. There are many local legumes in Indonesia, such as cowpeas. Cowpeas may be potential for increashing protein of traditional staple food, such as oyek. In the preliminary research was known that oyek with addition of cowpeas sprout flour 30% could increased protein, that was the same as rice (Kanetro and Luwihana, 2015).

The potency of legumes as functional food could be increased by germination. Germination of soybean increased arginine (Kanetro et al, 2008) that was known as hypocholesterolemic amino acid (Damasceno etal, 2000).The protein of cowpeas sprout contained high of arginine (Arg) that was the same as protein soybean (Kanetro and Dewi, 2013). In the preliminary research also showed that the arginine /lisine ratio of cowpeas sprout protein was no significant different with protein of soybean (Kanetro and Dewi, 2013). The ratio of arginine/lysine was important to controle cholesterol level (Damasceno et al.,2000). Protein isolate of soybean had been known as functional food due to hypocholesterolemic effect (Damasceno et al., 2000). Protein isolate of cowpeas sprout had hypocholesterolemic properties based on in vivo bioassay by using Sprague Dawley male (Kanetro, 2015). Therefore oyek with addition of cowpeas sprout might has hypocholesterolemic effect, so it was potential as functional food. This research was aimed to determine hypocholesterolemic properties of the oyek in normal and diabetic rats through in vivo bioassay..

MATERIALS AND METHOD

Material

The cowpea (*Vigna unguiculata*) were obtained from Beringharjo market in Yogyakarta. Chemical agents, such as aloxan, corn starch, casein, vitamin mix, mineral mix, sucrose, choline bitartat, soy oil, and kholesterol kit (*DiaSys Diagnostic System GmBH & Co*), were purchased from Sigma Chemical Co. The other material was rats that were obtained from Animal Experiment Development Unit, Gadjah Mada University, Yogyakarta.The methods of experimental activities were performed as follows:

Preparation of Cowpeas Sprout Flour and Oyek

Cowpea seeds were soaked for 8h, and then germinated for 36h. The germinated cowpeas were dried and milled to make flour (Kanetro and Dewi, 2013). The cowpeas sprout flour were prepared for increashing protein of oyek. Oyek was made of cassava through many step process according to Kanetro and Luwihana (2015). The first step, cassava was peeled, washed, and size reduced. After that cassava was fermented by soaking in water for 5 days. Then the fermented cassava are pressed to remove water, mixed with 30% cowpeas sprout flour, formed, steamed, and dried. The dried product was called oyek that was prepared to in vivo bioassay.

In Vivo Bioassay

The *in vivo* bioassay was done to determine the potency of hypocholesterolemic of oyek by using 24 *Sprague Dawley* male rats. The experiment sequences of the steps were adaptation of rats for 3 days, diveded rats into 4 groups, treated rats for 18 days with the condition of rat and feed treatments, and analysed the blood triglyceride, cholesterol total, High Density Lipoprotein (HDL) cholesterol , and Low Density Lipoprotein (LDL) cholesterol for the treatment of rats on 3^{th}, 18^{th} days and before treatment as control (0^{th}). The experimental design of this research was randomized complete design with 2 factors. The first factors were rat condition treatments, that were normal rats and diabetic rats which was induced by aloxan injection. The second factors were feed treatments, that were standart feed according to AIN–93 (Reeves et al, 1993) and oyek feed which was prepared by subtitution of corn starch in standard feed with the oyek. The data of this experiments was statistical analysed by Anova (analysis of varian) and DMRT (Duncan Multiple Range Test). The in vivo bioassay of this research had passed ethical clearance that was approved by Center Research Laboratorium of Gadjah Mada University, Yogyakarta, Indonesia.

RESULTS AND DISCUSSION

Trygliceride

Table 1 showed that the trygliceride of normal rats treatment were no significant differences between standart feed and oyek feed treatment. While the trygliseride of diabetic rats teratment showed significantly differences, especially for the treatment of rats on 18^{th}. The trygliseride of diabetic rats with protein isolate feed treatment increased after injection of aloxan at 3^{th} days of the tratment, and then decreased until below the standart of normal for the treatment of rats on 18^{th}.

The normal trygliceride of human according to US National Cholesterol Education Program (NCEP) was < 150 mg/dl (Anonim, 2007). While the normal trygliceride of rat was < 120 mg/dl (Herlina et al., 2013). Based on this data indicated that oyek could inhibit increashing trygliceride that was ussual happen in complication of diabetic.

Table 1. The effect of oyek feed treatment on blood tryglyceride of normal and diabetic rats (mg/dL) *)

Rats condition	Feed treatment	0^{th} days	3^{th} days	18^{th} days
Normal	Standard	72,79a	72,92a	79,33a
	Oyek	67,15a	69,07a	75,82a
Diabetic	Standard	69,52a	117,45b	120,67c
	Oyek	69,33a	113,60b	90,30b

*) The same notation of statistic in the table showed not significantly differences at the same column

Total cholesterol

The total cholesterol of normal and diabetic rats increased during the feed trealment but the increase of cholesterol of diabetic rats were higher than normal rats, that was seen at Table 2.

Table 2. The effect of oyek feed treatment on blood total cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0^{th} days	3^{th} days	18^{th} days
Normal	Standard	106,28a	112,15a	112,59a
	Oyek	106,46a	108,66a	112,53a
Diabetic	Standard	104,11a	152,49b	154,31c
	Oyek	103,40a	156,54b	133,45b

*) The same notation of statistic in the table showed not significantly differences at the same column

Data of the table showed that the increase of cholesterol was inhibited by oyek feed treatment. The cholesterol of diabetic rats reduced 20.5% after oyek feed treatment for 3th days until 18th days. The data indicated that protein isolate of oyek was potential to controle cholesterol of diabetic patient. However the cholesterol level of all rats were still normal, that were < 200mg/dl (Anonim, 2007; Herlina et al., 2013).

HDL cholesterol

Decreashing HDL of diabetic rats could be avoided by oyek treatment, that was seen at Table 3. The HDL of diabetic rats after oyek treatment for18th days increased significantly. Although the HDL of all rat was abnormal. The normal HDL cholesterol of human according to US National Cholesterol Education Program (NCEP) was > 60mg/dl (Anonim, 2007). While the normal HDL cholesterol of rat was > 45mg/dl (Herlina et al., 2013). This fact indicated that oyek with additionof cowpeas sprout could induce the formation of HDL, so the complication of diabetic could be prevented. The result conformed with Airliss and Biermann (2002) who showed that protein isolate of soybean feed treatment could increase 50% HDL and decrease 30-40% total cholesterol. Kanetro (2015) also showed that protein isolate of cowpeas sprout could increase HDL through in vivo bioassay.

Table 3. The effect of oyek feed treatment on blood HDL cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0th days	3th days	18th days
Normal	Standard	44,16b	45,08b	40,12c
	Oyek	41,69b	40,84b	38,50bc
Diabetic	Standard	35,91a	16,16a	14,76a
	Oyek	39,34ab	19,49a	30,86b

*) The same notation of statistic in the table showed not significantly differences at the same column

LDL cholesterol

The LDL of all rats were normal that was seen at Table 4, The normal LDL cholesterol of human according to US National Cholesterol Education Program (NCEP) was < 100mg/dl (Anonim, 2007). While the normal LDL cholesterol of rat was < 135mg/dl (Herlina *et al.*, 2013). The increase of LDL after injection of aloxan at 3th conformed with the increase of total cholesterol (Table 2).

The trend was same as the diabetic rats after oyek feed treatment. The LDL of this treatment increased at 3th days and then decreased until treatment for 18th days. The LDL of diabetic rats after standard feed treatment for 18th days increased 84,4% compared with control treatment for 0th days. While The LDL of diabetics rats after oyek feed treatment increased 36% compared with control treatment for 0th days. The result indicated that oyek with addition of cowpeas sprout could inhibit the increase of LDL cholesterol.

Table 4. The effect of oyek feed treatment on blood LDL cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0th days	3th days	18th days
Normal	Standard	57,54b	62,91a	59,59a
	Oyek	58,09b	58,19a	61,59a
Diabetic	Standard	46,75a	86,20b	87,18
	Oyek	49,01a	82,78b	66,67ab

*) The same notation of statistic in the table showed not significantly differences at the same column

The Ratio of Total Cholesterol/HDL and LDL/HDL

The ratio was computed from the data of Table 2 and 3 for total cholesterol/HDL ratio and the data of Table 3 and 4 for LDL/HDL ratio, that was seen at Table 5 and 6 respectively. The ratio indicated the risk of coronary heart disease (Fernandez and Webb. 2008). The normal level of the ratio of total cholesterol/HDL was < 5 and LDL/HDL was <3.2 for women and < 3.5 for men (Chandler and Zamora, 2011). Based on the ratio of cholesterol total/HDL and LDL/HDL were known that the ratio of all the rats including diabetic rats with oyek feed treatment for 18 days were normal, exception diabetic rats with standard feed treatment.

Table 5. The effect of oyek feed treatment for 0thand 18th days on the ratio of total cholesterol/HDL *)

Rats condition	Feed treatment	0th days	18th days
Normal	Standard	2.41	2.81

	Oyek	2.55	2.92
Diabetic	Standard	2.90	10.45
	Oyek	2.63	4.32

*computed according to Table 2 and 3.

Table 6. The effect of oyek feed treatment for 0^{th} and 18^{th} days on the ratio of LDL/HDL cholesterol *)

Rats condition	Feed treatment	0^{th} days	18^{th} days
Normal	Standard	1.30	1.49
	Oyek	1.39	1.60
Diabetic	Standard	1.30	5.91
	Oyek	1.25	2.16

*computed according to Table 3 and 4.

CONCLUSIONS

The potency of hypocholesterolemic of oyek were shown by decreasing of blood triglyceride, cholesterol total, LDL, and increasing of blood HDL in all rats treatments, especially diabetics rats with oyek feed treatment. Based on the ratio of cholesterol total/HDL and LDL/HDL showed that the ratio of all the rats including diabetic rats with oyek feed treatment for18 days were normal. This result indicated that oyek with addition of cowpeas sprout flour had the potency of hypocholesterolemic and might be used to prevent diabetic complication. Oyek with addition of cowpeas sprout flour could be potential as functional food.

ACKNOWLEDGEMENTS

The Authors are grateful to Directorate of Higher Education, Republic of Indonesia that has given financial support for this research.

REFERENCES

Anonim, (2007), "Normal and high cholesterol value", available at: http://www.prolipid.com/cholesterol-reference-levels/normal-and-high-cholesterol-values.html/ (accessed 25 September 2012)

Arliss**,** R.M. and Biermann, C.A**. (**2002), "Do soy isoflavon lower cholesterol inhibit atherosclerosis and play a role in cancer prevention", Holistic Nursing Practice, Vol. 16 No. 5, pp. 40-48.

Chandler ,S. and Zamora, A. 2011, "Cholesterol-LDL-HDL-Ratio". available at: http://www.scientificpsychic.com/health/cholesterol-LDL-HDL.html/ (accessed 25 september 2012)

Damasceno, N.R., Goto, H., Fernada, M.D.R., Dias, C.T.S., Okawabata, F.S., Dulcinela, S.P., Abdalia, D.S. and Gidlund, M.A. (2000), "Soy protein isolate reduces the oxidizability of ldl and generation of oxidized ldl autoantibodies in rabbit with diet-induced atherosclerosis", Journal of Nutrition, Vol. 130 No. 11, pp. 2641-2647.

Eduardo,M., Svanberg,U., Oliveira,J., dan Ahrné,L. (2013), "Effect of cassava flour characteristics on properties of cassava-wheat-maize composite bread types". available at: http://www.hindawi.com/journals/ijfs/2013/305407/ (accessed 16 May 2016)

Fernandez,M.L. and Webb, D. (2008), "The LDL to HDL cholesterol ratio as a valuable toolto evaluate coronary heart disease risk", Journal of the American College of Nutrition Vo 27 No.1, pp.1–5.

Garrison R, 2013. Normal Rat Blood Glucose Level. http://www.ehow.com/facts_5990203_normal-rat-blood-glucose-level.html 25/9/2012

Herlina, H., Subagio A., and Estiasih T. (2013), "Hipolipidemic Potential of Water Soluble Polysaccharides from Gembili Tuber (*Dioscoreaesculenta* L.) in Hiperlipidemia Rats", Agritech J Teknol Pertanian Vol.33 No. 1, pp.8-15

Kanetro, B., Noor, Z., Sutardi, and Indrati, R. (2008), "Potency of soybean sprout protein to stimulate insulin secretin of pancrease in normal and diabetic rat", Agritech J Teknologi Pertanian Vol 28 No. 2, pp. 50-57

Kanetro, B. and Dewi,S.H.C. (2013), "Effect of various local legume sprouts as raw materials of meat analog on the physical (texture), preference and arginine/lysine ratio characteristics", Agritech J Teknologi Pertanian 33:1-7.

Kanetro, B. (2015), "Hypocholesterolemic properties of protein isolate from cowpeas (*vigna unguiculata*) sprout in normal and diabetic rats", Procedia Food Science Vol 3 No.1, pp.112 – 118.

Kanetro, B., Pujimulyani, D., Sahrah, A., and Luwihana, S. (2015), "Community empowerment of kalirejo kulonprogo special region of yogyakarta for developing oyek into artificial rice as staple food", proceedings of the international conference in University of Mercu Buana Yogyakarta, 2015, Directorate of Development and Cooperation, Yogyakarta, pp....

Reeves, P.G., Nielsen, F.H., and Fahey, G.C. (1993), "AIN-93 Purified diets for laboratory rodents: final report of the american institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet", Journal of Nutrition Vol. 123 No.11, pp.1939-1951.

ATTACHMENT :

Table 1. The effect of oyek feed treatment on blood tryglyceride of normal and diabetic rats (mg/dL) *)

Rats condition	Feed treatment	0th days	3th days	18th days
Normal	Standard	72,79a	72,92a	79,33a
	Oyek	67,15a	69,07a	75,82a
Diabetic	Standard	69,52a	117,45b	120,67c
	Oyek	69,33a	113,60b	90,30b

*) The same notation of statistic in the table showed not significantly differences at the same column

Table 2. The effect of oyek feed treatment on blood total cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0^{th} days	3^{th} days	18^{th} days
Normal	Standard	106,28a	112,15a	112,59a
	Oyek	106,46a	108,66a	112,53a
Diabetic	Standard	104,11a	152,49b	154,31c
	Oyek	103,40a	156,54b	133,45b

*) The same notation of statistic in the table showed not significantly differences at the same column

Table 3. The effect of oyek feed treatment on blood HDL cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0^{th} days	3^{th} days	18^{th} days
Normal	Standard	44,16b	45,08b	40,12c
	Oyek	41,69b	40,84b	38,50bc
Diabetic	Standard	35,91a	16,16a	14,76a
	Oyek	39,34ab	19,49a	30,86b

*) The same notation of statistic in the table showed not significantly differences at the same column

Table 4. The effect of oyek feed treatment on blood LDL cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0^{th} days	3^{th} days	18^{th} days
Normal	Standard	57,54b	62,91a	59,59a
	Oyek	58,09b	58,19a	61,59a
Diabetic	Standard	46,75a	86,20b	87,18
	Oyek	49,01a	82,78b	66,67ab

*) The same notation of statistic in the table showed not significantly differences at the same column

Table 5. The effect of oyek feed treatment for $0^{th}$and 18^{th} days on the ratio of total cholesterol/HDL *)

Rats condition	Feed treatment	0^{th} days	18^{th} days
Normal	Standard	2.41	2.81
	Oyek	2.55	2.92
Diabetic	Standard	2.90	10.45
	Oyek	2.63	4.32

*computed according to Table 2 and 3.

Table 6. The effect of oyek feed treatment for 0^{th} and 18^{th} days on the ratio of LDL/HDL*)

Rats condition	Feed treatment	0^{th} days	18^{th} days
Normal	Standard	1.30	1.49
	Oyek	1.39	1.60
Diabetic	Standard	1.30	5.91
	Oyek	1.25	2.16

*computed according to Table 3 and 4.

Phenolic contents and antioxidant activities of *Sonneratia caseolaris*

Johnny Tang Jock Lee[1], Angelie Dely[2], Madushika Ramanayake[3], Isuriy Adasuriyya[4], Irine Runnie Ginjom[5]

Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 Kuching, Sarawak, Malaysia, (johnnytang714@gmail.com)
Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 Kuching, Sarawak, Malaysia, (7440359@students.swinburne.edu.my)
Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 0 Kuching, Sarawak, Malaysia, (4315065@students.swinburne.edu.my)
Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 Kuching, Sarawak, Malaysia, (iadasuriyya@swinburne.edu.my)

Corresponding author:
Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 Kuching, Sarawak, Malaysia, (ihenry@swinburne.edu.my)

ABSTRACT

Plant-based antioxidants are playing emerging role in the prevention of chronic degenerative diseases such as cancers, cardiovascular diseases and neurodegenerative conditions. Borneo Island is home to many exotic tropical fruits, where most remain underutilised. As with most fruits and vegetables, these tropical fruits are good source of nutrients and bioactive compounds. In this paper, three edible local fruits, namely, Sonneratia caseolaris (mangrove apple or `pedada'), Lepisanthes alata (`perupok' or `engkilili'), and Baccaurea angulata (wild star fruit or `gerumin') were evaluated for their total phenolic content (TPC) and total flavonoid content (TFC). Their antioxidant activities were measured using DPPH and ABTS assays. Different sections of these fruits (pericarp and seed or aril) were lyophilised, ground into powder, and subjected to 80% methanol extraction. All results were calculated in terms of 1 g dry weight (DW) sample. The results demonstrated that S. caseolaris seed consistently demonstrated the highest phenolic contents (TPC: 104 ± 0.8 mg gallic acid eq./g; TFC: 5.6 ± 0.3 mg quercetin eq./g), and antioxidant activities (DPPH EC_{50}: 16 ± 3 mg/L; ABTS: 502 ± 22 mg Trolox eq./g), while B. angulata aril sample consistently elicited the lowest values for all assays (TPC: 2.1 ± 0.2 mg gallic acid eq./g; TFC: 1.3 ± 0.3 mg quercetin eq./g; DPPH EC_{50}: 3,338 ± 42 mg/L; and ABTS: 32 ± 3 mg Trolox eq./g). The antioxidant activity (ABTS) values are well-correlated with the total phenolic contents (TPC) for all samples. It is thus recommended that S. caseolaris should be further studied to evaluate its potential as a dietary source of antioxidant.

Keywords: Phenolic, Flavonoid, Antioxidant activity, DPPH, ABTS, *Sonneratia caseolaris, Lepisanthes alata, Baccaurea angulata.*

INTRODUCTION

Fruits and vegetables are notably known for their health promoting properties specifically due to their micronutrient, fibre and antioxidant contents. Dietary antioxidants are the exogenous antioxidants that protect the body against the damaging effects of free radicals on biological cells. As free radicals are unstable, they react readily with other molecules to achieve stability. This leads to tissue damages that can contribute toward the aetiology of several chronic diseases such as cardiovascular and inflammatory diseases, cataracts, and cancer. Antioxidant is a substance that has ability to prevent or slow down the oxidation process when present in minute amounts (Fusco *et al*., 2007). While the evidence supporting the benefit of antioxidant supplement on human health is still inconclusive, antioxidants is widely used in the food industry as a preservative (e.g. BHT and BHA) to protect food ingredients against oxidative damages. BHT and BHA are both synthetic antioxidants. While extremely effective, there are concerns over their toxicity effects upon their long term consumptions.

Plants naturally produce antioxidants, mostly in the form of polyphenols, vitamin E and C as part of their defence mechanism against UV radiation and pathogens (Kasote *et al*., 2015). These secondary metabolites are synthesized for the normal growth, development and defence of plants (Kasote *et al*., 2015). Both *in vitro* and *in vivo* studies suggest that phenolic compounds are significantly promising antioxidants (Kasote *et al*., 2015). These are generally classified into phenolic acids, flavonoids, lignans, stilbenes and tannins with flavonoids and phenolic acids making up the largest group (Scalbert and Williamson, 2000). Based on their structures, flavonoids can be further classified as anthocyanidins (e.g. cyanidin, delphinidin), flavan-3-ols (e.g. catechin, epicatechin), flavonols (e.g. quercetin, kaempferol, myricetin), flavones (e.g. apigenin, luteolin), flavanones (e.g. hesperetin, naringenin) and isoflavones (e.g. genistein, daidzein). They are excellent antioxidants through their synergistic effects with endogenous antioxidants, electron donation, reducing power and metal ion chelating abilities (Kasote *et al*., 2015).

Skin and seed of fruits are rich source of phenolic compounds (Duda-Chodak and Tarko, 2007, Xu *et al*., 2010). The skin of Black Pearl grapes can contain up to 40 mg GAE/g of total phenolic contents while the seeds of Cabernet Sauvignon grapes can have up to 99 mg GAE/g (Xu *et al*., 2010). Most local edible plants are naturally nutritious, and some could be excellent source of antioxidants. Tropical berries such as Surinam (40 mg GAE/g) and Acerola (290 mg GAE/g) cherries are also excellent source of phenolic compounds and antioxidants (da Silva *et al*., 2014). Commonly found tropical fruits that are good source of phenolic compounds are cashew apple, soursop, guava, and papaya (53 mg GAE/g, 29 mg GAE/g, 17 mg GAE/g, 13 mg

GAE/g, respectively) (da Silva *et al.*, 2014). With so much variety of less commercialised fruits remains underutilised in Sarawak, there is so much opportunities to discover the next super-antioxidant fruit.

Three underutilized local fruits, namely *Baccaurea angulata, Lepisanthes alata* and *Sonneratia caseolaris* were selected in this study. To date, there is very limited information on the phytochemical information on all fruits. *Baccaurea angulata* or wild star fruit is widely distributed in Borneo. The fruit is covered in red angular pericarp with white tangy aril. The fruit contains about 5.2 mg GAE/g of total phenolic contents (Ahmed *et al.*, 2015). Its ethanolic extract was effective against Gram negative bacteria *K. pneumoniae* (Momand *et al.*, 2014) while its juice was reported to reduce lipid peroxidation and increase antioxidant enzyme activities in rabbits fed with high-cholesterol diet (Mikail *et al.*, 2016). *Lepisanthes alata* is indigenous to Southeast Asia, commonly grown as ornamental tree in Malaysia, Indonesia and the Phillipines. Locally known as `perupok' or `engkilli', the fruit has dark reddish purple pericarp with edible white pulp. To date, there is no reported medicinal property on this fruit (Lim, 2013). Mangrove apple or `pedada' (*Sonneratia caseolaris*) has persimmon-like fruit with sepals and usually grows in deep muddy soils of mangrove in tropical and sub-tropical areas worldwide. The leaves and fruits of *S. caseolaris* contain luteolin and its glycosides (Sadhu *et al.*, 2006; Wu *et al.*, 2009). These phenolic compounds are known for their antioxidant, anti-inflammatory, anticancer, neuroprotective, and cardioprotective effects (Nabavi *et al.*, 2015). These underutilized fruits may contain a significant amount of phytochemicals which might have high antioxidant capacities, and thus, health properties.

This paper discusses the results from the study on the phenolic contents and antioxidant activities of *B. angulata*, *L. alata* and *S. caseolaris* fruits.

MATERIALS AND METHODS

A. Sample collection and preparation

Mature fruits of *L. alata and B. angulata* were collected from their natural habitats in Bau, Sarawak, while *S. caseolaris* fruit was collected from Sarikei, Sarawak. The collected fruits were immediately rinsed with clean water and air-dried. The fruits were then manually separated into sections: (i) *B. angulata* – pericarp (BAP) and aril, including seed (BAA); (ii) *L. alata* - pericarp (LAP) and seed (LAS); and (iii) *S. caseolaris* - pericarp (SCP) and seed, including placenta (SCS); cut into smaller pieces and air-dried for another 1.5 hours. The samples were weighed and stored in tight containers at -22℃ overnight. The frozen samples were lyophilised for 48 hours and the

final (dry) weights of the samples were recorded to determine their moisture contents. These were then ground into powder and stored in air-tight containers, in the dark at -22℃ until analysis.

B. Chemicals

C. Gallic acid, quercetin, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All the other chemicals used were of analytical grade.

D. Sample extraction

On the day of analysis, 10 mL of 80% aqueous methanol was added to 0.2 g of dry sample powder. The mixture was swirled briefly, sonicated for 30 minutes at room temperature, centrifuged for 10 minutes at 2500 rpm (Eppendorf centrifuge 5702, Hamburg, Germany) and finally filtered with Whatman No.1 filter paper. The extract volume was accurately adjusted to 10 mL with distilled water to make a 20,000 mg/L extract solution. All of the phenolic and antioxidant assay results were based on the dry weight mass of the powder used in the extract preparation.

When not immediately used, the extract solution was stored in tight container, in the dark at -22^{o}C, and analysed within one week. On the day of analysis, the frozen extracts were thawed and diluted (as necessary) with distilled water. Triplicate extractions were prepared for each sample (on separate weeks), and subsequently, each was separately analysed for the TPC, TFC and antioxidant activities. A batch of the extracts was dried to completeness in a refrigerated CentriVap centrifugal vacuum concentrator (Labconco, Kansas City, Mo) to determine the crude phenolic extract yield estimation (dry weight basis). The % of extraction yield was calculated as below:

$$\text{Yield (\%)} = \frac{\text{Weight of dry extract (g)}}{\text{Weight of dry sample (g)}} \times 100$$

A. Determination of total phenolic content (TPC)

The TPC of the extracts was determined using Folin-Ciocalteu assay as described by Amado *et al*. (2014) with slight modification. Briefly, on the day of analysis, the frozen extract solution was thawed and subsequently diluted (0-1000 mg/L) with distilled water. In duplicates, exactly 1 mL of the diluted extract was mixed with 100µl of Folin-Ciocalteu reagent and mixed. After 5 min, 1 ml of 7% of sodium carbonate (Na_2CO_3) solution was added and mixed. After incubation for 1 h at room temperature in the dark, the absorbance read at 760 nm using Genesys 20 Visible Spectrophotometer (ThermoFisher Scientific, Germany) against a blank (0 mg/L). This was repeated for

gallic acid standard solution (0-25mg/L) which was used to construct the reference calibration curve. The TPC was expressed as mg of gallic acid equivalent per g of lyophilised sample (mg GAE/g).

B. Determination of total flavonoid content (TFC)

The TFC of the extracts was estimated using aluminium-complexation reaction assay as described by Amado *et al.* (2014) with slight modification. Firstly, each of the frozen sample extracts was thawed and subsequently diluted (0-8000 mg/L) with distilled water. In duplicates, an aliquot of 1 mL of the diluted extract solution was accurately transferred into a disposable cuvette. Then, 1mL of 2% $AlCl_3$ was added into all cuvettes and vortexed 5s. The mixture was allowed to stand in the dark for 10 mins, and absorbance was then read at 415 nm against a blank (0 mg/L). The steps were repeated for quercetin solution (0-60 mg/L) which is the reference standard. The TFC was expressed as mg of quercetin equivalent per g of dry sample (mg QE/g).

C. Determination of antioxidant activity: DPPH assay

The antioxidant activity of the extracts was determined by using DPPH assay described by Miladi and Damak (2008) with slight modification. Briefly, a 0-10,000mg/L of extract dilutions were prepared from the 20,000 mg/L extract solution with distilled water as a diluent. Exactly 1 mL of DPPH solution (80 mg/L or 2 mM, in methanol) was incubated with 1 mL of the diluted samples. The mixture was vortexed, and kept in the dark at room temperature for 30 minutes. Then, the absorbance was measured at 517 nm against a blank (distilled water). Control sample was the sample containing 0 mg/L sample dilution. For positive comparisons, the steps were repeated for gallic acid solution (0-10 mg/L) and quercetin solution (0-15 mg/L). The radical scavenging effect (RSE) was measured as a decrease in the absorbance of DPPH after 30 min incubation. RSE was calculated using the following equation:

$$\text{RSE}\ (\%) = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

The extract concentration providing 50% RSE (effective concentration, EC_{50}) was calculated from the graph of RSE against the extract concentration. The EC_{50} was expressed as the extract concentration at 50% RSE (mg of dry sample per L of solution or mg/L).

D. Determination of antioxidant activity: ABTS assay

The antioxidant activity of the extracts was determined by using ABTS assay as described by De Beer *et al*. (2003) and Re *et al*. (1999) with slight modification. Each of the sample extracts was thawed and diluted (0-100mg/L) with distilled water. To 0.5 ml of the diluted sample, 1 mL of working ABTS radical solution (~7.73 x 10^{-2} mM) was added. After mixing and 30 mins incubation in the dark at room temperature, absorbance was measured at 734 nm against a blank (distilled water). The ABTS value was determined using standard curve prepared with Trolox (0-10 mg/L) and was expressed as mg of Trolox equivalent per g of dry sample (mg TE/g).

E. Statistics

Except for the moisture contents and extraction yield, all data collected are means of three replicates along with standard deviations. Student's T-test was performed on the means to determine whether they differed significantly ($p<0.05$). As the data was not normally distributed, Spearman's correlation coefficients were calculated in order to characterize the relationship between antioxidant capacities detected by different assays, phenolics and flavonoids content. All statistical analysis was conducted using Excel 2010 (Microsoft Office Professional Plus 2010).

RESULTS AND DISCUSSION

Moisture content and extraction yield

The moisture content of the fruit samples ranged from 66% to 92% (Table 1). For *B. angulata* and *L. alata*, the moisture contents were higher in the pericarps (BAP, LAP) and lower in the sections containing the seeds (BAA, LAS). It was the opposite for *S. caseolaris*. The SCS section has more moisture content possibly due to the presence of the moisture-rich placenta and the relatively smaller seed sizes. The difference in moisture content or solid content led to the usage of dry weights basis in comparing the results of each chemical analysis.

Table 1 here

From these dry samples, crude phenolic compounds were extracted using 80% aqueous methanol. The extraction yield ranged from 17% to 38% (Table 1), with the highest recorded in BAP and the lowest in SCP. Overall, the extraction yield for *B. angulata* in this study was slightly lower than those reported by Ahmed *et al*., (2015) who recorded

31% to 61% yield from different fruit sections. The main differences were in the use of 100% methanol and 24 h incubation at 37°C by Ahmed *et al*., (2015).

While it is generally beneficial to have greater extraction yield, the potency of those extracts in terms of phenolic contents and antioxidant activities vary, as shown in the following analytical results.

A. Total phenolic contents (TPC) and total flavonoid contents (TFC)

Table 2 shows the TPC and TFC values of the six samples. There were significant (p<0.05) differences between the TPC and TFC in the extracts of each sample. The ranking of the sample was quite consistent with SCS possessing the highest TPC and TFC, while BAA eliciting the lowest TPC and TFC (Table 2).

Table 2 here

The results suggest that the seeds and placenta of *S. caseolaris*, a `mangrove apple' are rich in phenolic compounds. Its TPC and TFC values (104 mg GAE/g and 5.6 mg QE/g, respectively) are almost two times more that the TPC and TFC values of the second ranking sample, LAP (63 mg GAE/g and 3.4 mg QE/g, respectively). This value is comparable to those found in the seed of Cabernet Sauvignon seeds (99 mg GAE/g) and higher than those found in cashew apple (53 mg GAE/g) (Xu *et al*., 2010; da Silva *et al*., 2014). *S. caseolaris* is a mangrove plant. Banerjee *et al*. (2008) stated that mangroves plants have unique adaptations to overcome the environmental stress conditions such as high salinity, high temperature, low nutrients and excessive radiation. Therefore, it is possible that the high phenolic content of *S. caseolaris* fruit is an adaptation to the extreme environment.

Based on the TFC results, the flavonoids make up 5% to 62% of the total phenolics. The different in flavonoid fraction is most likely due to the different distribution and types of phenolic compounds in different fruit sections and in different plant species (Xu *et al*., 2010; da Silva *et al*., 2014). It is also possible that the assay did not evaluate all of the flavonoids present in the sample. According to Pekal and Pyrzynska (2014), the method employed in this study was selective only for flavonols and flavones luteolin. Thus, to imply that the assay determines the total flavonoid content is actually misleading. Other flavonoids that may be present but not determined in this study include anthocyanidins, flavan-3-ols (e.g. catechins), flavanones, and isoflavones. Phenolic compounds are also made up of non-flavonoids which were not specifically measured in this study such as phenolic acids (e.g. gallic acid), and stilbenes (e.g. resveratrol).

For *B. angulata*, the recorded values of TPC and TFC in this study were relatively lower than those reported in a similar study (Ahmed *et al*., 2015), possibly due to the

variances in the fruit samples. Difference in agriculture practices, soil nutrients, weather, fruit maturity level, biotic and abiotic factors will affect the phenolic contents the fruits (Briskin and Gawienowski, 2001; Huang *et al.*, 2014; Jaafar *et al.*, 2014, Mokhtar *et al*., 2014).

B. Antioxidant activity

For the DPPH assay, lower EC_{50} values signify higher antioxidant activities, whereas for the ABTS assay, higher values mean higher antioxidant activities. Table 3 summarizes the results obtained from the antioxidant assays. Based on the results, the ranking order of antioxidant activities of the samples closely resembles those obtained for the phenolic content assays. For both DPH and ABTS antioxidant assays, SCS topped the antioxidant values of both assays (16 mg/L, 501 mg TE/g, respectively), whilst BAA ranked the lowest (3,338 mg/L, 32 mg TE/g, respectively).

Table 3 here

By ranking all the data for Spearman's correlation calculation, all variables showed positive correlations ($r_s \geq 0.600$) (Table 4). However, only TPC and ABTS values showed significant correlation at p=0.01. At p=0.05, only DPPH and TPC, and DPPH and ABTS showed significant correlations. The data strongly suggested that the four variables, especially the TPC and the ABTS, were significantly and positively correlated to each other. Similar findings was reported by Floegel et al (2011), who recommended that ABTS assay is a more useful antioxidant assay than DPPH assay in assessing antioxidant capacity in a variety of foods. This is encouraging as TPC and ABTS assays are simple to conduct and can be modified into a high throughput assay using microplate readers. Rapid preliminary screening allows for more new potential antioxidant sources to be discovered and investigated.

Table 4 here

CONCLUSIONS

With total phenolic contents equivalent to Cabernet Sauvignon grape seeds, the seed and placenta section (SCS) of *S. caseolaris* fruit can be a rich source of antioxidants. Other fruits (*L. alata* and *B. angulata*) and *S. caseolaris* pericarp also possessed phenolic compounds and antioxidant activities, but at much lower levels. While all variables show correlations with each other, it was the TPC and ABTS that showed the strongest and most significant positive correlations. This led to the conclusion that the

TPC and ABTS assays are sufficient for the preliminary screening of antioxidant capacity in food samples.

This study also showed that local fruits have equivalent, if not higher contents of phenolic compounds and antioxidants than those widely reported fruits. As these fruits are readily available at much cheaper price, they should be promoted as an alternative source of antioxidant instead of the more expensive imported fruits or supplements. As they are more widely utilised, more studies can be done on their cultivation and sustainability. This, ultimately provide a positive economic outcome for the local community and the environment.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Swinburne University of Technology Sarawak Campus for providing the funds (SSRG 2014), materials and facilities to complete this study. The authors would also like to thank Sarawak Biodiversity Centre for the permits (SBC/RES/I/0003) for plant samples collections.

REFERENCES

Ahmed, I.A., Mikail, M.A., Ibrahim, M., Hazali, N., Abdul Rasad, M.S.B., Abdul Ghani, R., Abdul Wahab, R., Arief S.J., and Adros Yahya, M.N. (2015), "Antioxidant activity and phenolic profile of various morphological parts of underutilised *Baccaurea angulata* fruit", *Food Chemistry*, Vol.172, pp. 778-787.

Amado, I.R., Franco, D., Sanchez, M., Zapata C, and Vazquez JA (2014), "Optimisation of antioxidant extraction from *Solanum tuberosum* potato peel waste by surface response methodology", *Food Chemistry*, Vol. 165, pp.290-299.

Benerjee, D., Chakrabarti, S., Harza, A.K., Banerjee, S., Ray, J., and Mukherjee, B. (2008), "Antioxidant activity and total phenolic of some mangroves in Sundarbans", *African Journal of Biotechnology*, Vol. 7 No. 6, pp.805-810.

Briskin, D.P. and Gawienowski, M.C. (2001), "Differential effects of light and nitrogen on the production of hypericins and leaf glands in *Hypericum perforatum*", *Plant Physiology*, Vol. 39, pp.1075-1081.

da Silva, L.M.R, de Figueiredo, E.A.T., Ricardo, N.M.P.S., Vieira, I.G.P., de Figueiredo, R.W., Brasil, I.M., and Gome, C.L. (2014), "Quantification of bioactive compounds in pulps and by-products of tropical fruits from Brazil", *Food Chemistry*, Vol.143, pp. 398-404.

De-Beer, D., Joubert E., Gelderblom, W.C.A., and Manley, M. (2003), "Antioxidant activity of South African red and white cultivar wines: free radical scavenging", *Journal of Agricultural and Food Chemistry*, Vol. 51, pp. 902-909.

Duda-Chodak, A. and Tarko, T. (2007), "Antioxidant properties of different fruit seeds and peels", *ACTA Scientiarum Polonorum Technologia Alimentaria*, Vol. 6 No. 3, pp. 29-36.

Floegel, A., Dae-Ok, K., Sang-Jin, C., Sung-I, K.. and Ock-K, C. (2011), "Comparison of ABTS/ DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods", *Journal of Food Composition and Analysis*, Vol. 24 No. 7, pp. 1043-1048.

Fusco, D., Colloca, G., Monaco, L.M.R. and Cesari M (2007), "Effects of antioxidant supplementation on the aging process", *Clinical Interventions in Aging*, Vol. 2 No. 3, pp. 377-387.

Huang, H., Sun, Y., Lou, S., Li, H. and Ye, X. (2014), "*In vitro* digestion combined with cellular assay to determine the antioxidant activity in Chinese bayberry (*Myrica rubra* Sieb. Et Zucc.) fruits: A comparison with traditional methods", *Food Chemistry*, Vol. 146, pp. 363-370.

Jaafar, H.Z.E., Mohamed, H.N.B. and Rahmat, A. (2008), "Accumulation and partitioning of total phenols in two varieties of *Labisia pumila* Benth. under manipulation of greenhouse irradiance", *Acta Horticulturae*, Vol. 797, pp. 387-392.

Kasote, D.M., Katyare, S.S., Hegde, M.V., Bae, H. (2015), "Significance of antioxidant potential of plants and its relevance to therapeutic applications", *International Journal of Biological Sciences*, Vol. 11 No. 8, pp. 982-991.

Lim, T.K. (2013), "*Lepisanthes alata*", in Lim, T.K. (Ed.), Edible medicinal and non-medicinal plants, Springer, Dordrecht Heidelberg New York London, pp. 39-41.

Mikail, M.A., Ahmed, I.A., Ibrahim, M., Hazali, N., Rasad, M.S.B.A.R., Ghani, R.A., Wahab, R.A., Arief, S.J., Isa, M.L.M., Draman, S., Ishola, A.A. and Yahya, M.N.A.

(2014), “Changes in the markers of atherosclerosis following administration of Belimbing Dayak (*Baccaurea Angulata)* fruit juice in experimental rabbits fed with cholesterol diet”, *Journal of Advances in Agricultural and& Environmental Engineering*, Vol. 1 No. 1, pp. 151-154.

Miladi, S. and Damak, M. (2008), “*In vitro* antioxidant activities of Aloe vera leaf skin extracts”, *Journal de la Societe Chimique de Tunisie*, Vol. 10, pp. 101-109.

Mokhtar, S.I., Leong, P.C., Ven, L.E. and Aziz, N.A.A. (2014), “Total phenolic contents, antioxidant activities and organic acids composition of three selected fruit extracts at different maturity stages”, *Journal of Tropical Resources and Sustainable Science*, Vol. 2, pp. 40-46.

Momand, L., Zakaria, R., Mikail M., Jalal, T., Ibrahim, M. and Wahab, R.A. (2014), “Antimicrobial effect of *Baccaurea angulata* fruit extracts against human pathogenic microorganisms”, *Merit Research Journal of Medicine and Medical Sciences*, Vol. 2 No. 10, pp. 229-237.

Nabavi, S.F., Braidy, N., Gortzi, O., Sobarzo-Sanchez, E., Daglia, M., Skalicka-Woźniak, K. and Nabavi, S.M. (2015), “Luteolin as an anti-inflammatory and neuroprotective agent”, *Brain Research Bulletin*, Vol. 119 Part A, pp. 1-11.

Pekal, A. and Pyrzynska, K. (2014), “Evaluation of aluminium complexation reaction for flavonoid content assay”, *Food Analytical Methods*, Vol. 7, pp. 1776-1782.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999), “Antioxidant activity applying an improved ABTS radical cation decolorization assay”, *Free Radical Biology and Medicine*, Vol. 26, pp.1231-1237.

Sadhu, S.K., Ahmed, F., Ohtsuki, T. and Ishibashi, M. (2006), “Flavonoids from *Sonneratia caseolaris*”, *Journal of Natural Medicine*, Vol. 60, pp. 264-265.

Scalbert, A. and Williamson, G. (2000), “Dietary intake and bioavailability of polyphenols”, *The Journal of Nutrition*, Vol. 130 No. 8, pp.2073-2085.

Wu, S., Wen, Y., Li, X., Zhao, Y., Zhao, Z , Hu, J. (2009), “Chemical constituents from the fruits of *Sonneratia caseolaris* and *Sonneratia ovata* (Sonneratiaceae)”, *Biochemical Systematics and Ecology*, Vol. 37 No 1, pp. 1-5.

Xu, C., Zhang, Y., Cao, L., Lu, J. (2010), “Phenolic compounds and antioxidant properties of different grape cultivars grown in China”, *Food Chemistry*, Vol. 119, pp. 1557-1565.

ATTACHMENTS: TABLES

Table 6 Moisture contents and extraction yields of different sections of *L. alata, B. angulata* and *S. caseolaris*.

Sample	Moisture content, (%)	Extraction yield, (%, d.w.b)
B. angulata pericarp (BAP)	92	38
B. angulata aril and seed (BAA)	70	28
L. alata pericarp (LAP)	79	32
L. alata seed (LAS)	66	25
S. caseolaris seed and placenta (SCS)	80	26
S. caseolaris pericarp (SCP)	75	17

Values were determined from a single determination.

Table 7 Total phenolic contents (TPC) and total flavonoid contents (TFC) of different sections of *L. alata, B. angulata* and *S. caseolaris*.

Sample	TPC (mg GAE/g dw)	TFC (mg QE/g dw)
S. caseolaris seed and placenta (SCS)	103.9 ± 0.8^{a}	$5.6 \pm 0.3^{a,c}$
L. alata pericarp (LAP)	62.7 ± 2.5^{b}	3.4 ± 0.1^{b}
L. alata seed (LAS)	38.1 ± 0.8^{c}	1.9 ± 0.1^{d}
S. caseolaris pericarp (SCP)	20.4 ± 0.2^{d}	4.4 ± 0.1^{e}
B. angulata pericarp (BAP)	6.30 ± 0.3^{e}	3.3 ± 0.1^{f}
B. angulata aril and seed (BAA)	2.10 ± 0.2^{f}	1.3 ± 0.3^{g}

Values are expressed as mean ± standard deviation (n=3). Means in a column followed by different letters differ significantly ($P<0.05$). All results are per g of dry sample weight.

Table 3 Antioxidant activity as determined using DPPH and ABTS assays of different sections of *L. alata, B. angulata* and *S. caseolaris*.

Sample	DPPH assay	ABTS assay

	EC_{50} value (mg/L)	(mg TE/g dw)
S. caseolaris seed and placenta (SCS)	16 ± 3^{a}	501.5 ± 21.9^{a}
L. alata seed (LAS)	$76 \pm 6^{c,d}$	178.8 ± 17.8^{c}
L. alata pericarp (LAP)	113 ± 24^{d}	$220.0 \pm 19.6^{c,d}$
S. caseolaris pericarp (SCP)	117 ± 11^{e}	143.6 ± 11.0^{e}
B. angulata pericarp (BAP)	$1{,}615 \pm 75^{f}$	41.8 ± 3.2^{f}
B. angulata aril and seed (BAA)	$3{,}338 \pm 42^{g}$	31.7 ± 2.5^{g}
Gallic acid	2.3 ± 0.3^{h}	-
Quercetin	5.4 ± 1.4^{i}	-

Values are expressed as mean ± standard deviation (n=3). Means in a column followed by different letters differ significantly ($P<0.05$).

Table 4 Spearman's correlation coefficients (r_s) for the association between antioxidant activities as measured by DPPH and ABTS assays, total phenolic contents (TPC) and total flavonoids (TFC) of different sections of *L. alata, B. angulata* and *S. caseolaris.*

	DPPH ($1/EC_{50}$)	ABTS	TPC	TFC
DPPH ($1/EC_{50}$)	1.000	0.943	0.943	0.600
ABTS	0.943	1.000	1.000	0.714
TPC	0.943	1.000	1.000	0.714
TFC	0.600	0.714	0.714	1.000

Alpha-Glucosidase Inhibitory Effect of Methanolic Extracts from Indonesian Plants

Filiana Santoso[1], Chelsea Soedarma[1], Gregorius Herdiazto Adiyoga[1], Maria Dewi Puspitasari Tirtaningtyas Gunawan-Puteri[1], Hery Sutanto[1]

[1]Department of Food Technology, Faculty Life Sciences and Technology, Swiss German University, EduTown-BSD City, Tangerang, 15339, Indonesia, (filiana.santoso@sgu.ac.id)

ABSTRACT

Alpha-glucosidase inhibitors (AGIs) are oral anti-diabetic drugs used for type-2 diabetes treatment by retarding the carbohydrate digestion. There are numerous natural products with AGI action, however, only limited number of Indonesian plants have been studied for their AGIs potency. In this research, 57 samples of plants naturally grown in Indonesia were tested in vitro for their AGIs activities. Some of them are traditionally utilized for diabetes treatment. The study showed that the methanolic extracts of gambir fruit (Uncaria gambir), banana leaf (Musa paradisiaca), lemongrass stem (Cymbopogon citratus), bitter gourd fruit (Momordica charantia), and onion bulb (Allium cepa L.) exhibited the highest AGIs activities at 0.02 g/ml concentration. The gradient extraction using n-hexane, ethyl acetate, and butanol resulted in ethyl acetate and butanol fractions showing high AGIs activity, indicating that the AGIs activities in these five samples were influenced by the existence of semi-polar to polar compounds. Antioxidant activity, total phenolic and flavonoid contents were also evaluated. Gambir fruit exhibited the highest antioxidant activity followed by onion bulb, banana leaf, bitter gourd fruit, and lemongrass stem with IC_{50} of 101.75 ppm, 1,963.57 ppm, 2,377.64 ppm, 5,859.14 ppm and 5,910.14 ppm, respectively. Total phenolic and flavonoid analysis showed that the activity of gambir fruit was supported by the high content of phenolic (77.56 mg GAE/g), while the onion bulb exhibited the highest flavonoid content (8.94 mg QE/g). Positive correlation of antioxidant activity and total phenolic content with AGIs was found, however, there was no correlation between AGIs and total flavonoid content.

Keywords : alpha-glucosidase inhibitors, Indonesian plants, diabetes, antioxidant

INTRODUCTION

Diabetes mellitus (DM) is a metabolic, chronic disease that has become a significant public health problem and is one of four priority non-communicable diseases targeted for action by world leaders. Over the past few decades both the number of cases and the prevalence of diabetes have been steadily increasing and is growing most rapidly in low- and middle-income countries (World Health Organization, 2016). The estimated number of adult suffering from diabetes worldwide in 2015 is 415 million and expected to increase to 642 million in 2040, if the rise is not halted (International Diabetes Federation, 2015). Indonesia is ranked one of the world's top five worst affected nations for people living with diabetes, together with China, India, Brazil and the United States (World Health Organization, 2016). One of well-known oral treatments for type 2 diabetes is the use of α-glucosidase inhibitors (AGIs) drugs. α-Glucosidase (EC 3.2.1.20), an enzyme located in the brush-border surface membrane of intestinal cells, is responsible for the hydrolysis of polysaccharide or disaccharides into monosaccharide in the small intestine. Studies showed that glucose absorption was retarded by inhibiting the catalytic activity of a-glucosidase and thus, lowered the effect on postprandial blood glucose and insulin levels (Robinson *et al.*, 1991; Braun *et al.*, 1995; Dwek *et al.*, 2002). Consumption of AGIs that reversibly inhibit α-glucosidases, such as maltase and sucrase in the intestine, delayed carbohydrate digestion and hence, sugar absorption from the gut (Campbell *et al.*, 1996; Kumar *et al.*, 2011). The effects of monotherapy with AGIs for patients with type 2 diabetes were reviewed (Laar *et al.*, 2005). No evidence for an effect on mortality or morbidity was found. Furthermore, AGIs exhibited significant beneficial effects on glycemic control and postload insulin levels, but not on plasma lipids. These indicate the possible use of AGIs as a first-line agent or in combination with other antihyperglycemic drugs.

In the prevention and treatment of diabetes and obesity, plant-based medicines and functional foods affecting beneficial physiological effects have gained high interest in the last decades. There were many studies conducted with the aim to search for effective and safe AGIs from natural sources (Matsui *et al.*, 2001; Tundis *et al.*, 2010; Gunawan-Puteri *et al.*, 2012; Yonemoto *et al.*, 2014). Many results showed significant antihyperglycaemic effect with slight or no side effects. Therefore, natural AGIs from plant sources offers a potential strategy for the control of hyperglycaemia. Indonesia is the second largest biodiversity in the world after Brazil, with around 40,000 endemic plant species including 6,000 medicinal plants. Unfortunately, numerous Indonesian medicinal plants potency still remain unknown until now. According to Aditama, until October 2014 only 41 standardized herbal medicines and 6 phytopharmaca were listed in the National Agency of Drug and Food Control (Aditama, 2015). As continuation of the screening of Indonesian plants for their AGIs potency, in this research 57 natural plant samples were

investigated. The determination of prospective plants were assessed *in vitro* for their inhibitory effect using rat intestinal glucosidase. Furthermore, the antioxidant activity, total phenolic and total flavonoid content were also examined, and their correlation to AGIs activity assessed.

MATERIALS AND METHOD

Materials

The fresh or dried leaves, barks, fruits, seeds, or bulbs of 57 samples were obtained from CV Sekar Utami, Jakarta, CV Karya Tama, Lampung and Pasar Modern BSD, Tangerang. Rat intestinal acetone powder and DPPH (2,2-diphenyl-1-picrylhydrazyl) were commercially available from Sigma Aldrich, Germany. K.tartaric acid, quercetin, gallic acid, NaOH, folin-ciocaltau phenol reagent, glucose and sucrose were supplied from Merck, Germany. All other chemicals used were purchased from PT. Bratachem, Indonesia, unless otherwise stated. The samples used are shown in Table 1.

Table 1. Samples for AGIs evaluation

Sample preparation

Fresh samples were firstly cut and dried at 50 °C for 24h. Five grams (dry weight) of each sample was subjected to 100 mL of 50% (v/v) aqueous methanolic extraction for 24h at room temperature. The crude extract was obtained by vacuum filtration through filter paper (Whatman No. 5C, 70 mm) and was evaporated to dryness using rotary evaporated under reduced pressure at 50 °C and redissolved with 50% (v/v) aqueous dimethyl sulfoxide. Samples were kept in a dark place at 4 ± 2 °C prior to the glucosidase inhibitory activity assay.

Glucosidase inhibitory activity assay

The rat intestinal glucosidase inhibitory activity was determined using the method described previously with slight revision (Jong-Anurakkun *et al.*, 2007). Rat intestinal acetone powder was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, and centrifuged at 10,000 rpm (4 °C, 60 min). The crude enzyme solution obtained from the supernatant was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0). The final crude enzyme solution showed specific activities (0.137 unit/mg protein), which was measured by using sucrose as a substrate. The inhibitory activity against sucrose hydrolysis was measured by the following procedures. Two test tubes, as sample and control, containing 200 µl sucrose solution (56 mM) in potassium phosphate buffer (0.1 M, pH 7) and two test tubes, containing 400 µl potassium phosphate buffer (0.1 M, pH 7) as each blank were pre-incubated at 37 ^{o}C for 5 min. The control and control blank defined as 100 % and 0 % enzyme activity, respectively. The working samples diluted in 50 % DMSO (100 µl) were added to the sample and sample blank test tubes while 50 % DMSO (100 µl) was added to the control and control blank test tubes. And then crude rat intestinal glucosidase (200 µl) was added only to the test tubes containing sucrose solution (sample and control). The reaction was carried out at 37 ^{o}C for 15 min

and stopped by adding Tris-HCl buffer (2 M, pH 6.3, 750 µl). Procedures for inhibitory activity assay against maltose hydrolysis were basically the same as above except for replacing sucrose solution (56 mM, 200 µl) with maltase solution (3.5 mM, 350 µl) and for reducing the amount of enzyme solution from 200 µl to 50 µl. The reaction mixtures were then passed through a short column of aluminium oxide 60 (5 x 500 mm, 1.5 g) for removing phenolics which may interfere with the following glucose quantification. Each mixture was taken 0.5 ml to other test tubes and was added with 0.5 ml DNS solution. All mixtures were incubated for 5 minutes in boiling water. The absorbance was measured using UV-Vis spectrophotometer at 540 nm wavelength. Inhibitory activity was calculated by the following equation:

$$Inhibitory\ activity\ (\%) = \frac{(Abs\ control - Abs\ control\ blank) - (Abs\ sample - Abs\ sample\ blank)}{(Abs\ control - Abs\ control\ blank)} \times 100\%$$

The experiments were done in duplicate, and the results were presented as % inhibition, which is defined as the AGIs activity at certain concentration under the assay conditions.

Analysis of α-glucosidase inhibiting principle in relation to its antioxidant activity, total flavonoid content and total phenolic content

Five samples with the highest inhibitory activity were further extracted in gradient extraction system and were analyzed for its antioxidant activity, total phenolic content, and total flavonoid content. Dried samples (100 g) was macerated for 24 hours in n-hexane. The crude extract was filtered using vacuum filtration and the filtrate was referred as hexane extract. The remaining solid materials were re-extracted again with ethyl acetate, and the remaining solid from ethyl acetate extract was then filtrated with n-butanol. The evaluation of AGIs activity was done using rat intestinal glucosidase inhibitory assay with the same procedure in the screening of AGIs. Antioxidant activity was analyzed using DPPH radical scavenging activity, total phenolic content was measured using Folin-Ciocalteau assay, while total flavonoid was measured using aluminium chloride assay.

RESULTS AND DISCUSSION

Screening of AGIs

In this study, 57 samples from 56 species and 43 families were examined. Among them, 8 samples showed high inhibitory activity at concentration of 0.10 g/ml and five samples have more than 50% AGIs activity (Figure 1.(a)). The five samples, namely UG, MP, CC, MC, and AC were further observed in gradient eluent systems. The gradient eluent using solvents with different polarity (hexane, ethyl acetate and butanol) allows rough separation of the samples based on its general polarity. AGIs activity in each extract allowed prediction of the nature of active compounds and suitable fractionation system. The extraction yield of the samples in different solvent ranged from 0.9 to 12.5 mg/g (Table 2). The amount of extracted yield indicates that many compounds inside the

samples were semi polar and polar compounds since ethyl acetate and butanol showed higher yield compared to hexane.

Figure 1. (a) AGIs activity of Uncaria gambir (UG), Musa paradisiaca (MP), Cymbopogon citratus (CC), Momordica charantia (MC), Allium cepa L. (AC), Brassica oleracea (BO), Piper cocatum (PC), Luffa acutangula (LA), and Typhonium flagelliform (TF). (b) AGIs activity of gradient-extracted fractions of UG, MP, CC, MC, and AC

AGIs activity assay for each extract in the gradient extraction of the five samples resulted in the finding of ethyl acetate and butanol extracts with higher AGIs activity compared to hexane extracts (p-value < 0.05, Figure 1.(b)). The result was in line with previous research in isolating the active compound for AGIs from several plants, which showed that ethyl acetate fraction showed the higher AGIs activity among other fractions (Laar, 2005; Dewoto, 2007). The finding indicates that the compound(s) responsible for AGIs was probably semi-polar or polar compound(s), since it has higher AGIs activities in ethyl acetate and butanol extracts. However, the hexane extract of MC showed the highest AGIs activity, followed by ethyl acetate extract and butanol extract of MC with AGIs activity value of 76.70, 63.65 and 0 % respectively. Matsuur *et al.* found that the active compound from methanolic extract of the bitter melon seeds that effectively inhibited alpha-glucosidase was D-(+)-Trehalose (Matsuur *et al.*, 2002). This indicates that there might be other non-polar compound responsible for AGIs activity in hexane fraction.

Table 8.Yield gradient extraction of UG, MP, CC, MC and AC

Antioxidant activity, total flavonoid content and total phenolic content

Compound(s) that is responsible for AGIs activity might also contribute to antioxidant activity (Apea-Bah *et al.*, 2009). Therefore, DPPH radical scavening activity assay was used to determine the antioxidant activity of the samples. IC_{50} value indicates the concentration of extracts that is needed to reach 50% inhibition to the DPPH radical. The smaller IC_{50} value means less amount of concentration needed to reach 50% inhibition or in other words the antioxidant activity is stronger. Statistical analysis was used to determine the samples with the highest antioxidant activity and the result showed that UG has the higest antioxidant activity followed by AC and MP, afterwards CC and MC (p-value < 0.05). The result was in line with Apea-Bah *et al.* that catechin, which is a powerful antioxidant in neutralizing free radicals (Braichu *et al.*, 2013), was the major bioactive compound present in UG (Apea-Bah *et al.*, 2009). In addition, there was a strong correlation between AGIs and antioxidant activity (r = 0.973). This indicates that the increase in the overall AGIs activity of the samples can be attributed to the increase of antioxidant activity. This also indicates that the chemical compound(s) contributing to AGIs activity also exhibits antioxidant activity. This was in agreement with previous research that there was a strong correlation of AGIs activity and antioxidant activity of some selected medicinal plants in Malaysia (Sugiwati *et al*, 2009). Compared to UG, MC, CC and MP, AC has the lowest AGIs activity. However, the antioxidant activity is moderately high compared to other extracts. Therefore, AC was not included in the correlation of AGIs and antioxidant activity. It could be assumed that in AC, the compound(s) responsible for AGIs activity was not the same with the compound(s) responsible for antioxidant activity.

Table 9. AGIs activity (0.02 g/ml), phenolic content, flavonoid content, and antioxidant activity of UG,MP, CC, MC, and AC

Phyoconstituents of plants such as phenolic and flavonoid compound also contribute to AGIs activity (Kumar *et al.*, 2011). The total phenolic content of the samples extracts ranged between 6.14 to 67.57 mg GAE/g (Table 3). ANOVA single factor showed that the total phenolic content between the crude extracts has highly significant difference with p-value <0.05. The plant extracts which has the highest phenolic content was UG fruit extract, followed by AC, MC and MP, and the least was CC extract (p-value <0.05). The total phenolic content obtained by Kassim *et al.* from methanolic extract of UG leaves was higher (99.25 mg GAE/g) than that of UG fruit obtained in this study (67.60 mg GAE/g). This confirms the fact that different part of plant contains different amount of phenolic compounds. The correlation between AGIs activity and total phenolic content showed moderate positive correlation with r = 0.798. The positive correlations indicate that the increase in AGIs activity can be attributed with the amount of phenolic compounds. This was in agreement with previous research that there was a significant correlation between AGIs and total phenolic content in selected medicinal plants in Nigeria (Manaharan, *et al.*, 2012).

The flavonoid content of the extracts ranged between 1.00 to 8.94 mg QE/g (Table 3). The statistical analysis indicated that the amount of flavonoid content in AC, MP, UG were significantly different to MC and CC (p-value < 0.05). Among the samples, AC bulb extract had the highest flavonoid content. The correlation between AGIs activity and total flavonoid content value showed a moderate negative correlation (r = -0.538). However, the statistical analysis revealed that the correlation was not significant (p-value > 0.05). Therefore, no correlation between AGIs activity and total flavonoid content was found. This indicates flavonoid compound do not contribute to AGIs activity. This was in agreement with previous research that there was no correlation between total flavonoid content and AGIs activity (Adefegha *et al.*, 2012).

CONCLUSIONS

Among those plants, the methanolic extract of Uncaria gambir, Musa paradisiaca, Cymbopogon citratus, Momordica charantia and Allium cepa L. showed highest AGIs activities 100%, 83%, 73%, 71%, and 62% respectively at concentration 0.02 g/ml. The gradient extraction of the five samples with the highest AGIs activity showed that the ethyl acetate fraction extract had the highest AGIs. This indicates that the compound(s) responsible for the inhibitory effect is most likely to be semi-polar or polar. UG also had the strongest antioxidant activity (DPPH IC_{50} = 101.75 ppm) and highest total phenolic content (67.57 mg GAE/g). This confirmed the positive correlation between AGIs activity with antioxidant activity and total phenolic content, which indicates that there might be the same compound(s) responsible for both activities. On the other hand, no correlation was found between AGIs activity and total flavonoid content. *The AGIs activity of Allium cepa L. was indicated to be supported by the high content of flavonoid (8.94 mg QE/g).*

REFERENCES

Adefegha. S.A., Oboh, G. (2012), "In vitro inhibition activity of polyphenol-rich extracts from *Syzygium aromaticum* (L.) Merr. & Perry (clove) buds against carbohydrate hydrolyzing enzymes linke to type 2 diabetes and Fe-induced lipid peroxidation in rat pancreas", *Asian Pacific Journal of Tropical Biomedicine*, Vol. 2 No. 10, pp. 774-781.

Aditama, T.Y. (2015), "Jamu dan Kesehatan Edisi II", Badan Penelitian dan Pengembangan Kesehatan, available at: http://terbitan.litbang.depkes.go.id/penerbitan/index.php/blp/catalog/download/160/179/372-1 (accessed 9 June 2016)

Apea-Bah, F.B., Hanafi, M., Dewi, R., Fajriah, S., Darmawan, A., Artanti, N., *et al.* (2009), "Assessment of the DPPH and a-glucosidase inhibitory potential of gambier and qualitative identification of major bioactive compound", *Journal of Medicinal Plants Research*, Vol. 3 No. 10, pp. 736-757.

Braichu, C., Ladomery, M.R>, Chedea, V.S. (2013). "The relationship between the structure and biological actions of green tea catechins", *Food Chemistry*, Vol. 141 No. 3, pp. 3282-3289.

Braun, C., Brayer, G.D., Withers, S.G. (1995), "Mechanism-based inhibition of yeast α-glucosidase and human pancreatic α-amylase by a new class of inhibitors. 2-Deoxy-2,2-difluoro-α-glycosides", *The Journal of Biological Chemistry*, Vol. 270, pp. 26778-26781.

Campbell, L.K., White, J.R., Campbell, R.K. (1996), "Acarbose: Its role in the treatment of diabetes mellitus", *The Annals of Pharmacotherapy*, Vol.30, pp. 1255-1262.

Dewoto, H.R. (2007), "Pengembangan obat tradisional Indonesia menjadi fitofarmaka", *Majalah Kedokteran Indonesia*, Vol. 57.

Dwek, R.A., Butters, T.D., Platt, F.M., Zitzmann, N. (2002), "Targeting glycosylation as a therapeutic approach", *Nature Reviews Drug Discovery*, Vol. 1, pp. 65-75.

Gunawan-Puteri, M.D.P.T., Kato, E., Kawabata, J. (2012), "α-Amylase inhibitors from an Indonesian medicinal herb, *Phyllanthus urinaria*", *J. Sci. Food. Agric.*, Vol. 92 No. 3, pp. 606-609.

International Diabetes Federation (2015), "IDF Diabetes Atlas 7th Edition", International Diabetes Federation, available at: http://www.diabetesatlas.org/ (accessed 9 June 2016)

Jong-anurakkun, N., Bhandari, M.R., Kawabata, J. (2007), "α-Glucosidase inhibitors from Devil tree (*Alstonia scholaris*)", *Food Chemistry* Vol. 103, pp. 1319-1323.

Kassim, M., Hussin, M., Achmad, A., Dahon, H., Suan, K., Hamdan, H. (2011), "Determination of total phenol, consensed tannin and flavonoid contents and antioxidant activity of *Uncaria gambier* extracts", *Majalah Farmasi Indonesia*, Vol. 22, pp. 50-59

Kumar, S., Narwal, S., Kumar, V., Prakash, O. (2011), "α-Glucosidase inhibitors from plants: A natural approach to treat diabetes", *Pharmacogn. Rev.* Vol. 5, pp. 19-29.

Laar, F.A. van de, Lucassen, P.L., Akkermans, L.P., Lisdonk, E.H. van de, Rutte, G.E., Weel, C. (2005), "α-Glucosidase inhibitors for patients with type 2 diabetes", *Diabetes Care*, Vol. 28 No. 1, pp. 154-163.

Manaharan, T., Palanisamy, U.D., Ming, C.H. (2012), "Tropical plant extracts as potential antihyperglycemic agents", *Molecules*, Vol. 17, pp. 5915-5923.

Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N., Matsumoto, K. (2001), "α-Glucosidase inhibitory action of natural acylated anthocyanins. 1. Survey of natural pigments with potent inhibitory activity", *Journal of Agricultural and Food Chemistry*, Vol. 49, pp. 1948-1951.

Matsuur, H., Asakawa, C., Kurimoto, M., Mizutani, J. (2002), "Alpha-glucosidase inhibitor from the seeds of balsam pear (Momordica charantia) and the fruit bodies of Grifola frondosa", *Bioscience Biotechnology and Biochemistry*, Vol. 66 No. 7, pp 1576-1578.

Robinson, K.M., Begovic, M.E., Rhinehart B.L., Heineke, E.W., Ducep, J.B., Kastner, P.R., *et al.* (1991), "New potent α-glucohydrolase inhibitor MDL 73945 with long duration of action in rats", *Diabetes*, Vol. 40, pp. 825-830.

Sugiwati, St., Setiasih, S., Afifah, E., (2009), "Antihyperglycemic activity of the mahkota dewa leaf extracts as an alpha-glucosidase inhibitor", *Makara Kesehatan*, Vol. 13 No. 2, pp. 74-78

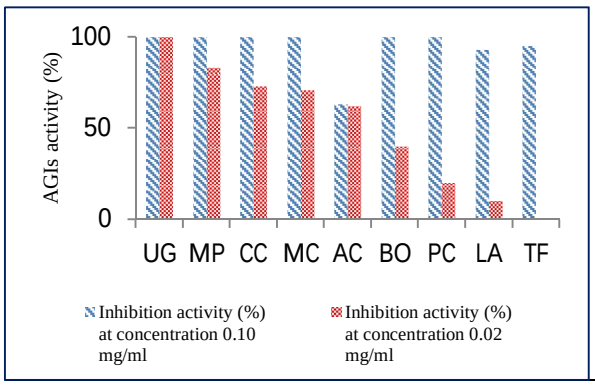
Tundis, R., Loizzo, M.R., Menichini, F. (2010), "Natural products as α-amylase and α-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: an update", *Mini Rev. Med. Chem.*, Vol. 10 No. 4, pp. 315--331

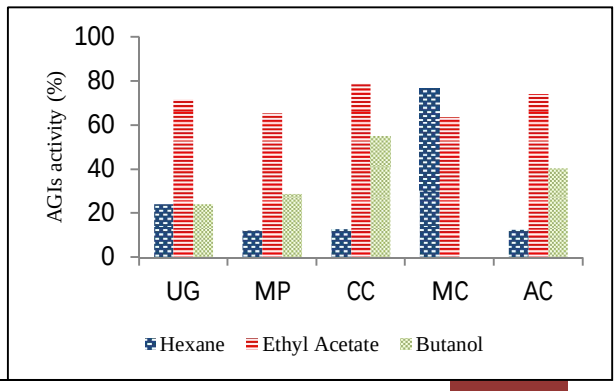
Wild, S., Roglic, G., Green, A., Sicree, R., King, H. (2004), "Global prevalence of diabetes", *Diabetes Care*, Vol. 27 No. 5, pp. 1047-1053.

World Health Organization (2016), "Global report on diabetes", World Health Organization, available at: http://apps.who.int/iris/bitstream/10665/204871/1/9789241565257_eng.pdf (accessed 8 June 2016).

Yonemoto, R., Shimada, M., Gunawan-Puteri, M.D.P.T., Kato, E., Kawabata, J. (2014), "α-Amylase inhibitory triterpene from *Abrus precatorius* leaves", *Journal of Agricultural and Food Chemistry*, Vol. 62, No. 33, pp. 8411-8414.

Attachment : Figures and Tables





(a) (b)

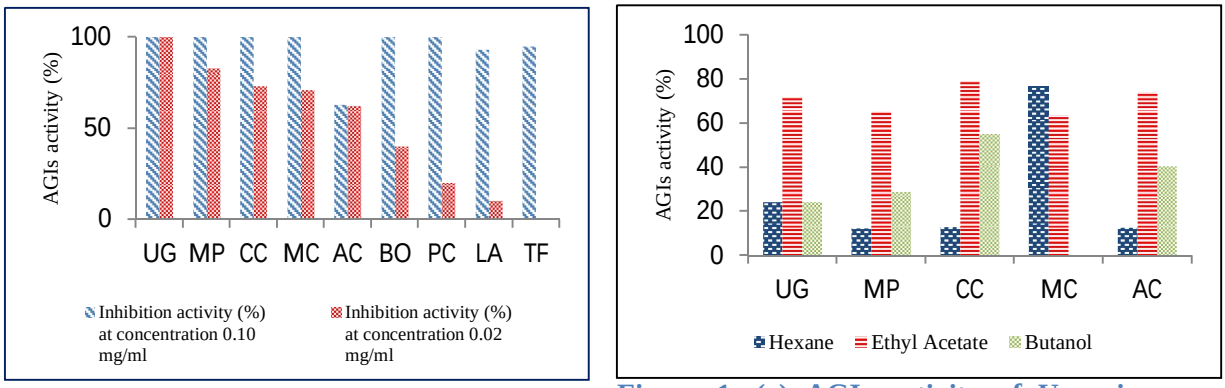


Figure 1. (a) AGIs activity of Uncaria gambir (UG), Musa paradisiaca (MP), Cymbopogon citratus (CC), Momordica charantia (MC), Allium cepa L. (AC), Brassica oleracea (BO), Piper cocatum (PC), Luffa acutangula (LA), and Typhonium flagelliform (TF). (b) AGIs activity of gradient-extracted fractions of UG, MP, CC, MC, and AC

Table 10. Samples for AGIs evaluation

Scientific name	Parts	Scientific name	Parts	Scientific name	Parts
Abelmochus esculentus	Fruit	*Coriandrum sativum L.*	Fruit	*Phaleria macrocarpa*	Fruit
Aleurites moluccana	Fruit	*Cuminum Cyminum L*	Seed	*Phyllanthus niruri*	Leaf
Allium Cepa	Bulb	*Cymbopogon citrates*	Stem	*Physalis angulata L.*	Leaf
Allium cepa L.(var Aggregatum)	Bulb	*Durio zibethinus*	Skin	*Piper betle L.*	Leaf
Aloe vera	Skin	*Eichhornia crassipes*	Leaf	*Piper cocatum*	Leaf
Amomum compactum	Fruit	*Elettaria cardamomum*	Fruit	*Plantago major L*	Leaf
Ananas comocus	Leaf	*Euphoria Longana*	Skin	*Quisqualis indica L*	Leaf
Andrographis paniculata	Leaf	*Garcinia mangostana*	Skin	*Ruellia tuberosa L.*	Leaf
Annona muricata L	Leaf	*Guazuma ulmifolia*	Leaf	*Ruellia tuberosa L.*	Leaf
Artocarpus altilis	Leaf	*Gynura procumbens*	Leaf	*Senna Alata*	Leaf
Azadirachta indica	Leaf	*Illicium Verum*	Flower	*Senna Alexandrina*	Leaf

Brassica oleracea	Stem	*Luffa acutangula*	Fruit	*Stachytarpheta mutabilis*	Leaf
Catharanthus roseus L.	Leaf	*Momordica charantia*	Fruit	*Swietenia macrophylla*	Leaf
Centella asiatica	Leaf	*Morus alba L.*	Leaf	*Tinospora crispa L.*	Leaf
Cinnamomum verum	Bark	*Musa paradisiacal*	Skin	*Trigonella foenum-graecum*	Seed
Citrullus lanatus	Skin	*Nicotiana tabacum*	Leaf	*Typhonium flagelliforme*	Leaf
Citrus nobilis (var. Microcarpa)	Seed	*Ocimum sanetum*	Leaf	*Uncaria gambir*	Fruit
Clinacanthus nutans L.	Leaf	*Persea americana*	Skin	*Zingiber zerumbet*	Leaf
Clitoria ternatea	Stem	*Persea Americana*	Seed	*Ziziphus mauritiana*	Leaf

Table 11.Yield gradient extraction of UG, MP, CC, MC and AC

	Yield (mg/g)		
Name	Hexane	Ethyl Acetate	Butanol
UG	1.30±0.10	7.60±0.20	9.77±0.01
MP	2.40±0.20	12.77±0.20	29.50±0.20
CC	8.00±0.20	12.00±0.20	16.70±0.50
MC	7.10±0.20	12.40±0.20	5.60±0.10
AC	0.90±0.20	15.10±0.40	11.60±0.04

Table 12. AGIs activity (0.02 g/ml), phenolic content, flavonoid content, and antioxidant activity of UG,MP, CC, MC, and AC

Name	AGIs (%)	Phenolic Content mg GAE/g	Flavonoid Content mg QE/g	Antioxidant Activity IC_{50} (ppm)
UG	100.00	67.57[a]	1.57[c]	101.75[a]
MP	83.21	14.95[c]	2.40[b]	2377.64[b]
CC	73.11	6.14[d]	1.00 [d]	5910.14[c]
MC	71.00	15.20[c]	0.99[d]	5859.14[c]
AC	62.34	19.90[b]	8.94[a]	1963.57[b]

Spectrophotometric quantification of collagen in commercial beauty drinks

Atittaya Tandhanskul*[1] and Napat Srisiam[2]

[1]Department of Food Technology, Faculty of Biotechnology, Assumption University, THAILAND (atittayatnd@au.edu)

[2] Department of Food Technology, Faculty of Biotechnology, Assumption University, THAILAND, (b.belle197@gmail.com)

ABSTRACT

Nowadays, collagen-infused drinks are another trend in global market. Several beverages have claimed to have health and beauty benefits from added collagen. Five collagen-containing drinks were randomly selected from local supermarket. Since collagen is thermally labile, the amount of collagen as claimed on the label was investigated. Different spectrophotometric determinations of collagen were applied and discussed on the specificity and applicable of the tests. Bradford protein assay showed lower specificity, even with the addition of 0.0035% sodium dodecyl sulfate, than bicinchoninic acid protein assay (BCA) in the detection of collagen in beauty drinks. Four out of five samples were found that collagen content was less than claimed. This suggests proper storage of these products.

Keywords : beauty drinks; collagen; functional drinks

INTRODUCTION

The expansion of functional drinks market has been increased dramatically in the past 5 years. People, especially in Asia, become more aware of their health and beauty. Collagen, fibrous proteins responsible for firmness of human skin, has shown to be one of the important ingredients in the food and beverage industries. It is mostly used in the form of collagen fiber and has been applied as protein dietary supplements, carriers in the meat processing, edible film and coating of products, and food additive to improve products' quality (Hashim *et al.*, 2015). In addition, collagen may help in boosting the health and nutritional value of the products.

Recently, there are a lot of products released by the manufacturers such as collagen beauty drinks, soy collagen, cocoa collagen, cappuccino collagen, juice with collagen and bird nest drink with collagen. Generally, the collagen drink claims to stimulate the collagen making mechanism in the body, which in turn will promote the body tissues and reduce the skin wrinkle and sagging (Hashim *et al.*, 2015). As a food ingredient, oral ingestion of hydrolyzed collagen has been reported as safe (Wu *et al.*, 2004). Hydrolyzed collagen, usually from fish, porcine or bovine origin, is currently used in various fields including functional food, beverages and dietary supplements because it is highly digestible.

Bradford assay is well-known and a common spectrophotometric method to detect the total protein concentration in the sample. It relies on the proportional binding of the dye Coomassie to proteins (Bradford, 1976). Bradford assay is widely used because it is quick

and simple and usually a first choice in protein concentration determination. The other protein-dye binding spectrophotometric method, the bicinchoninic acid (BCA) assay is a highly sensitive colorimetric assay. This method involves two-step reactions and less variability than the Bradford assay (Wiechelman *et al.*, 1988, Brown *et al.*, 1989).

However, the triple helix and rod like structure of collagen is reported to be thermally labile (Zhang *et al.*, 2005). The analysis of collagenous chains is also a difficult task due to the poor solubility (hydrophobicity) of these proteins. HPLC methods are not frequently used for the separation/characterization of individual chains of a collagen type (Miksik, 2007). Using spectrophotometric quantification of collagen is, thus, being utilized to investigate if the amount of added collagen is in a reasonable amount after reaching consumer's hands.

MATERIALS AND METHOD

Five beauty drinks containing collagen were randomly selected from a Thai supermarket. The samples were detected for the amount of collagen present by Bradford protein assay and Bicinchoninic acid (BCA) protein assay. Bovine serum albumin (BSA) was used as a standard protein (125, 250, 500, 750, 1000, 1500 and 2000 µg/ml). The absorbance should fall in the range 0.1-0.8. If lower, the sample was freeze-dried. If higher, the sample was diluted.

a) Bradford protein assay

The standard BSA was prepared as mentioned above. Distilled water was used as blank. Fifty µl of sample was mixed with 2.5 ml Bradford reagent (Biorad, USA). The mixture was incubated at room temperature for 10 min before measuring the absorbance at 595 nm. The modified Bradford protein assay was also perform with addition of 0.0035% SDS (Lopez *et al.*, 1993, Duhamel *et al.*, 1981).

b) Bicinchoninic acid protein assay (BCA)

The standard BSA was prepared as mentioned above. Standard working solution (SWR) was prepared by mixing reagent A (mixture of Sodium bicinchoninate, sodium tartrate, sodium hydroxide and sodium bicarbonate adjusted to pH 11.25) with reagent B (4% copper sulfate) in the ratio 50:1. The SWR could be kept for 1 week and green in color. Twenty-five µl of sample was mixed well with 200 µl SWR and incubated at 37°C for 30 min. The mixture was then cooled at room temperature for 5 min before measuring absorbance at 562 nm.

RESULTS AND DISCUSSION

Assuming that these beauty drinks contain negligible amount of proteins, with addition of collagen to the drink, the detection of protein here should come from collagen itself. Collagen is a protein forming triple helix of three polypeptide chains. Every chain is composed of thousands of amino acids based on the Gly-X-Y sequence while the X and

Y positions are mostly found to be proline and hydroxyproline (Liu *et al.*, 2012, Gelse *et al.*, 2003, Cheng *et al.*, 2009). These three amino acids account for 50% of the total protein content.

a) Bradford protein assay

Detection of collagen with Bradford protein assay was selected because of it is a simple and rapid protocol. Figure 1 shows the sample quantification from the standard curve. Most could be found that the values were not acceptable since they fell in the negative (undetectable) region. The improvement on the assay was performed by adding 0.0035% SDS according to the previous literatures (Lopez *et al.*, 1993, Duhamel *et al.*, 1981) and illustrated in figure 2. However, because collagen is deficient in aromatic amino acids, it displays low sensitivity of detection by spectrophotometric methods even with the modification.

b) Bicinchoninic acid protein assay (BCA)

The bicinchoninic acid protein assay (BCA) was developed to contain less interaction with many contaminants and buffer components compared to those in the Lowry assay (Smith *et al.*, 1985). It has less variability than the Bradford assay. In an alkaline environment, the BCA reagent forms a complex with Cu^{+}, which has a stronger absorbance at 562 nm. Figure 3 demonstrates the quantification of collagen using the BCA assay.

c) Quantification of collagen in commercial beauty drinks

Table 1 shows the results from different spectrophotometric methods for collagen determination. Bradford assay, even with modification, was definitely not recommended for collagen detection. If the amount of collagen added in the beauty drinks was not concentrated enough, it would be difficult to detect. When employing BCA assay, the sensitivity improved. The percent loss when compared to the amount as claimed on the label of each sample was calculated. Sample A, sold in a dark-colored glass bottle, contained highest collagen concentration and lost about 13%. Sample B, sold in the market in powder form, had potential to be adulterated with starch or other powder mix since, when dissolved, the concentration was less than claimed 67%. Sample C, D, and E were sold in a clear plastic bottle. But because the concentration was not that high, thus except for sample D, the percent loss was reported less than sample A or B.

CONCLUSIONS

The amount of collagen as claimed on the label was investigated by employing different spectrophotometric determinations. Bradford protein assay showed lower specificity, even with the addition of 0.0035% SDS, than bicinchoninic acid protein assay (BCA). The BCA assay proved to be a more rapid and sensitive spectrophotometric approach for collagen determination in commercial beauty drinks. Four out of five samples were found that collagen content was less than claimed. This suggests proper storage of these

products, including the packaging, temperature during transportation and storage to maintain the amount in the acceptable level.

ACKNOWLEDGEMENTS

The authors thank Prof. Gary M. Smith at University of California at Davis for his kind advice and support in completing the experiments.

REFERENCES

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72**,** 248-254.

BROWN, R. E., JARVIS, K. L. & HYLAND, K. J. 1989. Protein measurement using bicinchoninic acid: elimination of interfering substances. *Analytical Biochemistry,* 180**,** 136-139.

CHENG, F. Y., HSU, F. W., CHANG, H. S., LIN, L. C. & SAKATA, R. 2009. Effect of different acids on the extraction of pepsin-solubilised collagen melanin from silky fowl feet. *Food Chemistry,* 113**,** 563-567.

DUHAMEL, R. C., MEEZAN, E. & BRENDEL, K. 1981. The addition of SDS to the Bradford dye-binding protein assay, a modification with increased sensitivity to collagen. *Journal of Biochemical and Biophysical Methods,* 5**,** 67-74.

GELSE, K., POSCHL, E. & AIGNER, T. 2003. Collagens-structure, function and biosynthesis. *Advanced Drug Delivery Reviews,* 55**,** 113-136.

HASHIM, P., MOHD RIDZWAN, M. S., BAKAR, J. & MAT HASHIM, D. 2015. Collagen in food and beverage industries. *International Food Research Journal,* 22**,** 1-8.

LIU, D., LIANG, L., REGENSTEIN, J. M. & ZHOU, P. 2012. Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones, and swim bladders of bighead carp *(Hypophthalmichthysnobilis). Food Chemistry,* 133**,** 1441-1448.

LOPEZ, J. M., IMPERIAL, S., VALDERRAMA, R. & NAVARRO, S. 1993. An improved Bradford protein assay for collagen proteins. *Clinica Chimica Acta***,** 91-100.

MIKSIK, I. 2007. Collagen: HPLC and capillary electromigration methods. *Encyclopedia of Chromatography.*

SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. & KLENK, D. C. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry,* 150**,** 76-85.

WIECHELMAN, K. J., BRAUN, R. D. & FITZPATRICK, J. D. 1988. Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Analytical Biochemistry,* 175**,** 231-237.

WU, J., FUJIOKA M. & K., S. 2004. Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats. *Journal of Bone and Mineral Metabolism,* 22**,** 547-553.

ZHANG, Z., LI, G. & SHI, B. I. 2005. Physiochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *Journal of the Society of Leather Technologists and Chemists,* 90**,** 23-28.

ATTACHMENT: FIGURES AND TABLES

Table 1. Quantification of collagen in commercial beauty drinks using protein determination by different spectrophotometric methods

Sample	Type of sample	Concentration as claimed on the label (mg/ml)	Concentration as measured by Bradford technique (mg/ml)	Concentration as measured by modified Bradford technique (mg/ml)	Concentration as measured by BCA technique (mg/ml)	Percent loss*
A	Shot	100	0.029	0.808	87.362	-13%
B	Powder	100	0.638	1.987	32.613	-67%
C	Drink	11.11	N/A	0.151	10.55	-5%
D	Drink	5.55	N/A	0.418	3.895	-30%
E	Drink	4.86	N/A	0.031	5.095	+5%

Note: *Calculated using data from BCA technique

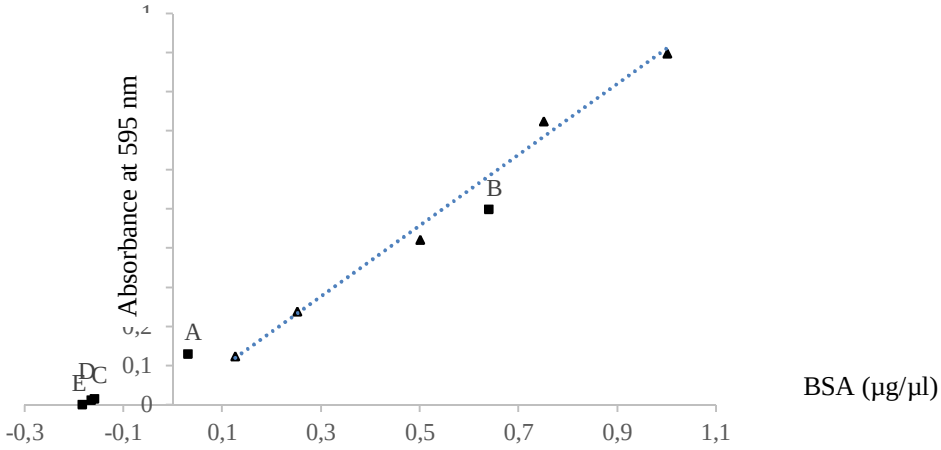


Figure 1. Data for a sample Bradford protein assay standard curve produced using BSA standard at 0.125, 0.25, 0.5, 0.75 and 1 µg/µl. The BSA (µg/µl) is plotted on the x axis and A_{595} is plotted on the y axis. The data are fit with a linear regression by the line y =

0.906x + 0.0067 with an R^2 value of 0.9927. Triangle points indicate the standard data points. Square points indicate the sample points.

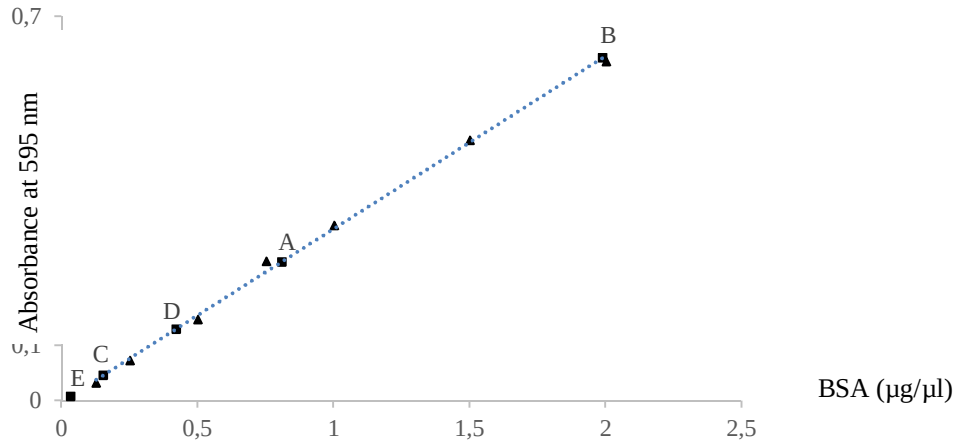


Figure 2. Data for a sample Bradford protein assay (with 0.0035% SDS addition) standard curve produced using BSA standard at 0.125, 0.25, 0.5, 0.75, 1, 1.5 and 2 µg/µl. The BSA (µg/µl) is plotted on the x axis and A_{595} is plotted on the y axis. The data are fit with a linear regression by the line y = 0.3147x - 0.00007 with an R^2 value of 0.9976. Triangle points indicate the standard data points. Square points indicate the sample points.

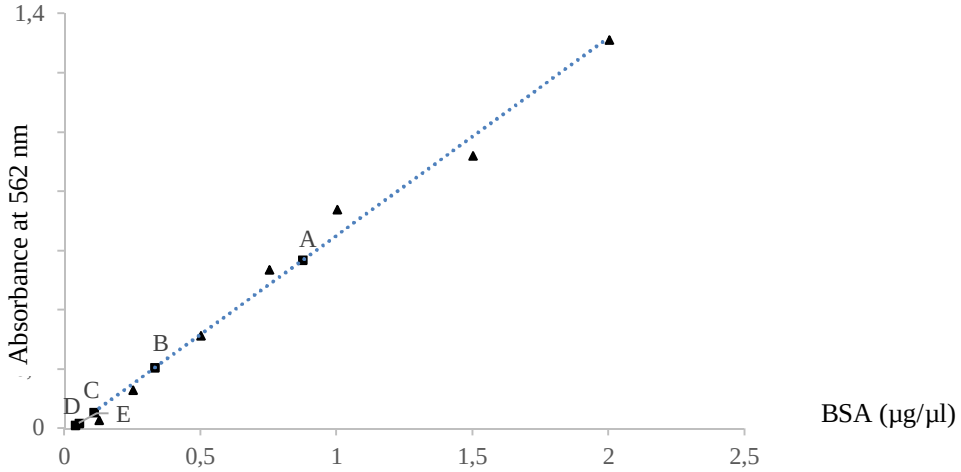


Figure 3. Data for a sample BCA protein assay standard curve produced using BSA standard at 0.125, 0.25, 0.5, 0.75, 1, 1.5 and 2 µg/µl. The BSA (µg/µl) is plotted on the x axis and A_{562} is plotted on the y axis. The data are fit with a linear regression by the line y = 0.6682x - 0.0147 with an R^2 value of 0.987. Triangle points indicate the standard data points. Square points indicate the sample points.

Impact of Citric Acid Immersion on the Physicochemical and Microbiological Characteristics of Curcuma Dried with Solar Tunnel Dryer

Victoria Kristina Ananingsih, R. Probo Y. Nugrahedi, Fransisca Sari
Food Technology Department, Soegijapranata Catholic University,Semarang-Indonesia
Email: kristina@unika.ac.id

Curcuma (*Curcuma xanthorrhiza* Roxb) is a kind of rhyzome that is usually used as raw material for making Jamu (traditional drink in Indonesia). Solar tunnel drying can be applied to extend the shelf life of fresh curcuma due to the reducing of its water content. Drying process could change the physicochemical properties of dried curcuma such as discoloration and degradation of chemical compounds because the use of high temperature during solar tunnel drying. To reduce the negative effects of drying process, pretreatment can be applied before drying process. Citric acid immersion is one of the drying pretreatment that could prevent the damage of curcuma during drying. The purpose of this study was to determine the effect of citric acid concentration and immersion time in maintaining the quality of dried curcuma. Two levels of citric acid concentration were applied, i.e. 0.5% and 1%, while 3 levels of immersion time were conducted, i.e. 10, 20 and 30 minutes. Curcuma was dried until its moisture content less than 7%. Curcuma processed with 1% citric acid and immersion time of 30 minutes provided superior qualities that resulted in the fastest drying time (255 minutes), produced the highest % inhibition (92.14%) and the highest curcumin content (5.34%), also showed the minimum possibility of fungal growth.

Keywords : curcuma, solar tunnel drying, pretreatment, citric acid

INTRODUCTION

Curcuma (*Curcuma xanthorrhiza* Roxb.) is a kind of rhyzomes grown in Indonesia which contains high antioxidant especially curcuminoid. It is used as natural colorant, and ingredients for making Indonesian traditional herbal drink (Sudrajad & Azar, 2004). The main bioactive compound of curcuma is curcuminoid, others are essential oil, flavoloid and tannin (Rukmana, 1995). Curcumin, one of the curcuminoid compounds, contributes to the yellow color (Tensiska., Nurhadi, B., dan Isfron, 2012) and it acts as antioxidant (Masuda, Isobe, Jitoe, & Nakatani, 1992). Curcumin is stable in the acid condition (Tonnesen & Karslen, 2007). Citric acid is an organic acid which is applied as drying pretreatment to prevent the physical and chemical changes of foodstuff after drying.

The shelf life of curcuma can be extended by drying process to reduce its water content and water activity. One of drying methods that is applicable for tropical countries is *Solar Tunnel Drying*. Temperature of Solar Tunnel Dryer is 60-70^{o}C which is suitable to dry the foodstuff. Before the drying process, pretreatment can be applied to improve the qualities of dried curcuma, which is immersion in the citric acid solution. Immersion in the citric acid solution is proposed to prevent the damage of dried curcuma after the drying process, accelerate the drying time and reduce the degradation of antioxidant. The purpose of this research is to study the impact of citric acid immersion on the qualities of curcuma dried by Solar Tunnel Dryer.

METHODS

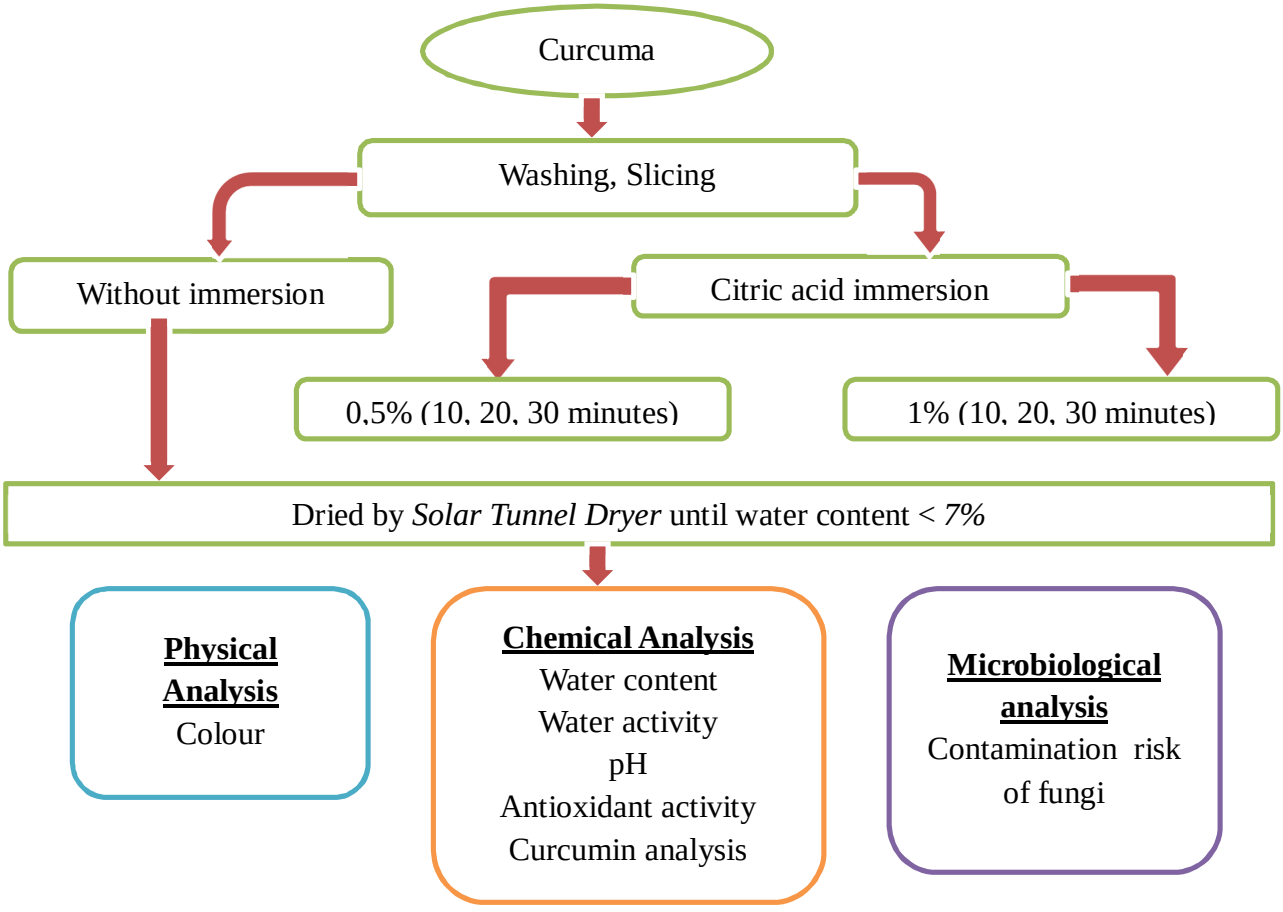


Figure 1. Research Design

Curcuma is washed, sliced and immersed in the 0.5% and 1% of citric acid for 10.20 and 30 minutes. Then, the samples were dried by Solar Tunnel Dryer until their water contents were lower that 7%. The dried curcuma was analyzed for the physical characteristic (color intensity), chemical characteristics (water content, water activity, pH, antioxidant activity and curcumin content), and microbiological characteristic (contamination risk of fungi). Antioxidant activity was analyzed by DPPH method, while curcumin was analyzed by spectrophotometric method.

Pretreatments conducted in this research were : B0 = Control/without immersion; B1 = 0,5% Citric acid + 10 min immersion; B2 = 0,5% Citric acid + 20 min immersion; B3 = 0,5% Citric acid + 30 min immersion; B4 = 1% Citric acid + 10 min immersion; B5 = 1% Citric acid + 20 min immersion; B6 = 1% Citric acid + 30 min immersion;

RESULTS AND DISCUSSION

WATER CONTENTS DURING DRYING

Degradation of water contents during solar tunnel drying can be seen in Table 1.

Tabel13. Degradation of water contents during Solar Tunnel Drying

Waktu	B0	B1	B2	B3	B4	B5	B6
0	72,70±0,13	80,77±0,05	82,16±0,02	84,92±0,01	81,25±0,02	81,93±0,03	82,04±0,01
15	66,24±0,12	72,94±0,07	76,21±0,04	78,88±0,05	76,45±0,05	77,04±0,06	78,32±0,04
30	62,16±0,11	67,36±0,10	73,34±0,06	75,25±0,06	72,62±0,05	72,89±0,07	73,39±0,05
45	59,18±0,10	63,34±0,10	71,15±0,06	70,82±0,07	69,48±0,06	69,17±0,07	69,69±0,04
60	57,66±0,10	59,57±0,10	68,75±0,06	67,42±0,07	65,00±0,05	65,43±0,07	63,67±0,05
75	54,23±0,10	55,22±0,11	66,23±0,06	62,77±0,05	60,79±0,06	60,57±0,06	57,23±0,08
90	51,06±0,09	52,40±0,10	62,94±0,06	57,76±0,06	55,84±0,06	53,15±0,05	49,53±0,10
105	49,11±0,10	49,13±0,09	59,57±0,07	50,46±0,76	50,97±0,07	43,85±0,04	40,80±0,11
120	45,86±0,09	43,97±0,09	53,83±0,07	42,44±0,08	44,83±0,07	34,22±0,04	34,21±0,10
135	36,08±0,04	37,99±0,08	47,15±0,07	35,10±0,08	38,70±0,07	27,46±0,04	28,72±0,09
150	28,19±0,03	32,87±0,08	41,32±0,08	29,35±0,06	33,57±0,07	21,47±0,05	23,94±0,09
165	23,74±0,02	28,11±0,07	35,84±0,07	23,85±0,05	28,02±0,07	17,11±0,04	18,52±0,07
180	18,19±0,02	22,96±0,06	29,73±0,07	19,21±0,04	22,27±0,06	13,68±0,04	14,41±0,05
195	14,85±0,02	18,65±0,04	25,17±0,06	15,73±0,03	17,77±0,05	11,55±0,03	10,86±0,03
210	12,62±0,02	15,51±0,03	20,86±0,05	11,82±0,01	13,51±0,04	9,34±0,02	8,11±0,02
225	10,61±0,02	12,48±0,02	16,65±0,05	9,89±0,02	9,94±0,02	7,49±0,02	6,30±0,02
240	8,39±0,01	10,25±0,02	13,00±0,04	6,85±0,01	8,08±0,01	6,15±0,01	5,70±0,01
255	8,05±0,02	8,15±0,02	9,55±0,03	6,41±0,01	6,36±0,01	-	-
270	6,95±0,01	6,19±0,01	6,67±0,01	-	-	-	-

Curcuma was dried until the water content was lower than 7%. It is purposed to reduce the water content and inhibit the growth of fungi. Table 1 revealed that the fastest drying time was B6 (immersion in the 1% citric acid for 30 minutes). This pretreatment would open the pores of the cell wall hence the water could be evaporated faster (Pangavhane, Sawhney, & Sarsavadia, 1999). The results showed that higher temperature of solar tunnel dryer produced higher drying rate. Velic, Darko (2007) said that higher drying temperature resulted in higher drying time (Velić, Tomas, & Bucić-kojić, 2007).

PH OF CURCUMA

Table 14. pH of curcuma before and after drying

Condition	Pretreatments	pH
Before drying	B0	$4,70\pm0,34^{ef}$
	B1	$4,36\pm0,34^{cd}$
	B2	$4,28\pm0,17^{c}$
	B3	$4,13\pm0,18^{abc}$
	B4	$4,08\pm0,11^{abc}$
	B5	$3,91\pm0,20^{ab}$
	B6	$3,89\pm0,28^{a}$
After drying	B0	$5,42\pm0,34^{g}$
	B1	$4,88\pm0,48^{f}$
	B2	$4,68\pm0,31^{e}$
	B3	$4,58\pm0,28^{de}$
	B4	$4,36\pm0,38^{cd}$
	B5	$4,27\pm0,30^{c}$
	B6	$4,19\pm0,13^{bc}$

Table 15. pH of citric acid solution

Pretreatments	pH
B1	$2,39\pm0,11^{c}$
B2	$2,31\pm0,07^{b}$
B3	$2,27\pm0,06^{b}$
B4	$2,05\pm0,07^{a}$
B5	$2,04\pm0,04^{a}$
B6	$2,01\pm0,04^{a}$

It can be seen in Table 2 that pH of fresh curcuma was lower than dried curcuma. This is due to the immersion pretreatment in the citric acid. Citric acid reduced the pH and retained the color of dried curcuma because it inhibited the activity of phenolaze enzyme. This enzyme contributes to the browning process. The immersion solution had the pH

around 2.01-2.39. At this range of pH, the activity of phenolaze enzyme is low; hence it reduces the browning reaction.

CURCUMIN CONTENTS

Table 16. Curcumin contents before and after drying

Condition	Pretreatments	Curcumin contents (ppm)	
		Wet Basis	*Dry Basis*
Before Drying	B0	540,51± 26,67[a]	1093,63±334,45[b]
	B1	392,80±86,94[e]	2225,38±898,34 [c]
	B2	181,56±17,90[b]	1025,16±134,83 [b]
	B3	168,72±27,23 [b]	1121,38±209,02 [b]
	B4	253,212±8,93[c]	1375,33±226,18 [b]
	B5	350,213±145,80[d]	2059,35±1101,33[c]
	B6	361,62±10,98[de]	2028,42±211,53 [c]
After Dyring	B0	502,78±22,61[e]	540,51±26,66[a]
	B1	509,82±27,46 [f]	547,60±27,58 [a]
	B2	516,55±38,71 [f]	553,33±27,48 [a]
	B3	513,85±26,13 [f]	549,26±31,04 [a]
	B4	512,60±32,66 [f]	547,46±35,71 [a]
	B5	507,35±23,78 [f]	540,70±26,89 [a]
	B6	533,840±7,61 [f]	565,97±40,23 [a]

Table 4 showed that curcumin content (dry basis) decreased significantly due to it was easily degraded during drying. The curcumin content in the dried curcuma was higher than the fresh one. During drying, the cell wall of oleoresin was broken hence curcumin was detected higher.

ANTIOXIDANT ACTIVITY (% INHIBITION)

Table 17. % Inhibition of curcuma before and after drying

Condition	Pretreatments	% *Inhibibition*	
		Wet Basis	*Dry Basis*
Before drying	B0	62,07±3,00[b]	271,68±106,37 [c]
	B1	75,08±6,1[c]	407,06±90,15 [d]
	B2	75,31±0,86[c]	425,76±43,01 [d]
	B3	76,78±1,93[c]	510,11± 26,44 [e]
	B4	81,26±8,34[d]	434,88±21,64 [d]
	B5	89,75±1,63[ef]	514,20±108,07 [e]
	B6	91,31±3,48[ef]	510,85±35,10 [e]
After drying	B0	51,93±2,40[a]	55,84±3,2[a]
	B1	87,76±2,49[e]	94,28±2,71[b]

	B2	88,07±3,57^{e}	94,36±1,25^{b}
	B3	88,51±2,36^{e}	106,87±1,26^{b}
	B4	88,86±1,94^{ef}	94,89±2,30^{b}
	B5	90,95±0,85^{ef}	96,92±1,18^{b}
	B6	92,14±2,61^{f}	97,73±2,74^{b}

Table 4 showed the degradation of antioxidant which caused by the drying process. Pretreamen B6 (immersion in the 1% citric acid for 30 minutes) produced the highest % inhibition. Citric acid caused the fastest drying time, therefore it could retained the active compound of dried curcuma.

WATER ACTIVITY (AW)

Table 18. Water activity of curcuma

Condition	Pretreatments	Aw
Before drying	B0	0,982±0,01^{e}
	B1	0,976±0,02^{e}
	B2	0,973±0,02^{e}
	B3	0,980±0,01^{e}
	B4	0,964±0,03^{e}
	B5	0,969±0,03^{e}
	B6	0,964±0,03^{e}
After drying	B0	0,577±0,06^{d}
	B1	0,466±0,10^{c}
	B2	0,403±0,10^{abc}
	B3	0,417±0,12^{abc}
	B4	0,389±0,08^{ab}
	B5	0,442±0,09^{bc}
	B6	0,348±0,10^{a}

The water activity of curcuma reduced significantly after drying, which were around 0,348 – 0,984. This low water activity can extend the shelf life of dried curcuma. Labuza (1980) said that dried food which has the water activity lower than 0.6 is categorized as dried food that resistant to the growth of microorganisms.

CONTAMINATION RISK OF FUNGI

Table 19. Contamination risks of fungi of dried curcuma(%)

Condition	Pretreatment	Total fungi (%)
After drying	B0	27,78±9,72 [a]
	B1	26,67±15,00 [a]
	B2	17,78±4,41 [a]
	B3	23,38±13,23 [a]
	B4	24,44±11,30 [a]
	B5	16,67±8,66 [a]
	B6	15,56±5,27 [a]

Contamination risks of fungi for all samples were not significantly different. Pretreatment B6 had the lowest contamination risk of fungi which was 15,56%. Citric acid inhibits the grow of fungi due to its low pH and it acts as *chellating agent* which binds the metal ion which functions to absorb the nutrition through the cell wall of fungi. Therefore, the fungi will difficult to grow. Moreover, the low water activity inhibits the growth of fungi. In the low water activity, the fungi does not has enough water, hence they can not grow (Guo et al., 2016).

COLOR INTENSITY

Tabel 20. Color intensity of curcuma before and after drying

Condition	Pretreatments	Values		
		L	a*	b*
Before drying	B0	59,07±2,83^{a}	12,13 ± 1,06^{cd}	51,61 ± 3,70^{d}
	B1	66,91±1,80^{cd}	13,82±1,34^{ef}	64,39±6,3e
	B2	67,55±2,99^{cd}	14,15±1,07^{efg}	65,14±4,05^{e}
	B3	68,23±1,7^{cd}	15,02±1,41^{g}	65,73±5,92^{ef}
	B4	69,62±1,90^{d}	13,12±1,22^{de}	66,65±4,73^{ef}
	B5	69,80±0,74^{d}	14,26±1,23^{fg}	68,47±4,40^{f}
	B6	69,83±1,40^{d}	14,53±1,30^{fg}	71,46±2,83^{g}
After drying	B0	60,87±5,04^{ab}	10,47±0,51^{ab}	32,92±2,42^{a}
	B1	64,32±5,97^{bc}	9,51±0,82^{a}	35,51±1,45^{ab}
	B2	64,55±4,27^{bc}	11,40±1,02^{bc}	37,15±0,93^{b}
	B3	64,69±6,18^{bc}	11,73±0,74^{cd}	37,07±0,74^{b}
	B4	65,81±5,35^{cd}	12,12±1,19^{c}	41,38±3,10^{c}
	B5	66,18±3,96^{cd}	11,55±1,07^{c}	41,02±3,30^{c}
	B6	66,98±4,45^{cd}	12,36±1,15^{cd}	41,63±4,04^{c}

Table 8 showed that measurement of color intensity of dried curcuma produced higher L*, a* b* values compared to those of fresh curcuma. The higher L* and b* values showed the increase level of lightness and yellow color of dried curcuma. Citric acid could reduce the browning reaction because it contributed to the lower pH (Nurdjannah & Hoerudin, 2008). The low pH of curcuma can be seen in Table 2.

CONCLUSION

Pretreatment B6 which was immersion in the 1% citric acid solution for 30 minutes could accelerate the drying time (240 minutes) compared to that without pretreatment (270 minutes). This pretreatment could also produce dried curcuma that had high % inhibition (97,73%, dry basis), high curcumin content (565,97 ppm, dry basis) and low contamination risk of fungi (15,56%)

REFERENCES

Guo, J., Wang, W., Hu, J., Xie, D., Gerhard, E., Nisic, M., … Yang, J. (2016). Synthesis and characterization of anti-bacterial and anti-fungal citrate-based mussel-inspired bioadhesives. *Biomaterials*, *85*, 204–217.

Labuza, T. P. (1980). Effect of water activity on reaction kinetics of food deterioration. Food Technol. New York.

Masuda, T., Isobe, J., Jitoe, A., & Nakatani, N. (1992). Antioxidative curcuminoids from rhizomes of Curcuma xanthorrhiza. *Phytochemistry*, *31*(10), 3645–3647.

Nurdjannah, N., & Hoerudin. (2008). Pengaruh Perendaman Dalam Asam Organik Dan Kering, *XIX*(2), 181–196.

Pangavhane, D. R., Sawhney, R. L., & Sarsavadia, P. N. (1999). Effect of various dipping pretreatment on drying kinetics of Thompson seedless grapes. *Journal of Food Engineering*, *39*(2), 211–216.

Rosidi, A., Khomsan, A., Setiawan, B., & Briawan, D. (2004). Potensi temulawak (c, (1995).

Rukmana, Ir Rahmat. 1995. Temulawak: Tanaman rempah dan obat. Penerbit Kanisius. Yogyakarta

Singleton V.L. & Rossi J.A. Jr. Colorunetry of Total Phenolic With Phosphomolybdic-Phosphotungstic Acid Reagent. Amer. J. Enol. Viticult. 16:144-58, 1965.

Sudrajad, H., & Azar, F. Al. (2004). Uji Aktivitas Antifungi Minyak Atsiri Rimpang Temulawak (Curcuma xanthorriza Roxb.) secara In Vitro Terhadap Candida albicans. *Balai Besar Penelitian Dan Pengembangan Tanaman Obat Dan Obat Tradisional*, 84–89.

Tensiska., Nurhadi, B., dan Isfron, A. . (2012). Kestabilan warna kurkumin terenkapsulasi dari kunyit (, *14*(3), 198–206.

Tonnesen, H. H., & Karslen, J. (2007). Studies on curcumin and curcuminoids. *International Journal of Pharmaceutics*, *338*(1-2), 27–34.

Velić, D., Tomas, S., & Bucić-kojić, A. (2007). Study of the Drying Kinetics of " Granny Smith " Apple in Tray Drier. *Agriculturae Conspectus Scientificus*, *72*(4), 323–328.

AGRICULTURE AND FOOD SECURITY

Bioprospecting of Brown Macroalgae From Java Island's Southern Coast, Gunung Kidul Coast of Yogyarta and Binuangeun Coast of Banten as Source of Lectins

Choiroel Anam[1], Danar Praseptiangga[1], Nurrahmi Dewi Fajarningsih[2], Naomi Carissa Intaqta[1]

[1)] Department of Food Science and Technology, Sebelas Maret University Surakarta
Jl Ir Sutami 36 A Kentingan Surakarta 57126 , Indonesia
Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, DKP Jakarta,
Jl KS Tubun Petamburan VI Jakarta 10260
Email : Dikchoir@yahoo.com

ABSTRACT

Gunungkidul coast of Yogyakarta and Binuangeun coast of Banten are parts of Java Island's Southern coast which tangent to Indian Ocean and have high spreading of brown algae. Bioactive compounds named lectins which are able to extract from algae are valuable and bio-prospective. Lectins are carbohydrate-binding proteins, of which can agglutinate cell or precipitate glycoconjugate. Many algae lectins have several distinct properties compared to lectins of higher plants such as low-molecular size, monomeric form, having no affinity for monosaccharides, thermostable, and divalent cation-independent for its hemagglutination activities. Generally, algae lectins have no affinity for monosaccharides but for glycoproteins. Thirteen species of brown marine algae were extracted by phosphate buffered saline and the ammonium sulphate-precipitates were tested for hemagglutination activities using native and enzyme-treated rabbit and human erythrocytes (A, B and O types). Among thirteen species, *Dictyota dichotoma*, *Himanthalia elongata*, *Padina australis*, *Padina minor*, *Padina pavonica*, *Sargassum crassifolium*, *Sargassum cristaefolium*, *Sargassum gracillimum*, *Sargassum oligocystum*, *Sargassum polycystum*, *Sargassum turbinarioides*, *Turbinaria conoides*, dan *Turbinaria ornata* showed the activity by hemagglutinantion activity assay. The strongest activity was obtained from *Dictyota dichotoma*. These results shown that Gunungkidul and Binuangeun brown algae were potential as useful sources of novel lectins.

Keywords: Lectins, Brown macroalgae, Screening, Gunungkidul coast, Binuangeun coast, Indonesia

INTRODUCTION

Indonesia known as a maritime nation has large marine areas (Witikta, 2014) of 5,8 million km^2 and a coast-line of 95.181 km (Rosyid, 20**1**5). Indonesia is one of three countries having the biggest number and varieties of **n**atural resources in the world, including its marine biota (Supriatna, 2008). Ministry of Marine Affairs and Fisheries has set seaweed as one of the ultimate marine commodities in Indonesia (Kasim dan Asnani, 2012). Seaweed has bioactive components (polysaccharides, protein, PUFAs, pigments, polyphenols, mineral, and plant growth hormones) which produce beneficial effects to human, animal, and plant such as protecting organisms from biotic and abiotic pressure as well as being applied as pharmaceutical components, feed, and fertilizer (Chojnacka *et al*., 2012). Brown algae contain bioactive compounds which can be used as medicine, such as cancer, detoxification agents, activities of antibacterial, antiviral, antimicrobial, antitumor, antidiabetic, antiosteoporotic, gastro-protective, hyalurodinase, antioxidant, and immunological property activities (Kumar *et al.*, 2011; Nursid *et al.*, 2013).

Seaweed has been used in Indonesia as vegetables and traditional medicine such as fever reducer(Wikanta *et al.*, 2008). Brown algae have an important mineral such as potassium which is higher than minerals of red and green algae. (Setyawati *et al*., 2014). The use of seaweed in Indonesia has increased, but it is still limited in the forms of carrageenan, agar-agar, and alginate (polysaccharide) only (Anonim, 2011). The structure and biological properties of protein extracts of seaweed were not reported as large as polysaccharides. The very important bioactive protein compounds which could be extracted of macroalgae were lectins(Chojnacka *et al.*, 2012).

"Lectins" is derived from Latin word *legere* which means *to select* (Pietrzyk *et al.*, 2015). Hemaglutinins or lectins are carbohydrate-binding proteins of non-immune origin, cell-agglutinating or glycoconjugate-precipitating (Goldstein *et al.*, 1980), which are separated from antibody and enzymes (Barondes *et al.*, 1981-1988 in Gabius, 2009). Lectins are redefined by Peumans and Van Damme (1995) as proteins posessing at least one *non-catalytic* domain, which binds reversibly to a specific mono- or oligosaccharide (Kim *et al.*, 2005). By the end of 1960's, it was found the fact that glucose which was in the cell surface was important toward cell growth and differentiation, cell interaction with envireonment, and many pathology processes (Sharon, 2005). Due to its chemical properties, lectins are useful in the field of biological researches (immunology, cell biology, membrane structure, cancer research and genetic technologies) (Oliveira *et al*., 2002) and become modern glycobiology subject (Sharon dan Lis, 2004).

Lectins can also be found in organisms in general, from virus to human. Unlike antibodies, lectins show diversity in their molecular structures and carbohydrate-binding spesificities, depending on their organisms of origin (Hung, *et al*., 2009). Several types of sea algae have lectins (Boyd *et al*., 1966). Algal lectins are different from higher plant lectins at various types of characteristics. In general, they have lower molecule masses, monomeric forms, heat-stable proteins, and no divalent cations dependence. Algal lectins do not have affinity for simple sugars but are more spesific for complex oligosaccharides), have glycoproteins and high amino acids proportion with isoelectric point from 4 to 6 (Necib *et al*, 2015;

Praseptiangga *et al.*, 2012). The low molecule weight provides lower antigenic effects. Due to its benefits, algal lectins are more suitable for medicine making (Rogers dan Hori, 1993).

Seaweed lectins research has been done in certain countries. Puerto Rico (Boyd *et al*., 1966), England (Blunden *et al.*, 1975, 1978; Rogers *et al.*, 1980) Japan (Hori *et al*., 1988), Spain (Fabregas *et al*., 1985, 1992), United States (Chiles dan Bird, 1989; Bird *et al*., 1993), Brazil (Ainouz dan Sampaio, 1991; Ainouz *et al*., 1992; Freitaz *et al*., 1997), Pakistan (Alam and Usmanghani, 1994), India (Kumar and Barros, 2010), and Vietnam (Hung *et al.*, 2009) were some quotations by Hung *et al.*, (2012). More than 250 species of algae have been researched, but their purification and characteristics have not yet been done much. (Hung *et al*., 2012). The use of abundant algae in Indonesia has not yet been done optimally, particularly in term of proteins. The research about algal lectins has not yet been studied often, thus, the research on the filter and early characteristics of bioactive lectins compounds of brown macroalgae in Gunungkidul coast and Binuangeun coast was conducted.

Research Methods

Samples were collected from the Java Island's southern coast, Gunung Kidul coast of Yogyakarta and Binuangeun coast of Banten, and were washed and stored at -20 ^{o}C until use. The sample identification was conducted to identify each species of brown macroalgae. The stage of algae lectins extraction and crude lectins fraction preparation began by taking the frozen samples of brown algae out from freezer at -20^{o}C, thawing, and weighing for 50 grams each. Each species of brown algae were cut into small pieces and extracted in the presence of liquid nitrogen. 20 mM phosphate buffer containing 0,85% NaCl and 0,02% NaN_3 (PBSA, pH 7.0) was used for the sample extraction. The mixture of samples and buffer (PBSA) was then was stirred by using a magnetic stirrer overnight at 4^{o}C. After that, the mixture was centrifuged 8000 rpm at 4^{o}C for 30 minutes. Solid ammonium sulfates were grounded and then were added into the obtained supernatant to attain the concentration of 75% saturation. The mixture was kept for one night at 4^{o}C and was centrifuged 8000 rpm at 4^{o}C for 30 minutes. The precipitate was then dissolved by buffer addition as little as possible and was dialyzed. After being dialyzed, the inner fraction was further centrifuged 10,000 rpm at 4^{o}C for 30 minutes and the obtained supernatant was called as crude lectins fractions. These crude lectins fractions were kept in the freezer at -20^{o}C and was used to be analyzed further, namely hemagglutination activities and protein contents.

In preparing the hemagglutination activity, erythrocytes of rabbit and human were used (with blood types A, B, and O). Enzyme-treated erythrocytes were prepared from rabbit erythrocytes and human erythrocytes A, B, and O, whereas the enzymes used was trypsin. Erythrocytes were washed three times with 50 volume of saline (0,85 % NaCl) and were suspended into saline to give 2 % (v/v) suspension from native erythrocytes. After that, 0,5 % (w/v) of enzymes(trypsin and papain for each) in saline (1/10 volumes) was added into 2 % suspension of native erythrocytes and the mixture were incubated at 37^{o}C for 60 minutes. Next, the mixture was washed for four times in saline and 2% suspension of enzyme-treated erythrocytes were prepared in saline.

The hemagglutination activity assay was determined based on Hori *et al*. (1986) methods in Praseptiangga *et al*. (2012). This assay was conducted in 96-well microtiter V-plate by using

enzyme-treated and native erythrocytes. Hemagglutination activity was observed macroscopically and was scored as positive when more than 50 % of TRBC in the well was agglutinated. This activity was expressed as titer, the reciprocal of the highest twofold dilution exhibiting positive hemagglutination (Hori *et al*, 1986a). Proteins were determined by using BCA Protein Assay (Thermo Scientific™ Pierce™) method.

Results and Discussion

Gunungkidul coast of Yogyakarta and Binuangeun coast of Banten are located at the Southern part of Java Island with the highest brown algae spreading (Nurmiyati, 2013; Kusumawati and Murdinah, 2012). 11 species of brown macroalgae were found and identified, namely *Dictyota dichotoma* (Krakal Beach, Watulawang, Siung, and Wedi Ombo), *Himanthalia elongata* (Watulawang), *Padina minor* (Watulawang, Pok Tunggal, Siung and Wedi Ombo), *Padina pavonica* (Watulawang), *Sargassum crassifolium* (Krakal, Watulawang, Siung, and Wedi Ombo), *Sargassum cristaefolium* (Krakal, Watulawang, Siung, and Wedi Ombo), *Sargassum gracillimum* (Watulawang and Pok Tunggal), *Sargassum oligocystum* (Krakal), *Sargassum turbinarioides* (Siung), *Turbinaria conoides* (Wedi Ombo), and *Turbinaria ornata* (Krakal) taken from Gunungkidul coast and two species brown macroalgae such as *Padina australis* and *Sargassum polycystum* taken from Binuangeun coast.

The starting materials of extraction was chosen to the samples with minimum 50 grams (starting material). Thus, 10 out of 13 species were chosen to be extracted on this preliminary study. They were *Dictyota dichotoma*, *Padina australis*, *Padina minor*, *Sargassum crassifolium*, *Sargassum cristaefolium*, *Sargassum gracillimum*, *Sargassum oligocystum*, *Sargassum turbinarioides*, *Sargassum polycystum* and *Turbinaria ornata*.

Protein Contents

Table 1. Protein Content of Crude Lectins Fractions

No	Species	Protein Content (µg/ml)
1	Dictyota dichotoma	1.540,764*
2	Padina australis	995,972*
3	Padina minor	1.260,972*
4	Sargassum crassifolium	1.107,683
5	Sargassum cristaefolium	1.387,167
6	Sargassum gracillimum	862,471*
7	Sargassum oligocystum	1.590,7
8	Sargassum polycystum	764,931
9	Sargassum turbinarioides	723,65
10	Turbinaria ornata	1.298,45

Note: * = the results of 5x dilution

There were four thick samples having concentration more than 2000 µg/ml, which were diluted for 5x in order to be able to read. Based on Table 1. *Sargassum oligocystum* had the highest protein content of 1.590,7 µg/ml and *Sargassum turbinarioides* had the lowest protein content of 723,65 µg/ml. When associated with rendement of crude lectins fraction,

the protein content could not be directly proportional to the result of rendement since the tested protein content was total protein content, therefore it was not only lectins which were detected as protein. There are many factors which can influence the of algae, such as light, season and temperature, salt content, water movement, and nutrients (Achmad dan Atmadja, 1988). Some proteins are very sensitive to changes in environment. The protein activity depends a lot on the structure and the molecule conformation of protein. When the molecule conformation of protein changes, therefore the activity of its biochemical activities will reduce (Poedjiaji, 1994). When proteins are denaturated, therefore the unique structures of polypeptide will be distracted and cause the molecule to open into random structures (Poedjiaji, 1994). The factors influencing the stability of protein structures are pH, radiation, temperature, organic solvent media and surfactant (Goeswin, 2009).

Hemagglutination Activity Assay

Rabbit erythrocytes were used for Hemagglutination Activity (HA) assay. Native (RBC) and trypsin-treated enzyme (TRBC) was tested for 2 times of repetition. The obtained means were designated a titer. A titer is the reciprocal of the highest two-fold dilution showing positive hemagglutination (Hori *et al.*, 1981). It was determined to be positive when there were similar layers on the bottom of the hole and to be negative when there was central circle formed from the erythrocytes surrounded by clean concentric zones with the same measurement (John *et al.*, 2013). According to Hori, *et al.* (1981) researching 53 species of Japanese seaweed, algal lectins were sensitive rabbit erythrocytes than human. Moreover, according to Fabregas, *et al.* (1986) researching 28 brown seaweeds, rabbit erythrocytes were mostly suitable for seaweed lectins filtering compared to other animals (sheep, chicken, pig, horse, and calf).

Table 2. Hemagglutination Activities on Rabbit Erythrocytes

No	Species	RBC		Titer Means	TRBC		Titer Means
1	Dictyota dichotoma	2^{12}	2^{12}	4096	2^{16}	2^{16}	65536
2	Padina australis	2^{3}	2^{3}	8	2^{9}	2^{8}	384
3	Padina minor	2^{4}	2^{4}	16	2^{8}	2^{9}	384
4	Sargassum crassifolium	2^{2}	2^{2}	4	2^{4}	2^{4}	16
5	Sargassum cristaefolium	2^{5}	2^{5}	32	2^{6}	2^{6}	64
6	Sargassum gracillimum	2^{3}	2^{3}	8	2^{3}	2^{3}	8
7	Sargassum oligocystum	2^{6}	2^{5}	48	2^{6}	2^{5}	48
8	Sargassum polycystum	2^{4}	2^{4}	16	2^{8}	2^{8}	256
9	Sargassum turbinarioides	2^{4}	2^{4}	16	2^{5}	2^{5}	32
10	Turbinaria ornata	2^{3}	2^{3}	8	2^{7}	2^{6}	96

Based on Table **2,** all samples had hemagglutination activities, that 8 out of 10 species treated with trypsin enzymes on erythrocytes showing higher hemagglutination activities than native erythrocytes (without trypsin enzymes treatment). Rogers, *et al.* (1980) and Hori, *et al.* (1988) reported that hemagglutination activity was more readly detected when erythrocytes were enzyme-treated. Furthermore, Rogers and Hori (1993) reported that the principle effect caused by proteolytic enzyme treatment toward erythrocytes was the sialoglycoprotein release from cell surface, therefore it decreased negative contents of the surface in cells and facilitated hemagglutinations by lectins. Samples of *Sargassum gracillimum* and *Sargassum oligocystum* showed constant hemagglutination activities with or without trypsin enzymes treatment. According to Mangaiyarkarasi, *et al.* (2014), in recent studies, a treatment using proteolytic on erythrocytes enzyme did not indicate results having the same form (higher, constant, lower) toward hemagglutination activities with RBC, depending on blood types and the seaweed extract which was used.

Dictyota dichotoma lectins had hemagglutination activities in titer 4.096 toward rabbit RBC. A similar result was reported by Fabregas, *et al.* (1986) researching *Dictyota dichotoma* from Spain. Rabbit TRBC indicated the hemagglutination activity titer of 65.536. Hung *et al.* (2012) also reported the existence of *Dictyota dichotoma* lectins which agglutinated rabbit TRBC, but on titer of 4 there was no existance for rabbit RBC. There was a difference of extraction methodes of Hung, *et al.* (2012) and Fabregas, *et al.* (1986) with the methodes of Praseptiangga, *et al.* (2012) which were used in this research. Hung, *et al.* (2012) mentioned the ratio of PBS 3:1 samples weight and centrifugation speeds at 6000 rpm. Fabregas, *et al.* (1986) mentioned starting material (10 gram), preparation processes of size reduction (4 times washing with 0.5 N HCl, 10 times with aquades, and drainage with oven at 180^0 C for 6 hours before crushing using mortar), centrifugation speeds at 1000 rpm, the resulted crude lectins fraction (the supernatant which was filtered at a pore size of 0.22 µm as *yield*) and HA testing methods was the incubation time(2 hours).

Padina australis lectins had hemagglutination activities on titer of 8 toward rabbit RBC and 384 toward rabbit TRBC. Fajarningsih, *et al.* (2014) and Rizki (2014) reported *Padina austalis* of the same beach agglutinated rabbit TRBC, on titer of 12. There was a difference about the extraction methodes of Fajarningsih, *et al.* (2014) and Rizki (2014) with the methodes of Praseptiangga, *et al.* (2012) which were used in this research. The extraction methodes used by Fajarningsih, *et al.* (2014) as well as by Rizki (2014) only got into the first centrifugation process (there was no ammonium sulfates precipitation and dialysis to obtain salting out fraction).

Padina minor lectins had hemagglutination activities on titer of 16 toward rabbit RBC and of 384 toward rabbit TRBC. *Sargassum crassifolium* lectins had hemagglutination activities on titer 4 toward rabbit RBC and 16 toward rabbit TRBC. Rizki (2014) reported *Sargassum crassifolium* of the same beach agglutinated rabbit RBC and TRBC, on titer of 1024. *Sargassum cristaefolium* lectins had hemagglutination activities on titer of 32 toward rabbit RBC and of 64 toward rabbit TRBC. *Sargassum gracillimum* had hemagglutination activities on titer of 8 toward rabbit RBC as well as TRBC.

Sargassum oligocystum lectins had hemagglutination activities on titer of 48 toward rabbit RBC as well as TRBC. Hung, *et al.* (2009) reported *Sargassum oligocystum* from Vietnam positively agglutinated rabbit erythrocytes, on titer of 4 for RBC and 32 for TRBC. *Sargassum polycystum* lectins had hemagglutination activities on titer of 16 toward rabbit RBC and 256 toward TRB. Hung, *et al.* (2009) reported *Sargassum polycystum* from Vietnam agglutinated rabbit TRBC, on titer of 8 and there was nothing for RBC. Rizki (2014) reported *Sargassum polycystum* from the same beach agglutinated rabbit RBC, on titer of 1024 and titer of 80 for TRBC. *Sargassum turbinarioides* lectins had hemagglutination activities on titer of 16 toward rabbit RBC and of 32 toward rabbit TRBC. *Turbinaria ornata* lectins had hemagglutination activities on titer of 8 toward rabbit RBC and of 96 toward TRBC. Rizki (2014) reported *Turbinaria ornata* from the same beach agglutinated rabbit erythrocytes, on titer of 1024 for RBC and of 256 for TRBC.

When compared to the results of the research conducted previously, the hemagglutination activities of lectins owned by the same type of species but having different habitat and different ways of extraction might provide different scores of activities. Ismet (2007) reported the difference of water environment might influence the metabolism of organisms, including its bioactivity significantly. According to Wibowo, *et al*. (2014), environmental factors such as light, salinities, water movement, water pH, and particularly temperature influenced growth, reproduction, photosynthesis, and respiration of seaweed. For example, extreme high and low temperatures might denaturate protein and break cell membranes, whereas current influenced the amount of nutrient obtained by algae, various for each kind of algae. Many reports discussed about composition of seaweeds and its function. In conclusion, the extract composition of seaweeds was influenced by the condition of geograpical location as well as the cultivation location and also its methods of extraction (Chojnacka *et al.*, 2012).

Hemagglutionation Activities Test of Human Erythrocytes

Specific hemagglutinins toward blood types plays important roles in the early investigation of specificities of basic structures on antigen related to ABO blood types. Walter J. T. Morgan and Winifred M. Watkins 1950s at Institut Lister, London found the conglomeration of type A erythrocytes by *lima bean* (*P. limensis*) lectins inhibited by specific sugars α-*linked* N-asetil-D-galaktosamin and type O erythrocytes by *asparagus pea* (*L. tetragonolobous*)lectins inhibited by specific sugars α-*linked* L-fukosa. They were the pioneers of sugar existence in cell surface and its potential as an identity marker was accepted themed *glycobiology* modern (Sharon and Lis, 2004). Erythrocytes agglutination of human with blood type B occured due to a very strong binding by lectins of the *D-galactose* on its surface(Khan *et al.*, 2002).

Table 4. Hemagglutination Activities on Human Erythrocytes

No	Names of Spesies	Erythrocyte A						Erythrocyte B						Erythrocyte O					
		Native		Titer Means	Trypsin-Treated		Titer Means	Native		Titer Means	Trypsin--Treated		Titer Means	Native		Titer Means	Trypsin-Treated		Titer Means
1	D. dichotoma	0	0	0	2^6	2^6	64	2^1	2^1	2	2^4	2^4	16	0	0	0	2^4	2^4	16
2	P. australis	0	0	0	2^6	2^5	48	0	0	0	2^5	2^5	32	0	0	0	2^6	2^5	48
3	P. minor	2^5	2^5	32	2^9	2^{10}	768	2^5	2^5	32	2^1	2^1	2	2^6	2^6	64	2^{14}	2^{14}	16384
4	S. crassifolium	2^2	2^2	4	2^2	2^2	4	0	0	0	0	0	0	0	0	0	0	0	0
5	S. cristaefolium	2^3	2^3	8	2^6	2^6	64	2^4	2^4	16	2^6	2^6	64	2^8	2^8	256	2^8	2^8	256
6	S. gracillimum	2^1	2^1	2	0	0	0	0	0	0	2^4	2^3	12	2^2	2^2	4	0	0	0
7	S. oligocystum	2^3	2^3	8	2^4	2^5	24	2^2	2^3	6	2^6	2^6	64	2^5	2^5	32	2^5	2^4	24
8	S. polycystum	2^6	2^6	64	2^{10}	2^{11}	1536	2^4	2^3	12	2^7	2^7	128	2^8	2^8	256	2^{10}	2^{10}	1024
9	S. turbinarioides	2^5	2^5	32	2^4	2^4	16	2^3	2^3	8	2^6	2^6	64	2^5	2^5	32	2^5	2^4	24
10	T. ornate	2^5	2^4	24	2^5	2^5	32	2^5	2^6	48	2^6	2^7	96	2^1	2^1	2	2^{10}	2^{10}	1024

The hemagglutination test toward human erythrocytes was done to ascertain the antigenic effects when applied as medicine as well as functional food. Based on Table 4, all samples show hemagglutination activities on human erythrocytes. Hemagglutination activities on human treated with trypsin enzymes indicated higher results than native erythrocytes, but some were not influenced or lower. *Dictyota dichotoma* lectins had lectins hemagglutination activities on titer 2 toward human RBC, only blood type B. The same result was reported by Fabregas, *et al.* (1986) researching *Dictyota dichotoma* from Spain. Meanwhile, a different result was reported by Hung, *et al.* (2012) researching *Dictyota dichotoma* from Vietnam which could not agglutinate human erythrocytes with or without trypsin treatment. Meanwhile, toward human TRBC, *Dictyota dichotoma* lectins agglutinated blood type A on titer of 64 and were similar to blood type B as well as blood type O on titer of 16.

Padina australis lectins had hemagglutination activities toward human TRBC manusia only, on titer of 32 for blood type B and of 48 for blood types A and O. Fajarningsih, *et al.* (2014) reported *Padina austalis* from the same beach agglutinated human TRBC, on titer of 64 for blood type A, 8 for blood type B, and 4 for blood type O. Rizki (2014) also reported *Padina austalis* from the same beach agglutinated human TRBC, on titer of 32 for blood type A and of 12 for blood types B and O. In this research, it was found that *Padina australis* did not

agglutinate human RBC, whereas Fajarningsih, *et al.* (2014) and Rizki (2014) reported it was able to agglutinate RBC blood types B and O on titer of 1024.

Padina minor lectins had hemagglutination activities toward human RBC as well as TRBC. RBC blood type O was agglutinated on titer of 64, blood types A and B on titer of 32. Meanwhile, on TRBC treatment, blood type O was agglutinated strongly on titer of 16.384, blood type A on titer of 768 and blood type B on titer of 2. Boyd, *et al.* (1966) reported that in the same genus but different species, *Padina vickersiae* from Puerto Rico agglutinated human erythrocytes blood types A and O strongly but did not agglutinate blood type B. There was a difference between methods of extraction of Boyd, *et al.* (1966) and the methods of Praseptiangga, *et al.* (2012) which were used in this research, which was to get lectins only to the supernatant filter as a result of size reduction by using waring blender.

Sargassum crassifolium lectins had specific hemagglutination activities toward human erythrocytes blood type A only, which was on titer of 4 for both RBC and TRBC. Rizki (2014) reported *Sargassum crassifolium* from the same beach agglutinated human erythrocytes both RBC and TRBC. On titer of 4, for RBC blood types A, B, and O, whereas TRBC on titer of 64 for blood type A, 32 for blood type B, and 48 for blood type O. *Sargassum cristaefolium* lectins had hemagglutination activity toward both human RBC and TRBC. RBC blood type A was agglutinated on titer of 8 and blood type B on titer of 16. In the TRBC treatment, it showed a higher result which was on titer of 64. Meanwhile, the HA toward blood type O was agglutinated on titer of 256 for both TRBC and RBC.

Sargassum gracillimum lectins had hemagglutination activities toward human RBC blood type A on titer of 2 and blood type O on titer of 4, and there was no activity for blood type B. Meanwhile, *Sargassum gracillimum* lectins agglutinated blood type B only toward TRBC treatment on titer of 12. *Sargassum olygocystum* lectins had hemagglutination activity toward both human TRBC and RBC. RBC blood type A was agglutinated on titer of 8, blood type B on titer of 6, and blood type O on titer of 32. Meanwhile, toward TRBC treatment, blood type B was agglutinated on titer of 64, blood types A and O on titer of 24. Hung, *et al.* (2009) reported *Sargassum oligocystum* from Vietnam agglutinated both human RBC and TRBC, on titer of 8 for TRBC blood type A and on titer of 32 for RBC blood type A as well as RBC and TRBC blood types B dan O.

Sargassum polycystum lectins had hemagglutination activity toward both human RBC and TRBC. On RBC blood type A on titer of 64, blood type B on titer of 12, and blood type O on titer of 256. Meanwhile, toward TRBC, on titer of 1,536 for blood type A, blood type B on titer of 128, and blood type O on titer of 1,024. Hung, *et al.* (2009) reported *Sargassum oligocystum* from Vietnam did not agglutinate human erythrocytes. Different results were reported by Rizki (2014) that *Sargassum polycystum* lectins from the same beach agglutinated blood type A on titer of 3 for RBC and 4 for TRBC, blood type B on titer of 2 for RBC and on titer of 3 for TRBC, and blood type O on titer of 1,024 for RBC only.

Sargassum turbinarioides lectins had hemagglutination activities toward human RBC and TRBC. RBC blood type B agglutinated on titer of 8, blood types A and O on titer of 32. Meanwhile, in TRBC treatment, blood type A agglutinated on titer of 16, blood type B on

titer of 64 and blood type O on titer of 24. *Turbinaria ornata* lectins had hemagglutination activities toward human RBC and TRBC. RBC blood type A agglutinated on titer of 24, blood group B on titer of 48, and blood type O on titer of 2. In TRBC treatment, blood type A agglutinated on titer of 32 and blood type B on titer of 96, while blood type O strongly agglutinated on titer of 1024. Rizki (2014) reported *Turbinaria ornata* from the same beach positevely agglutinated human RBC and TRBC, on titer of 10 to RBC blood type A, 8 to RBC blood type B, 1024 to RBC blood typee O, and titers equal to 64 TRBC blood types A, B, and O.

Thus, the test results showed the crude fraction lectins of brown macroalgae samples had hemagglutination activities toward human erythrocytes. In native human erythrocytes, the highest hemagglutination activity was produced by species of *Sargassum polycystum* on titer of 64 toward blood type A, *Turbinaria ornata* on titer 48 toward blood type B, and *Sargassum cristaefolium* and *Sargassum polycystum* on titer of 256 toward blood type O. In human TRBC, the highest hemagglutination activity was produced by species of *Sargassum polycystum* 1,536 titers toward blood type A, *Turbinaria ornata* on titer of 128 toward blood type B, and *Padina minor* on titer of 16,384 to blood type O. All species had hemagglutination activities toward RBC and TRBC blood type A. Meanwhile, *Sargassum crassifolium* species did not have hemagglutination activity toward blood types B and O, but it is specific to blood type A both RBC and TRBC on titer of 4.

Conclusion

There were 11 species of brown macroalgae from Gunungkidul coast, Yogyakarta and two species of brown macroalgae from Binuangeun coast, Banten. The 13 species of brown macroalgae were *Dictyota dichotoma, Himanthalia elongata, Padina australis, Padina minor, Padina pavonica, crassifolium Sargassum, Sargassum cristaefolium, gracillimum Sargassum, Sargassum oligocystum, polycystum Sargassum, Sargassum turbinarioides, Turbinaria conoides and Turbinaria ornata.* 10 species of brown macroalgae from Gunungkidul coast and Binuangeun coast namely *D. dichotoma, P. australis, P. minor, S. crassifolium, S. cristaefolium, S. gracillimum, S. oligocystum, S. polycystum, S. turbinarioides* and *Turbinaria ornata* showed hemagglutination activities toward rabbit erythrocytes with the highest activity at *D. dichotoma*. In human erythrocytes, 6 brown macroalgae species, namely *P. minor, S. cristaefolium, S. oligocystum, S. polycystum, S. turbinarioides* and *Turbinaria ornata* had hemagglutination activity against all types (native or by treatment with trypsin), *D. dichotoma* and *P. australis* had hemagglutination activities toward all types with trypsin treatment where *D. dichotoma* also had activities toward native Type B, *S. gracillimum* had hemagglutination activities of the native type A and O and type B by trypsin treatment, and *S. crassifolium* specific to type A (native or treated with trypsin).

Acknowledgement

This work was financially supported by research project of PNBP UNS and from Sebelas Maret University, Indonesia and the Education and Cultural Office of Central Java Province Local Government of Central Java Province, the Office of Education and Culture which has provided Facilitation Program Universities in Central Java, so that this research can be conducted well.

REFERENCES

Anonim, (2011) Kementrian Perdagangan Republik Indonesia. Rumput Laut dan Produk Turunannya. Jakarta: Warta Ekspor Edisi Oktober 2011.

Ainouz, I. L., dan Sampaio, A. H. (1991). Screening of Brazilian Marine Algae for Hemagglutinins. *Journal Botanica Marina*, 32: 211-214.

Ainouz, I.L., Sampaio, A.H., Benevides, N.M.B., Freitas, A.L.P., Costa, F.H.F., Carvalho, M.C., Pinheiro-Joventino, F., 1992. *Agglutination of enzyme treated erythrocytes by Brazilian marine algae*. Bot. Mar. 35, 475–479.

Bird, K.T, Chiles, T.C., Longley, R.E., Kendrick, A.F., Kinkema, M.D., 1993. *Agglutinins from marine macroalgae of the Southeastern United States.* J. Appl. Phycol. 5, 212–213.

Blunden, G., D. J. Rogers, dan W. F. Farnham. 1975. Survey of British Seaweeds for Hemagglutinins. Lloydia, Vol. 38, pp. 162-168 dalam Fabregas J., A. Munoz, dan J. Llovo. 1986. Hemagglutinins in Brown Seaweeds. Journal Exp. Mar. Biol. Ecol., vol. 97, pp. 213-219.

Boyd, W. C., Almodovar, L. R., dan Boyd L. G. (1966). Agglutinins in Marine Algae for Human Erythrocytes. *Journal of Transfusion*, 6: 82-83.

Chiles, T. C., dan Bird, K. T. (1989). A Comparative Study of Animal Erythrocyte Agglutinins from Marine Algae. *Journal of Comp. Biochemical Physiology*, 94B (1):107-111.

Chojnacka, K., Saeid, A., Witkowska, Z., dan Tuhy, L. (2012). Biologically Active Compounds In Seaweed Extracts – The Prospects for The Application. *The Open Conference Proceedings Journal*, 3: 20-28.

Fabregas, J., Llovo, J., Muñoz, A., 1985. *Hemagglutinins in red seaweeds.* Bot. Mar. 28, 517-520.

Fabregas, J., Munoz, A., dan Llovo, J. (1986). Short Communication Hemagglutinins in Brown Seaweeds. *J. Exp. Mar. Biol. Ecol*, 97: 213-219.

Fajarningsih, N. D., Yamin, D. F., Yunita, Idha., Fahriza, Anisa., Praseptiangga, D., Sarnianto, Prih., dan Chasanah, E. (2014). Penapisan Senyawa Hemaglutinin dari Makroalga Asal Pantai Binuangeun, Banten.

Freitas, A.L.P., Teixeira, D.I.A., Costa, F.H.F., Farias, W.R.L., Lobato, A.S.C., Sampaio, A.H., Benevides, N.M.B., 1997. *A new survey of Brazilian marine algae for agglutinins.* J. Appl. Phycol. 9, 495–501.

Gabius, H. J. (Ed.). (2009). The Sugar Code: Fundamentals of Glycosciences. Jerman: Wiley-VCH. Weinhem.

Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T., dan Sharon, N. (1980). What Should be Called a Lectin?. *Nature Journal*, 285: 66.

Goeswin, A. (2009). Sediaan Farmasi Steril. Bandung: ITB Press.

Hori, K., Miyazawa, K., dan Ito, K. (1981). Hemagglutinins in Marine Algae. *Bulletin of the Japanese Society of Scientific Fisheries*, 47 (6): 793-798.

Hori, K., Miyazawa, K., dan Ito, K. (1986). Preliminary Characterization of Agglutinins from Seven Marine Algal Species. *Bulletin of the Japanese Society of Scientific Fisheries*, 52 (2): 323-331.

Hori, K., Ikegami, S., Miyazawa, K., dan Ito, K. (1988). Mitogenic and Antineoplastic Isoagglutinins from The Red Alga *Solieria robusta*. *Journal of Phytochemistry*, 27 (7).

Hung, L. D., Hori, K., dan Quang, N. H. (2009). Screening and Preliminary Characterization of Hemagglutinins in Vietnamese Marine Algae. *Journal of Applied Phycology*, 21.

Hung, L. D., Sato, T., Shibata, H., dan Hori, K. (2009). Biochemical Comparison of Lectins among Three Different Color Strains of The Red Alga *Kappaphycus alvarezii*. *Fisheries Science*, 75 (3): 723-730

Hung, L. D., Bui, M. L., Trang, V. T. D., Ngoc, N. T. D., Hoa, L. T., dan Trinh, P. T. H. (2012). A New Screening for Hemagglutinins from Vietnamese Marine Macroalgae. *Journal of Applied Phyclogy*, 22: 227.

Ismet, M. S. (2007). Penapisan Senyawa Bioaktif Spons *Aaptos aaptos* dan *Petrosia* sp. dari Lokasi yang Berbeda. Bogor: IPB Press.

John, S. A., dan Claimer, C. S. (2013). Isolation, Purification and Characterization of Galactose Spesifix Lectin from Adathoda vasica. *International Journal of Advanced Life Sciences* 6 (3): 238-245.

Kasim, M., dan Asnani. (2012). Penentuan Musim Reproduksi Generatif dan Preferensi Perekatan Spora Rumput Laut (*Eucheuma cottonii*). *Indonesian Journal of Marine Sciences/Ilmu Kelautan*, 17 (4).

Khan, F., Khan R. H., dan Sherwani, A. (2002). Lectins as Markers for Blood Grouping. *Journal of Medical Science Monitor*, 8: 293–300.

Kim, G. H., Klochkova, T. A., Yoon, K., Song, Y., dan Lee, K. P. (2005). Purification and Characterization of A Lectin, Bryohealin, Involved in The Protoplast Formation of A Marine Green Alga *Bryopsis Plumosa* (Chlorophyta). *Journal of Applied Phycology*, 42: 86-95.

Kumar, A. S., Nagarjuna, S., Venkatakullaisetty, N., Raju. C., dan Kashinath, M. (2011). A Comprehensive Review on Marine Algae and Their Multidisciplinary Activities. *International Journal of Advances in Pharmacy and Biological Sciences*, 1 (2): 44-50.

Kusumawati, R., dan Murdinah. (2012). Potensi Pemanfaatan Rumput Laut: Biodiversitas di Pantai Binuangeun. Prosiding Seminar Nasional Inovasi Teknologi. Balai Besar Litbang Pengolahan Produk dan Bioteknologi Kelautan dan Perikanan, Jakarta

Nurmiyati. (2013). Keragaman, Distribusi dan Nilai Penting Makro Alga di Pantai Sepanjang Gunungkidul. *Jurnal Bioedukasi*, 6 (1): 12-21.

Nursid, M., Wikanta, T., dan Susilowati, R. (2013). Aktivitas Antioksidan, Sitotoksisitas, dan Kandungan Fukosantin Ekstrak Rumput Laut Coklat dari Pantai Binuangeun, Banten. *Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan*, 8 (1): 73-84.

Oliveira, S. R. M., Nascimento, A. E., Lima, M. E. P., Leite, Y. F., dan Benevides, M. B. (2002). Purification and Characterisation of A Lectin from The Red Marine Alga *Pterocladiella capillacea* (S. G. Gmel.) Santel. & Hommers. *Revista Brasil. Bot.*, 25 (4): 397-403

Pietrzyk, A.J., Bujacz, A., Mak, P., Potempa, B., dan Niedziela, T. (2015). Structural Studies of Helix aspera Agglutinin Complexed with GalNAc:A Lectin that Serve as a Diagnostic Tool. *International Journal of biomac*, 81: 1059-1068.

Poedjiaji, A. (1994). Dasar-Dasar Biokimia. Jakarta: UI Press.

Praseptiangga, D., Hirayama, M., dan Hori, K. (2012). Purification, Characterization, and cDNA Cloning of a Novel Lectin from the Green Alga, *Codium barbatum*. *Journal of Bioscience, Biotechnology, Biochemical*, 76 (4): 805-811.

Rizki, I.Y. (2014). Uji Aktivitas Hemaglutinasi Lektin dalam Alga Cokelat (Phaeophyta) dari Perairan Pantai Binuangeun (Banten) dan Sepajang (Gunung Kidul). Jakarta: Universitas Pancasila Press.

Rogers, D.J., dan Hori, K. (1993). *M*arine Algal Lectins: New Developments. *Journal Hydrobiologia*, 260: 589-593.

Rogers, D. J., dan Blunden, G. (1980). Structural Properties of the anti-B Lectin from the Red Alga *Ptilota plumose* (Huds.) C. Agardh. *Journal Botanica Marina*, 23: 459-462.

Rosyid, M. (2015). Menggali Potensi Perairan dalam Al-Quran dengan Pendekatan Sejarah. *Hermeneutik*, 1 (1): 93-111.

Setyawati, E., Ma'arif, S., dan Arkeman, Y. (2014). Inovasi Hijau dalam Industri Pengolahan Rumput Laut Semi Refined Carrageenan (SRC). *Jurnal Teknik Industri*, ISSN: 1411-6340.

Sharon, N. (2005). Memories of a Senior Scientist a Life With Lectins. *Celluler and Molecular Life Sciences*, 62.

Sharon, N., dan Lis, H. (2004). Review – History of Lectins: from Hemagglutinins to Biological Recognition Molecules. *Jorunal of Glycobiology*, 14 (11): 53-62.

Supriatna, J. (2008). Melestarikan Alam Indonesia. Jakarta: Yayasan Obor Indonesia.

Wikanta, T., Damayanti, R., dan Rahayu, L. (2008). Pengaruh Pemberian K-Karagenan dan I-Karagenan terhadap Penurunan Kadar Glukosa Darah Tikus Hiperglikemia. *Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan*, 3 (2): 131-138.

Witikta, A. (2014). Kebijakan Pemerintah Indonesia dalam Penegakan Hukum Zona Ekonomi Eksklusif (ZEE) di Wilayah Negara Republik Indonesia Ditinjau dari Perspektif Hukum Internasional. Jember: UNEJ Press.

Wibowo, Singgih., Peranginangin, Rosmawaty., Darmawan, Muhamad., dan Hakim, Arif Rahman. (2014). Teknik Pengolahan ATC dari Rumput Laut *Eucheuma cottonii*. Jakarta: Penebar Swadaya.

NUTRITION
AND
CULINARY SCIENCE

Effect of Surfactant on Cellular Uptake of Human Serum Albumin Nanoparticles

Waralee Watcharin
Department of Agro-Industry, Faculty of Biotechnology, Assumption University, Hua Mak Campus, Bangkok 10240, Thailand

E-mail: waraleewtc@au.edu

ABSTRACT

Human serum albumin (HSA) nanoparticles appear to be a suitable carrier due to their safety and feasibility of functionalization. For drug and gene delivery applications, it is important to study the intracellular uptake and tissue distribution to optimize the efficacy of the encapsulated therapeutic agent. (Gd-DTPA)-conjugated HSA nanoparticles were prepared and coated with poloxamine as a surfactant. The effect of poloxamine on the uptake of nanoparticles was investigated by Fluorescence-activated cell sorting (FACS) analysis. The quantification of fluorescence-signal profiles of the particles indicated the difference in the *in vitro* cellular uptake of unmodified and modified nanoparticles.

Keywords: Human serum albumin, Poloxamine, Nanoparticles, Cellular uptake, Fluorescence-signal profiles

INTRODUCTION

The use of biodegradable human serum albumin (HSA) nanoparticles for drug and gene delivery has been extensively studied for a few decades (Lohcharoenkal et al., 2014; Look et al., 2015). HSA nanoparticles have been well employed as carriers for several anticancer agents such as paclitaxel, methotrexate, doxorubicin, and 5-fluorouracil (5-FU) (Cucinotto et al., 2013; Bae et al., 2012; Misak et al., 2013; Taheri et al., 2012). Due to its biodegradability, non-toxicity, and non-immunogenicity, the use of HSA nanoparticles in a combination of chemotherapy and gene therapy hold promise for the treatment of a number of cancers i.e. hepatocarcinoma, pancreatic, neuroblastoma, and prostate cancers (Cucinotto et al., 2013). A formulation of paclitaxel-HSA nanoparticles (Abraxane^{TM}) was approved by FDA for metastatic breast cancer in 2005 (Wacker 2013). Moreover, HSA nanoparticles with attached apolipoprotein E have successfully been used to transport drugs across Blood Brain Barrier (BBB) (Zensi *et al.* 2009). And HSA nanoparticles conjugated with Gd--based contrast agents and coupled with transferrin can also cross BBB (Korkusuz *et al.* 2013). In another application, Arnedo et al. reported the delay and decrease in cellular uptake of oligonucleotides by HSA nanoparticles in MRC-5 cell (Rhaese et al., 2003). HSA nanoparticles associated to DNA-PEI complexes could efficiently transfect and deliver DNA to target cells preventing the degradation of oligonucleotides.

Up to date, HSA nanoparticles are widely accepted as a platform which can offer a higher drug bioavailability compared to a reference solution (Loureiro et al., 2016). The major advantage of the nanoparticles for intravenous injection is their small particle size which enables the passage through very small capillaries in the blood circulation system. However, the rapid elimination from the blood circulation by phagocytosis is a major limitation of intravenous delivery of nanoparticles. When distributing in systemic circulation of body, the nanoparticles are coated with blood components on their surface as any foreign material through opsonization processes (Moghimi et al., 2012). The opsonizing molecules rapidly induce the phagocytosis by macrophages of the reticuloendothelial system (RES). Therefore, intravenous injected nanoparticles are mainly distributed into the liver (60-90%) and spleen (2-20%) within minutes after administration (Kreuter 1996).

The surface modification of nanoparticles has been mainly focused on development of long circulating nanoparticles. Surface absorption may be achieved *via* either hydrophobic interactions of drug moieties and polymer particle surfaces or electric charge interactions (Sakuma et al., 2002). The application of this method is more reasonable for coupling therapeutic agents to non-stealth carriers. Due to nature of stealth particles, surface coating cannot produce an effective loading and delivery as a result of insufficient loading capacity. Although surface absorption is limited as it does not protect the degradation of the drug bound within the blood or intracellular compartments (i.e. lysosomes), it may alter the drug release in a controlled way, prolong circulation time, and offer alternative tissue targeting. It has been established that surface-coating of nanoparticles can decrease the uptake by the macrophage cells of RES and increase the circulation time (Moghimi et al., 2012). The polymeric surfactants of PEG and poloxamine series could be a challenge possibility to investigate the prolonged circulation of surface-engineered nanoparticles to achieve stealth properties (Otsuka et al., 2003).

Nanoparticle-based magnetic resonance imaging (MRI) contrast agents, such as Gd-DTPA and Gd-DOTA, have been investigated as platforms for targeting imaging agents for the tumor detection *in vitro* and *in vivo*. In the present work Gd-DTPA covalently bond to HSA nanoparticles were developed as a potential MRI contrast agent for improving the detection of HCC (Watcharin et al., 2014). In this study, Gd-DTPA-loaded HSA nanoparticles were prepared and coated with poloxamine as a surfactant. The effect of poloxamine on the uptake of nanoparticles was evaluated by Fluorescence-activated cell sorting (FACS) analysis.

EXPERIMENTAL METHODS

Materials

Batch 028K7550 of HSA, fraction V, (96–99%), diethylenetriaminepentaacetic acid dianhydride (DTPAa) (98%), rhodamine 123, and $GdCl_3$ (anhydrous powder, 99.99%) were purchased from Sigma (Steinheim, Germany). Poloxamine 908 was obtained from Boehringer Ingelheim (Ingelheim, Germany). All other reagents were of analytical grade and used as received.

Methods

Preparation of nanoparticles

Preparation of HSA nanoparticles (HSA-NP) and Gd-DTPA labeled HSA nanoparticles (Gd-HSA-NP)

HSA-NP were prepared by a desolvation process (Weber et al., 2000). To prepare Gd-HSA-NP, an amount of 5 mg DTPAa was added to 1 mL HSA-NP. pH was adjusted to 9.2, and incubated at room temperature for 3 h under constant shaking 600 rpm. To separate the unbound DTPAa from the protein fraction, DTPA-conjugated HSA-NP were purified by three cycles of centrifugation (16,100×g, 10min) and redispersion in 1.0 mL water to yield HSA-DTPA conjugates. 20 µL 94.8 mM $GdCl_3$ was then added into HSA-DTPA and incubated at 21°C for 30 min under constant shaking 600 rpm. The Gd-HSA-NP was purified by four cycles of differential centrifugation (16,100×g, 10min) and redispersion 1.0 mL water.

Preparation of Fluorescently labeled (Gd-DTPA)-HSA nanoparticles (Gd-Rho-HSA-NP)

Gd-Rho-HSA-NP were prepared as described by Watcharin (Watcharin et al., 2013). The 1 mg of EDC in 0.1 ml PBS (pH 8) was added into the Gd-HSA-NP suspension, and incubated for 15 min. Then 50 µg of rhodamine 123 was added to the resulting nanoparticles, and continued to incubate for 3 h at room temperature. The particles were purified by centrifugation 3 times to obtain the rhodamine 123-labeled (Gd-DTPA)-HSA nanoparticles (Gd-Rho-HSA-NP).

Preparation of poloxamine-coated Gd-Rho-HSA-NP [po(Gd-Rho-HSA)-NP]

The prepared nanoparticles, Gd-Rho-HSA-NP, were mixed with an appropriate amount of 1% w/v solution of poloxamine 908 to obtain a final surfactant concentration of 0.5% w/v. The nanoparticle suspensions were incubated overnight under constant agitation (550 rpm at room temperature). Poloxamine-modified nanoparticles [po(Gd-Rho-HSA)-NP] were washed by centrifugation (16,100×g, 8 min) to remove the excess surfactant and redispersed to the original volume in water.

Particle characterization

Particle size and surface charge

The mean diameter and polydispersity of the resulting nanoparticles were measured by photon correlation spectroscopy (PCS); their zeta-potential was assessed using Laser Doppler micro-electrophoresis in a palladium electrode dip cell (Malvern Instruments). For the measurements the samples were diluted 1:50 with purified water; the scattering angle was 173°; the temperature was set to 25°C. All measurements were repeated 3 times.

Particle morphology

The morphology of the nanoparticles was examined by transmission electronic microscopy (TEM). The samples were prepared by placing a drop of nanoparticle suspension onto a copper grid. Then the grids were air-dried, and the particles were stained with 3% aqueous solution of uranyl acetate for contrast enhancement. The air-dried samples were then directly examined by TEM, JEM-1200EX (Japan)

Gd concentration

Gd(III) content was determined by total reflection X-ray fluorescence (TXRF) (S2 Picofox, Bruker nano, Germany) [154, 155]. Briefly, 45 µl of nanparticles was mixed with 5 µl of Gallium standard (1 mg/l). The solution mixture was carefully homogenized, subsequently 5 µl of solution was pipetted on a quartz carrier, and the excess solvent was evaporated. The air-dried samples were directly examined the amount of Gd in mg/l. The measurement was measured in triplicate for each sample.

Cellular uptake experiments

Cell culture preparation

The Huh-7 cells and HepG2 cells were cultured in DMEM medium supplemented with 10% FBS, streptomycin at 100 mg/ml and penicillin at 100 U/ml.

Cell uptake study

Comparison of cell uptake of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by Huh-7 cells

Cellular uptake of the nanoparticles into the Huh-7 cells was evaluated using flow cytometry. 100, 200 and 400 µg/ml of Gd-Rho-HSA-NP or po(Gd-Rho-HSA)-NP were added, and the cells were incubated for 24 h. The cells grown in the absence of the nanoparticles were used as control. Flow cytometry was performed on a FACS Calibur (Becton Dickinson Inc., USA).

Comparison of cell uptake of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by HepG2 cells

Cellular uptake of the nanoparticles into the HepG2 cells was evaluated using flow cytometry in the same way as previous experiments.

Comparison of cell uptake of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by hepatocytes

Cellular uptake of the nanoparticles into the hepatocytes was evaluated using flow cytometry in the same way as previous experiments. 100 µg/ml of Gd-Rho-HSA-NP or po(Gd-Rho-HSA)-NP were added, and incubated for 16 h in culture media. The cells grown in the absence of the nanoparticles were used as control.

Intracellular localization of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by hepatocytes

For the imaging of cellular uptake, the hepatocytes were cultured and 100 µg/ml of nanoparticles i.e. Gd-Rho-HSA-NP or po(Gd-Rho-HSA)-NP were added. After incubation

for 4 h, the cells were stained with rabbit polyclonal anti-EEA1 antibody, goat anti-rabbit IgG H&L (Chromeo™ 488), and Hoechst. The stained cells were then observed by CLSM, Fluoview FV1000, Olympus (Japan).

RESULTS AND DISCUSSION

Particle characterization

HSA-NP can be easily prepared by two basic methods; emulsification and desolvation (Weber et al., 2000). Using the emulsification technique, an aqueous solution of albumin is emulsified in a lipophilic phase, dispersed in high concentrations of surfactant combined with highly energetic homoginisation, and can additionally be thermally stabilized at high temperatures. Finally, the particles are washed several times to remove residual oil. To prepare HSA-NP by the desolvation technique, a desolvating agent is added to an albumin solution, followed by stabilization using chemical crosslinker and then is purified to eliminate excess albumin molecules. Later, lyophilization can be used to preserve HSA nanoparticles for a long storage (Anhorn et al., 2008).

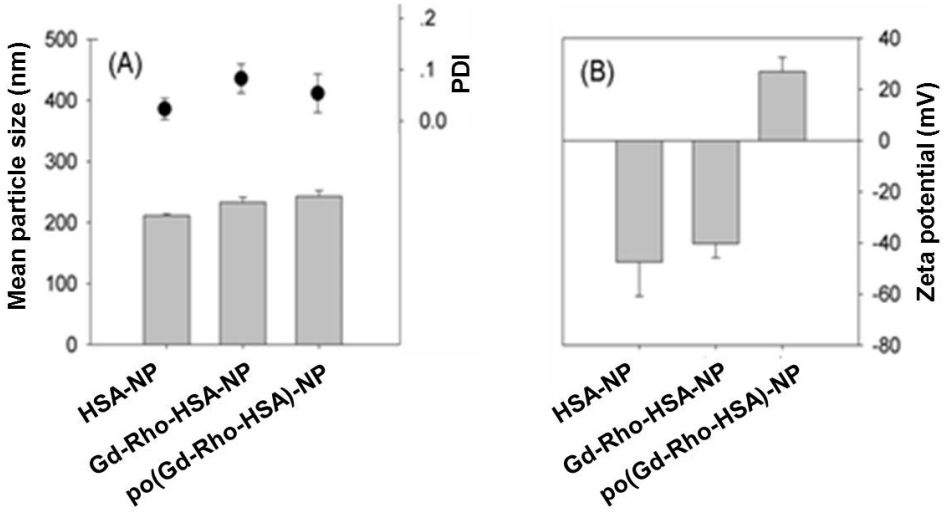


Figure 1: Particle size and polydispersity index (A) and zeta potential (B) of various HSA NPs

In this study, HSA-NP were prepared by desolvation and stabilised by crosslinking with glutaraldehyde. The physicochemical properties of unmodified and modified NPs, particle size and size-distribution, and zeta-potential were determined as shown in Figure 1. As determined by photon correlation spectroscopy (PCS), the empty nanoparticles had average diameters of ~ 200 nm, and the zeta-potential was -40 mV approximately. This was in agreement with a previous study (Ulbrich et al., 2011).

Gd-Rho-HSA-NP were modified by coating with poloxamine 908, containing four chains of both, PEO and PPO. The prepared particles were resuspended with 1% poloxamine 908 to obtain a final concentration of 0.5% poloxamine, i.e. po(Gd-Rho-HSA)-NP, subsequently incubated 3 h at room temperature and washed with dH2O to remove the excess surfactants. The modified nanopaticles were found to be in the

same size range of 244 ± 22 nm and a polydispersity of 0.054 ± 0.046. The zeta potential of poloxamine-coated nanoparticles strongly increased due to the physical adsorption of poloxamine to the nanoparticle surface. The binding of the Gd complex causes an increase of the particle size and polydispersity index as a result of the particle aggregation. The zeta potential of coated nanoparticles strongly increased due to the physical adsorption of poloxamine to the surface. The coating thickness of po(Gd-Rho-HSA)-NP was 4.78 ± 1.46. Therefore, coating of the surfactant can cause a change of the particle size and polydispersity index.

Gd-Rho-HSA-NP were investigated as novel contrast agents to detect HCC by T1-MRI in mice with endogenously formed HCC. In addition, the surface modification of the nanoparticles by coating them with poloxamine may reduce the RES uptake and alter the MRI signal due to their different distribution between tumor and liver.

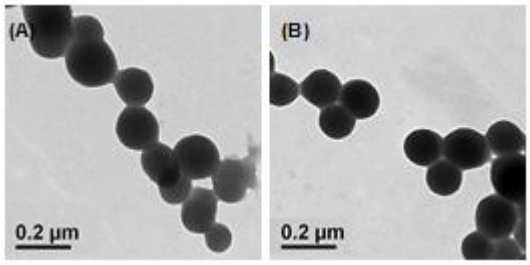


Figure 2: TEM images of Gd-Rho-HSA-NP (A) and po(Gd-Rho-HSA)-NP (B)

For the morphology of nanoparticles, TEM confirmed the morphology of NPs as a regular spherical shape (Figure 2). As shown by TEM, the Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP revealed a uniform size distribution and spherical shape similar to unmodified HSA-NP. The Gd content of HSA-Gd-Rh and po(Gd-Rho-HSA)-NP was 5.7 ± 1.0 mg Gd per g NP determined by TXRF.

***In vitro* cellular uptake**

Rhodamine 123 is a fluorescent dye widely used for general cellular staining in flow cytometry, fluorescent microscopy and immunohistochemistry. Indeed, Being a P-gp substrate, rhodamine 123 could serve as a marker of the P-glycoprotein-related efflux for materials in the hepatoma cells (Xu et al., 2012; Zastre et al., 2002; Lehne et al., 1996). The uptake of the Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by the Huh-7 (HCC cell line), HepG2 (HCC cell line) and primary hepatocyte cells was evaluated by flow cytometry. The concentration and uptake time are an essential factor which influence the efficiency of the cellular uptake of nanoparticles and affect their cytotoxicity. Time-dependent uptake also could decrease when the concentration of nanoparticles in the cells reach equilibrium as it promotes the intracellular lysosome proton sponge effect over time (Liu et al., 2013)

Cell uptake study of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by Huh-7

FACS analysis was carried out to study the *in vitro* cell uptake of Gd-Rho-HSA-NP or poloxamine-coated Gd-Rho-HSA-NP [po(Gd-Rho-HSA)-NP] at the concentration of 100 - 400 µg/ml. The suspensions of untreated Huh-7 cells were analyzed as a negative control which exhibited less than 0.5% cell uptake. As shown in Figure 3,

the cell uptake of Gd-Rho-HSA-NP by Huh-7 considerably increased in parallel with nanoparticle concentration whereas the cell uptake of po(Gd-Rho-HSA)-NP was found to increase with higher nanoparticle concentrations in a lower level after 24 h of incubation. Significant differences were observed between the % uptake of Gd-Rho-HSA-NP and modified po(Gd-Rho-HSA)-NP after incubation of each nanoparticle at the concentration of 100 - 400 µg/ml with the cells for 24 h.

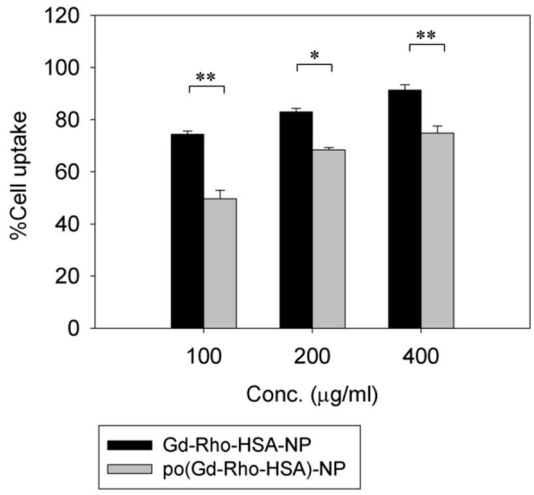


Figure 3: Cellular uptake of Gd-Rho-HSA-NP or poloxamine-coated Gd-RhoHSA-NP (po(Gd-Rho-HSA)-NP) by Huh-7 after 24 h of incubation at different NP concentrations (100 µg/ml, 200 µg/ml and 400 µg/ml) at 37ºC. The measurement was performed using a flow cytometric analysis (mean ± SD; n = 3). There was a statistically significant difference between uncoated and coated particles for each nanoparticle concentration (* $p < 0.05$; ** $p < 0.01$).

Cell uptake study of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by HepG2

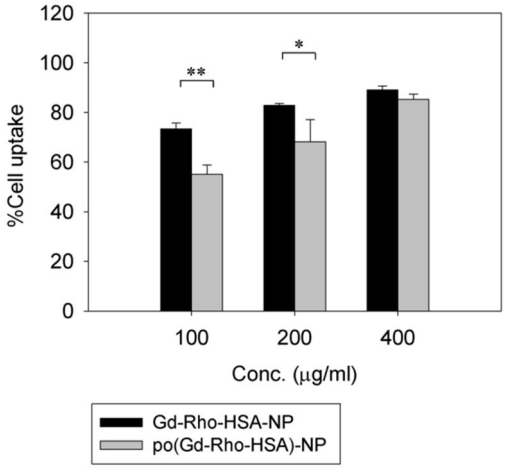


Figure 4. Cellular uptake of Gd-Rho-HSA-NP or poloxamine-coated Gd-Rho-HSA-NP (po(Gd-Rho-HSA)-NP) by HepG2 after 24 h of incubation at different NP concentrations (100 µg/ml, 200 µg/ml and 400 µg/ml) at 37ºC. The measurement was performed using a

flow cytometric analysis (means ± SD; n = 3). There was a statistically significant difference between uncoated and coated particles for each nanoparticle concentration (* $p < 0.05$; ** $p < 0.01$).

To evaluate the uptake of nanoparticles by HepG2 cells, FACS analysis was carried out in the same way as described for Huh-7 cells. As shown in Figure 4, the uptake of po(Gd-Rho-HSA)-NP was considerable lower than that of Gd-Rho-HSA-NP at nanoparticle concentration of 100 and 200 µg/ml as a result of particle modification. However, po(Gd-Rho-HSA)-NP showed only a small difference in the cell uptake by HepG2 compared to that of Gd-Rho-HSA-NP at a concentration of 400 µg/ml after incubation for 24 h. At this higher concentration the flexibility of the poloxamine and of poloxamer chains on the surface of the nanoparticles is restricted leading to a higher packing and less polymer chain mobility of the poloxamine side arm and as a consequence to a more shielding corona. It has been reported that the strength of polymer adsorption and the crowding around each hydrophilic chain depend on the proportion and the size of both POP and POE segments, surface concentration and adlayer thickness, as well as hydrophobic forces among the polymer chains and polymer–solvent interactions (Moghimi and Hunter, 2000) The quantification of fluorescence-signal profiles of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by FACS analysis indicated that the difference in cellular uptake of nanoparticles by Huh-7 and HepG2 during the incubation time of 24 h can be attributed to the presence of poloxamine. Therefore, poloxamine coated-nanoparticles could reduce the cell uptake by the human HCC cell line. This could lead to an increase in blood circulation time *in vivo*.

Cell uptake study of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by Hepatocytes

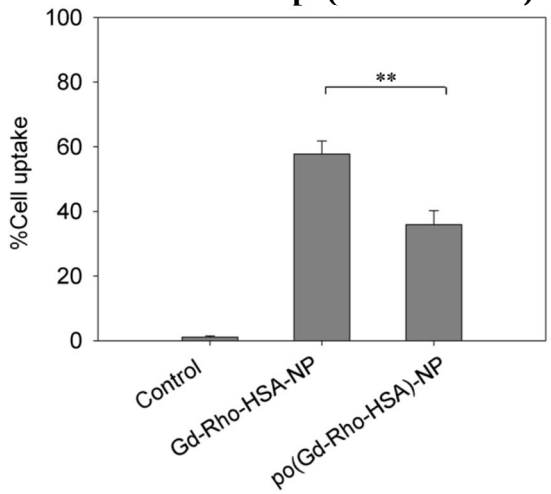


Figure 5. Cellular uptake of Gd-Rho-HSA-NP or poloxamine-coated Gd-RhoHSA-NP (po(Gd-Rho-HSA)-NP) by hepatocytes after 16 h of incubation at a nanoparticle concentration of 100 µg/ml at 37ºC. The measurement was performed using a flow cytometric analysis (means ± SD; n = 3). There was a statistically significant difference between uncoated and coated particles (** $p < 0.01$).

To further investigate the effect of coated-poloxamine on the cell uptake by hepatocytes, the cells were isolated from healthy mice, cultured, and incubated with Gd-Rho-HSA-NP or po(Gd-Rho-HSA)-NP at a concentration of 100 µg/ml. After 16 h of incubation, the uptake of nanoparticles was determined by FACS analysis. The amount of po(Gd-Rho-HSA)-NP taken up was considerably lower than that of unmodified Gd-Rho-HSA-NP, i.e. approximately 40% (Figure 5). A significant difference was observed in the presence or absence of poloxamine similar to those with the HCC cell line at the same nanoparticle concentration of 100 µg/ml.

Intracellular localization of Gd-Rho-HSA-NP and po(Gd-Rho-HSA)-NP by hepatocytes

The decreased uptake of po(Gd-Rho-HSA)-NP by hepatocytes was confirmed by CLSM analysis. An intracellular localization of Gd-Rho-HSA-NP or po(Gd-Rho-HSA)-NP was observed after 4 h of incubation with nanoparticles (100 µg/ml).

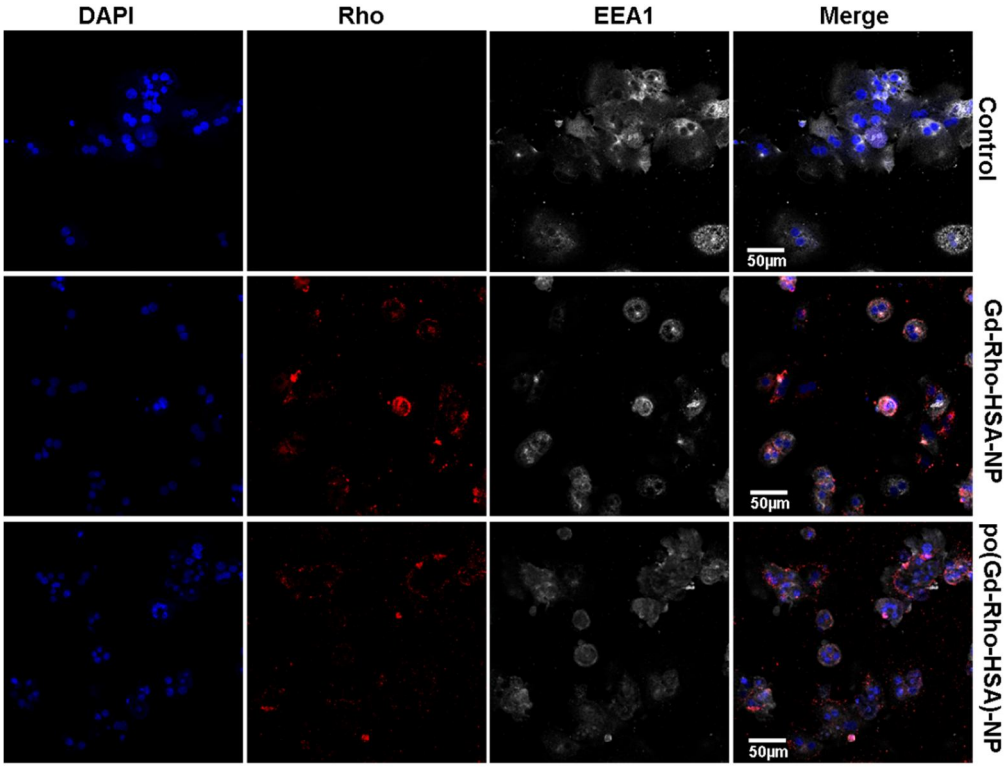


Figure 6. CLSM images of hepatocytes incubated with or without Gd-Rho-HSAor poloxamine-coated Gd-Rho-HSA-NP (po(Gd-Rho-HSA)-NP) (100 µg/ml of each formulation) after 4 h of incubation at 37oC. The fluorescence signals and cell images were obtained using three channels shown in each column: blue for cell nuclei (DAPI), red for rhodamence labeled NP (Rho), white for endosomes (EEA1). The merged images display the overlay of all fluorescence channels. Endosomes were stained with rabbit polyclonal anti-EEA1 antibody, and cell nuclei (blue) were stained with Hoechst 33342. Scale bar = 50 µm.

To study the cellular uptake, rhodamine 123 was chosen as fluorescent labeling dye not only to make the uptake easily detectable by CLSM but also being a P-pg substrate which can

overcome the P-glycoprotein-mediated efflux. As shown in Figure 6, the colocalization of rhodamine 123-labeled NP and EEA1 was observable as bright pink fluorescence after 4 h incubation at 37^{o}C indicating a uniform distribution in the endosomes of NP-incubated cells. It can be seen that the control cells (non-treated cells) exhibited no fluorescence at the same condition as a negative control. The cells incubated with Gd-Rho-HSA-NP exhibited a stronger fluorescence of rhodamine 123 than po(Gd-Rho-HSA)-NP, implying the decrease uptake of NPs coated with poloxamine by hepatocytes after 4 h incubation. It has been found that incorporating hydrophilic polymers on the surface of carriers can reduce opsonization and uptake (Gelperina *et al.* 2010). A multilayer of hydrophilic molecules on the surface can form a dynamic molecular cloud which could impart protein adsorption and the uptake by macrophages.

Among these cell types, hepatocytes, Huh7, and HepG2 cells, the uptake of the poloxamine-coated po(Gd-Rho-HSA)-NP was clearly reduced in comparison with the uncoated Gd-Rho-HSA-NP. Due to the large size and active surface area, the primary hepatocytes required a short incubation time for NP internalization by endocytosis. However, the lack of functionalization and active cellular targeting can be an obstacle to promote the receptor-mediated endocytosis of nanoparticles.

CONCLUSION

Gd-Rho-HSA-NP were prepared as a MRI contrast agent and fluorescent cell tracking. For modification with surfactant, poloxamine coated-(Gd-Rho-HSA-NP) could be obtained by a simple method. Zeta potential results revealed a surface charge reversal after modification. The cellular uptake of nanoparticles was analyzed by flow cytometry and Huh7, HepG2 cells and hepatocytes were used as an *in vitro* model. It was shown that poloxamine-coated Gd-Rho-HSA-NP had a significantly lower level of the cellular uptake compared with unmodified Gd-Rho-HSA-NP at the same concentration and incubation period. It was found the coating with poloxamine 908 could reduce the uptake of (Gd-Rho-HSA)-NP. This demonstrated the relationship between uptake and surface properties including surface charge and hydrophilicity of poloxamine modified Gd-Rho-HSA-NP, showing that coating with poloxamine revealed a stealth effect to the nanoparticles at the optimum concentration. With functional properties and a non-toxicity of HSA nanoparticles, Gd-Rho-HSA-NP and poloxamine coated Gd-Rho-HSA-NP have a promising value to be further developed for drug delivery and *in vivo* MRI which would have significant clinical applications.

ACKNOWLECGEMENTS

This work was performed with support by Prof. Dr. Joerg Kreuter and Prof. Dr. Albrecht Piiper. We thank Dr. Thomas Pleli and Christian Schmithals (Department of Medicine 1, University of Hospital Frankfurt, Germany) for FACS and CLSM measurement.

REFERENENCES

W. Lohcharoenkal, L. Wang, Yi C. Chen, and Y. Rojanasakul, "Protein Nanoparticles as Drug Delivery Carriers for Cancer Therapy," BioMed Research International, vol. 2014, Article ID 180549, 12 pages, 2014. doi:10.1155/2014/180549

Jennifer Look, Nadine Wilhelm, Hagen von Briesen, Nadja Noske, Christine Günther, Klaus Langer, Erwin Gorjup, Ligand-Modified Human Serum Albumin Nanoparticles for Enhanced Gene Delivery, *Mol. Pharmaceutics*, 2015, *12* (9), pp 3202–3213. DOI: 10.1021/acs.molpharmaceut.5b00153

I. Cucinotto, L. Fiorillo, S. Gualtieri, M. Arbitrio, D. Ciliberto, N. Staropoli, A. Grimaldi, A. Luce, P. Tassone, M. Caraglia, P. Tagliaferri, Nanoparticle albumin bound Paclitaxel in the treatment of human cancer: nanodelivery reaches prime-time? J Drug Deliv 2013 (2013) 905091.

S. Bae, K. Ma, T.H. Kim, E.S. Lee, K.T. Oh, E.-S. Park, K.C. Lee, Y.S. Youn, Doxorubicin-loaded human serum albumin nanoparticles surface-modified with TNF-related apoptosis-inducing ligand and transferrin for targeting multiple tumor types. Biomaterials 33(5) (2012) 1536-1546.

H. Misak, N. Zacharias, Z. Song, S. Hwang, K.P. Man, R. Asmatulu, S.Y. Yang, Skin cancer treatment by albumin/5-Fu loaded magnetic nanocomposite spheres in a mouse model. Journal of Biotechnology 164(1) (2013) 130-136.

A. Taheri, R. Dinarvand, F. Atyabi, M.H. Ghahremani, S.N. Ostad, Trastuzumab decorated methotrexate human serum albumin conjugated nanoparticles for targeted delivery to HER2 positive tumor cells. European Journal of Pharmaceutical Sciences 47(2) (2012) 331-340.

M. Wacker, Nanocarriers for intravenous injection--the long hard road to the market. Int J Pharm 457(1) (2013) 50-62.

Zensi A, Begley D, Pontikis C, Legros C, Mihoreanu L, Wagner S, Büchel C, von Briesen H, Kreuter J, Albumin nanoparticles targeted with Apo E enter the CNS by transcytosis and are delivered to neurones, J Control Release, 137, 78–86 (2009)

H. Korkusuz *et al*., Transferrin-coated gadolinium nanoparticles as MRI contrast agent, Mol Imaging Biol, 15(2), 148-54 (2013)

S. Rhaese, H. von Briesen, H. Rubsamen-Waigmann, J. Kreuter, K. Langer, Human serum albumin-polyethylenimine nanoparticles for gene delivery. J Control Release 92(1-2) (2003) 199-208.

Loureiro A, Azoia NG, Gomes AC, Cavaco-Paulo A. Albumin-Based Nanodevices as Drug Carriers. Curr Pharm Des. 2016;22(10):1371-90.

S.M. Moghimi, A.C. Hunter, T.L. Andresen, Factors controlling nanoparticle pharmacokinetics: an integrated analysis and perspective. Annu Rev Pharmacol Toxicol 52 (2012) 481-503.

J. Kreuter, Nanoparticles and microparticles for drug and vaccine delivery. J Anat 189 (Pt 3) (1996) 503-505.

S. Sakuma, N. Suzuki, R. Sudo, K. Hiwatari, A. Kishida, M. Akashi, Optimized chemical structure of nanoparticles as carriers for oral delivery of salmon calcitonin. Int J Pharm 239(1-2) (2002) 185-195.

H. Otsuka, Y. Nagasaki, K. Kataoka, PEGylated nanoparticles for biological and pharmaceutical applications. Adv Drug Deliv Rev 55(3) (2003) 403-419.

W. Watcharin, C. Schmithals, T. Pleli, V. Köberle, H. Korkusuz, F. Huebner, S. Zeuzem, H.W. Korf, T.J. Vogl, C. Rittmeyer, A. Terfort, A. Piiper, S. Gelperina, J. Kreuter, Biodegradable human serum albumin nanoparticles as contrast agents for the detection of hepatocellular carcinoma by magnetic resonance imaging, Eur. J. Pharm. Biopharm. 87 (2014) 132–141.

C. Weber *et al*., Desolvation process and surface characterisation of protein nanoparticles, International Journal of Pharmaceutics, 194, 91–102 (2000)

M.G. Anhorn, H.-C. Mahler, K. Langer, Freeze drying of human serum albumin (HSA) nanoparticles with different excipients. International Journal of Pharmaceutics 363(1-2) (2008) 162-169.

K. Ulbrich, T. Knobloch, J. Kreuter, Targeting the insulin receptor: nanoparticles for drug delivery across the blood-brain barrier (BBB). Journal of Drug Targeting 19(2) (2011) 125-132.

G. Lehne, P. DeAngelis, O.P.F. Clausen, H.E. Rugstad, Human hepatoma cells rich in P-glycoprotein are sensitive to aclarubicin and resistant to three other anthracyclines. British Journal of Cancer 74(11) (1996) 1719-1729.

X. Xu, R. Li, M. Ma, X. Wang, Y. Wang, H. Zou, Multidrug resistance protein P-glycoprotein does not recognize nanoparticle C-60: experiment and modeling. Soft Matter 8(10) (2012) 2915-2923.

J. Zastre, J. Jackson, M. Bajwa, R. Liggins, F. Iqbal, H. Burt, Enhanced cellular accumulation of a P-glycoprotein substrate, rhodamine-123, by caco-2 cells using low molecular weight methoxypolyethylene glycol-block-polycaprolactone diblock copolymers. European Journal of Pharmaceutics and Biopharmaceutics 54(3) (2002) 299-309.

R. Senetta, G. Stella, E. Pozzi, N. Sturli, D. Massi, P. Cassoni, Caveolin-1 as a promoter of tumour spreading: when, how, where and why. Journal of Cellular and Molecular Medicine 17(3) (2013) 325-336.

S.M. Moghimi, A.C. Hunter, Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. Trends Biotechnol 18(10) (2000) 412-420.

Geperina S *et al*., Drug deliverz to brain using surfactant-coated poly(lactide-co-glycolide) nanoparticles: Influence of the formulation parameters, European Journal of Pharmaceutics and Biopharmaceutics, 76, (2010) 421–427.

Alpha-Glucosidase Inhibitory Effect of Methanolic Extracts from Indonesian Plants

Filiana Santoso[1], Chelsea Soedarma[1], Gregorius Herdiazto Adiyoga[1], Maria Dewi Puspitasari Tirtaningtyas Gunawan-Puteri[1], Hery Sutanto[1]

[1]Department of Food Technology, Faculty Life Sciences and Technology, Swiss German University, EduTown-BSD City, Tangerang, 15339, Indonesia, (filiana.santoso@sgu.ac.id)

ABSTRACT

Alpha-glucosidase inhibitors (AGIs) are oral anti-diabetic drugs used for type-2 diabetes treatment by retarding the carbohydrate digestion. There are numerous natural products with AGI action, however, only limited number of Indonesian plants have been studied for their AGIs potency. In this research, 57 samples of plants naturally grown in Indonesia were tested in vitro for their AGIs activities. Some of them are traditionally utilized for diabetes treatment. The study showed that the methanolic extracts of gambir fruit (Uncaria gambir), banana leaf (Musa paradisiaca), lemongrass stem (Cymbopogon citratus), bitter gourd fruit (Momordica charantia), and onion bulb (Allium cepa L.) exhibited the highest AGIs activities at 0.02 g/ml concentration. The gradient extraction using n-hexane, ethyl acetate, and butanol resulted in ethyl acetate and butanol fractions showing high AGIs activity, indicating that the AGIs activities in these five samples were influenced by the existence of semi-polar to polar compounds. Antioxidant activity, total phenolic and flavonoid contents were also evaluated. Gambir fruit exhibited the highest antioxidant activity followed by onion bulb, banana leaf, bitter gourd fruit, and lemongrass stem with IC_{50} of 101.75 ppm, 1,963.57 ppm, 2,377.64 ppm, 5,859.14 ppm and 5,910.14 ppm, respectively. Total phenolic and flavonoid analysis showed that the activity of gambir fruit was supported by the high content of phenolic (77.56 mg GAE/g), while the onion bulb exhibited the highest flavonoid content (8.94 mg QE/g). Positive correlation of antioxidant activity and total phenolic content with AGIs was found, however, there was no correlation between AGIs and total flavonoid content.

Keywords : alpha-glucosidase inhibitors, Indonesian plants, diabetes, antioxidant

Introduction

Diabetes mellitus (DM) is a metabolic, chronic disease that has become a significant public health problem and is one of four priority non-communicable diseases targeted for action by world leaders. Over the past few decades both the number of cases and the prevalence of diabetes have been steadily increasing and is growing most rapidly in low- and middle-income countries (World Health Organization, 2016). The estimated number of adult suffering from diabetes worldwide in 2015 is 415 million and expected to increase to 642 million in 2040, if the rise is not halted (International Diabetes Federation, 2015). Indonesia is ranked one of the world's top five worst affected nations for people living with diabetes, together with China, India, Brazil and the United States (World Health Organization, 2016). One of well-known oral treatments for type 2 diabetes is the use of □-glucosidase inhibitors (AGIs) drugs. □-Glucosidase (EC 3.2.1.20), an enzyme located in the brush-border surface membrane of intestinal cells, is responsible for the hydrolysis of polysaccharide or disaccharides into monosaccharide in the small intestine. Studies showed that glucose absorption was retarded by inhibiting the catalytic activity of a-glucosidase and thus, lowered the effect on postprandial blood glucose and insulin levels (Robinson *et al.*, 1991; Braun *et al.*, 1995; Dwek *et al.*, 2002). Consumption of AGIs that reversibly inhibit □-glucosidases, such as maltase and sucrase in the intestine, delayed carbohydrate digestion and hence, sugar absorption from the gut (Campbell *et al.*, 1996; Kumar *et al.*, 2011). The effects of monotherapy with AGIs for patients with type 2 diabetes were reviewed (Laar *et al.*, 2005). No evidence for an effect on mortality or morbidity was found. Furthermore, AGIs exhibited significant beneficial effects on glycemic control and postload insulin levels, but not on plasma lipids. These indicate the possible use of AGIs as a first-line agent or in combination with other antihyperglycemic drugs.

In the prevention and treatment of diabetes and obesity, plant-based medicines and functional foods affecting beneficial physiological effects have gained high interest in the last decades. There were many studies conducted with the aim to search for effective and safe AGIs from natural sources (Matsui *et al.*, 2001; Tundis *et al.*, 2010; Gunawan-Puteri *et al.*, 2012; Yonemoto *et al.*, 2014). Many results showed significant antihyperglycaemic effect with slight or no side effects. Therefore, natural AGIs from plant sources offers a potential strategy for the control of hyperglycaemia. Indonesia is the second largest biodiversity in the world after Brazil, with around 40,000 endemic plant species including 6,000 medicinal plants. Unfortunately, numerous Indonesian medicinal plants potency still remain unknown until now. According to Aditama, until October 2014 only 41 standardized herbal medicines and 6 phytopharmaca were listed in the National Agency of Drug and Food Control (Aditama, 2015). As continuation of the screening of Indonesian plants for their AGIs potency, in this research 57 natural plant samples were investigated. The determination of prospective plants were assessed *in vitro* for their inhibitory effect using rat intestinal glucosidase. Furthermore, the antioxidant activity, total phenolic and total flavonoid content were also examined, and their correlation to AGIs activity assessed.

Materials and method

Materials

The fresh or dried leaves, barks, fruits, seeds, or bulbs of 57 samples were obtained from CV Sekar Utami, Jakarta, CV Karya Tama, Lampung and Pasar Modern BSD, Tangerang. Rat

intestinal acetone powder and DPPH (2,2-diphenyl-1-picrylhydrazyl) were commercially available from Sigma Aldrich, Germany. K.tartaric acid, quercetin, gallic acid, NaOH, folin-ciocaltau phenol reagent, glucose and sucrose were supplied from Merck, Germany. All other chemicals used were purchased from PT. Bratachem, Indonesia, unless otherwise stated. The samples used are shown in Table 1.

Table 1. Samples for AGIs evaluation

Scientific name	Parts	Scientific name	Parts	Scientific name	Parts
Abelmochus esculentus	Fruit	*Coriandrum sativum L.*	Fruit	*Phaleria macrocarpa*	Fruit
Aleurites moluccana	Fruit	*Cuminum Cyminum L*	Seed	*Phyllanthus niruri*	Leaf
Allium Cepa	Bulb	*Cymbopogon citrates*	Stem	*Physalis angulata L.*	Leaf
Allium cepa L.(var Aggregatum)	Bulb	*Durio zibethinus*	Skin	*Piper betle L.*	Leaf
Aloe vera	Skin	*Eichhornia crassipes*	Leaf	*Piper cocatum*	Leaf
Amomum compactum	Fruit	*Elettaria cardamomum*	Fruit	*Plantago major L*	Leaf
Ananas comocus	Leaf	*Euphoria Longana*	Skin	*Quisqualis indica L*	Leaf
Andrographis paniculata	Leaf	*Garcinia mangostana*	Skin	*Ruellia tuberosa L.*	Leaf
Annona muricata L	Leaf	*Guazuma ulmifolia*	Leaf	*Ruellia tuberosa L.*	Leaf
Artocarpus altilis	Leaf	*Gynura procumbens*	Leaf	*Senna Alata*	Leaf
Azadirachta indica	Leaf	*Illicium Verum*	Flower	*Senna Alexandrina*	Leaf
Brassica oleracea	Stem	*Luffa acutangula*	Fruit	*Stachytarpheta mutabilis*	Leaf
Catharanthus roseus L.	Leaf	*Momordica charantia*	Fruit	*Swietenia macrophylla*	Leaf
Centella asiatica	Leaf	*Morus alba L.*	Leaf	*Tinospora crispa L.*	Leaf
Cinnamomum verum	Bark	*Musa paradisiacal*	Skin	*Trigonella foenum-graecum*	Seed
Citrullus lanatus	Skin	*Nicotiana tabacum*	Leaf	*Typhonium flagelliforme*	Leaf
Citrus nobilis (var. Microcarpa)	Seed	*Ocimum sanetum*	Leaf	*Uncaria gambir*	Fruit
Clinacanthus nutans L.	Leaf	*Persea americana*	Skin	*Zingiber zerumbet*	Leaf
Clitoria ternatea	Stem	*Persea Americana*	Seed	*Ziziphus mauritiana*	Leaf

Sample preparation

Fresh samples were firstly cut and dried at 50 °C for 24h. Five grams (dry weight) of each sample was subjected to 100 mL of 50% (v/v) aqueous methanolic extraction for 24h at room temperature. The crude extract was obtained by vacuum filtration through filter paper

(Whatman No. 5C, 70 mm) and was evaporated to dryness using rotary evaporated under reduced pressure at 50 °C and redissolved with 50% (v/v) aqueous dimethyl sulfoxide. Samples were kept in a dark place at 4 ± 2 °C prior to the glucosidase inhibitory activity assay.

Glucosidase inhibitory activity assay

The rat intestinal glucosidase inhibitory activity was determined using the method described previously with slight revision (Jong-Anurakkun *et al.*, 2007). Rat intestinal acetone powder was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, and centrifuged at 10,000 rpm (4 °C, 60 min). The crude enzyme solution obtained from the supernatant was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0). The final crude enzyme solution showed specific activities (0.137 unit/mg protein), which was measured by using sucrose as a substrate. The inhibitory activity against sucrose hydrolysis was measured by the following procedures. Two test tubes, as sample and control, containing 200 µl sucrose solution (56 mM) in potassium phosphate buffer (0.1 M, pH 7) and two test tubes, containing 400 µl potassium phosphate buffer (0.1 M, pH 7) as each blank were pre-incubated at 37 ºC for 5 min. The control and control blank defined as 100 % and 0 % enzyme activity, respectively. The working samples diluted in 50 % DMSO (100 µl) were added to the sample and sample blank test tubes while 50 % DMSO (100 µl) was added to the control and control blank test tubes. And then crude rat intestinal glucosidase (200 µl) was added only to the test tubes containing sucrose solution (sample and control). The reaction was carried out at 37 ºC for 15 min and stopped by adding Tris-HCl buffer (2 M, pH 6.3, 750 µl). Procedures for inhibitory activity assay against maltose hydrolysis were basically the same as above except for replacing sucrose solution (56 mM, 200 µl) with maltase solution (3.5 mM, 350 µl) and for reducing the amount of enzyme solution from 200 µl to 50 µl. The reaction mixtures were then passed through a short column of aluminium oxide 60 (5 x 500 mm, 1.5 g) for removing phenolics which may interfere with the following glucose quantification. Each mixture was taken 0.5 ml to other test tubes and was added with 0.5 ml DNS solution. All mixtures were incubated for 5 minutes in boiling water. The absorbance was measured using UV-Vis spectrophotometer at 540 nm wavelength. Inhibitory activity was calculated by the following equation:

$$Inhibitory\ activity\ (\%) = \frac{(Abs\ control - Abs\ control\ blank) - (Abs\ sample - Abs\ sample\ blank)}{(Abs\ control - Abs\ control\ blank)} \times 100\%$$

The experiments were done in duplicate, and the results were presented as % inhibition, which is defined as the AGIs activity at certain concentration under the assay conditions.

Analysis of □-glucosidase inhibiting principle in relation to its antioxidant activity, total flavonoid content and total phenolic content

Five samples with the highest inhibitory activity were further extracted in gradient extraction system and were analyzed for its antioxidant activity, total phenolic content, and total flavonoid content. Dried samples (100 g) was macerated for 24 hours in n-hexane. The crude extract was filtered using vacuum filtration and the filtrate was referred as hexane extract. The remaining solid materials were re-extracted again with ethyl acetate, and the remaining solid from ethyl acetate extract was then filtrated with n-butanol. The evaluation of AGIs activity was done using rat intestinal glucosidase inhibitory assay with the same procedure in the screening of AGIs. Antioxidant activity was analyzed using DPPH radical scavenging

activity, total phenolic content was measured using Folin-Ciocalteau assay, while total flavonoid was measured using aluminium chloride assay.

Results and Discussion

Screening of AGIs

In this study, 57 samples from 56 species and 43 families were examined. Among them, 8 samples showed high inhibitory activity at concentration of 0.10 g/ml and five samples have more than 50% AGIs activity (Figure 1.(a)). The five samples, namely UG, MP, CC, MC, and AC were further observed in gradient eluent systems. The gradient eluent using solvents with different polarity (hexane, ethyl acetate and butanol) allows rough separation of the samples based on its general polarity. AGIs activity in each extract allowed prediction of the nature of active compounds and suitable fractionation system. The extraction yield of the samples in different solvent ranged from 0.9 to 12.5 mg/g (Table 2). The amount of extracted yield indicates that many compounds inside the samples were semi polar and polar compounds since ethyl acetate and butanol showed higher yield compared to hexane.

(a) (b)

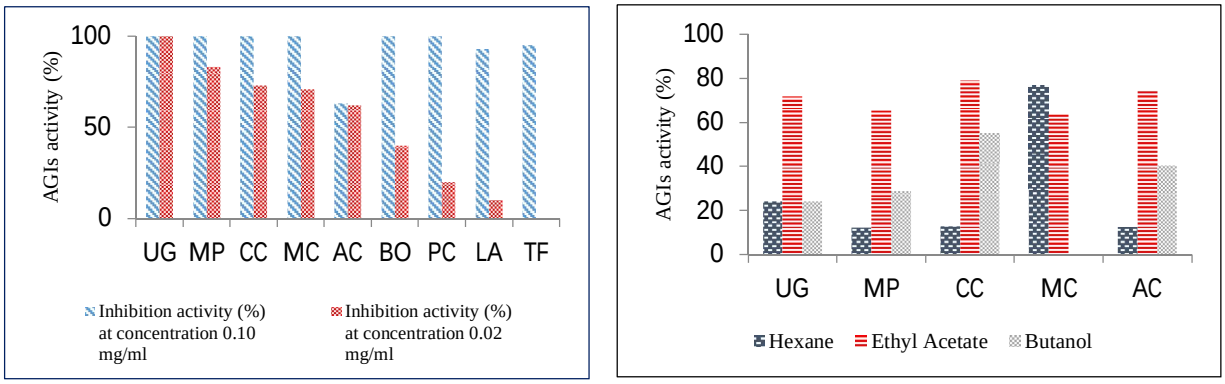


Figure 1. (a) AGIs activity of *Uncaria gambir* (UG), *Musa paradisiaca* (MP), *Cymbopogon citratus* (CC), *Momordica charantia* (MC), *Allium cepa L.* (AC), *Brassica oleracea* (BO), *Piper cocatum* (PC), *Luffa acutangula* (LA), and *Typhonium flagelliform* (TF). (b) AGIs activity of gradient-extracted fractions of UG, MP, CC, MC, and AC

AGIs activity assay for each extract in the gradient extraction of the five samples resulted in the finding of ethyl acetate and butanol extracts with higher AGIs activity compared to hexane extracts (p-value < 0.05, Figure 1.(b)). The result was in line with previous research in isolating the active compound for AGIs from several plants, which showed that ethyl acetate fraction showed the higher AGIs activity among other fractions (Laar, 2005; Dewoto, 2007). The finding indicates that the compound(s) responsible for AGIs was probably semi-polar or polar compound(s), since it has higher AGIs activities in ethyl acetate and butanol extracts. However, the hexane extract of MC showed the highest AGIs activity, followed by ethyl acetate extract and butanol extract of MC with AGIs activity value of 76.70, 63.65 and 0 % respectively. Matsuur *et al.* found that the active compound from methanolic extract of the bitter melon seeds that effectively inhibited alpha-glucosidase was D-(+)-Trehalose (Matsuur *et al.*, 2002). This indicates that there might be other non-polar compound responsible for AGIs activity in hexane fraction.

Table 2.Yield gradient extraction of UG, MP, CC, MC and AC

Name	Yield (mg/g)

	Hexane	Ethyl Acetate	Butanol
UG	1.30±0.10	7.60±0.20	9.77±0.01
MP	2.40±0.20	12.77±0.20	29.50±0.20
CC	8.00±0.20	12.00±0.20	16.70±0.50
MC	7.10±0.20	12.40±0.20	5.60±0.10
AC	0.90±0.20	15.10±0.40	11.60±0.04

Antioxidant activity, total flavonoid content and total phenolic content

Compound(s) that is responsible for AGIs activity might also contribute to antioxidant activity (Apea-Bah *et al.*, 2009). Therefore, DPPH radical scavening activity assay was used to determine the antioxidant activity of the samples. IC_{50} value indicates the concentration of extracts that is needed to reach 50% inhibition to the DPPH radical. The smaller IC_{50} value means less amount of concentration needed to reach 50% inhibition or in other words the antioxidant activity is stronger. Statistical analysis was used to determine the samples with the highest antioxidant activity and the result showed that UG has the higest antioxidant activity followed by AC and MP, afterwards CC and MC (p-value < 0.05). The result was in line with Apea-Bah *et al.* that catechin, which is a powerful antioxidant in neutralizing free radicals (Braichu *et al.*, 2013), was the major bioactive compound present in UG (Apea-Bah *et al.*, 2009). In addition, there was a strong correlation between AGIs and antioxidant activity (r = 0.973). This indicates that the increase in the overall AGIs activity of the samples can be attributed to the increase of antioxidant activity. This also indicates that the chemical compound(s) contributing to AGIs activity also exhibits antioxidant activity. This was in agreement with previous research that there was a strong correlation of AGIs activity and antioxidant activity of some selected medicinal plants in Malaysia (Sugiwati *et al*, 2009). Compared to UG, MC, CC and MP, AC has the lowest AGIs activity. However, the antioxidant activity is moderately high compared to other extracts. Therefore, AC was not included in the correlation of AGIs and antioxidant activity. It could be assumed that in AC, the compound(s) responsible for AGIs activity was not the same with the compound(s) responsible for antioxidant activity.

Table 3. AGIs activity (0.02 g/ml), phenolic content, flavonoid content, and antioxidant activity of UG,MP, CC, MC, and AC

Name	AGIs (%)	Phenolic Content mg GAE/g	Flavonoid Content mg QE/g	Antioxidant Activity IC_{50} (ppm)
UG	100.00	67.57^{a}	1.57^{c}	101.75^{a}
MP	83.21	14.95^{c}	2.40^{b}	2377.64^{b}
CC	73.11	6.14^{d}	1.00^{d}	5910.14^{c}
MC	71.00	15.20^{c}	0.99^{d}	5859.14^{c}
AC	62.34	19.90^{b}	8.94^{a}	1963.57^{b}

Phyoconstituents of plants such as phenolic and flavonoid compound also contribute to AGIs activity (Kumar *et al.*, 2011). The total phenolic content of the samples extracts ranged between 6.14 to 67.57 mg GAE/g (Table 3). ANOVA single factor showed that the total phenolic content between the crude extracts has highly significant difference with p-value <0.05. The plant extracts which has the highest phenolic content was UG fruit extract,

followed by AC, MC and MP, and the least was CC extract (p-value <0.05). The total phenolic content obtained by Kassim *et al.* from methanolic extract of UG leaves was higher (99.25 mg GAE/g) than that of UG fruit obtained in this study (67.60 mg GAE/g). This confirms the fact that different part of plant contains different amount of phenolic compounds. The correlation between AGIs activity and total phenolic content showed moderate positive correlation with r = 0.798. The positive correlations indicate that the increase in AGIs activity can be attributed with the amount of phenolic compounds. This was in agreement with previous research that there was a significant correlation between AGIs and total phenolic content in selected medicinal plants in Nigeria (Manaharan, *et al.*, 2012).

The flavonoid content of the extracts ranged between 1.00 to 8.94 mg QE/g (Table 3). The statistical analysis indicated that the amount of flavonoid content in AC, MP, UG were significantly different to MC and CC (p-value < 0.05). Among the samples, AC bulb extract had the highest flavonoid content. The correlation between AGIs activity and total flavonoid content value showed a moderate negative correlation (r = -0.538). However, the statistical analysis revealed that the correlation was not significant (p-value > 0.05). Therefore, no correlation between AGIs activity and total flavonoid content was found. This indicates flavonoid compound do not contribute to AGIs activity. This was in agreement with previous research that there was no correlation between total flavonoid content and AGIs activity (Adefegha *et al.*, 2012).

Conclusions

Among those plants, the methanolic extract of *Uncaria gambir, Musa paradisiaca, Cymbopogon citratus, Momordica charantia* and *Allium cepa L.* showed highest AGIs activities 100%, 83%, 73%, 71%, and 62% respectively at concentration 0.02 g/ml. The gradient extraction of the five samples with the highest AGIs activity showed that the ethyl acetate fraction extract had the highest AGIs. This indicates that the compound(s) responsible for the inhibitory effect is most likely to be semi-polar or polar. UG also had the strongest antioxidant activity (DPPH IC_{50} = 101.75 ppm) and highest total phenolic content (67.57 mg GAE/g). This confirmed the positive correlation between AGIs activity with antioxidant activity and total phenolic content, which indicates that there might be the same compound(s) responsible for both activities. On the other hand, no correlation was found between AGIs activity and total flavonoid content. The AGIs activity of *Allium cepa L.* was indicated to be supported by the high content of flavonoid (8.94 mg QE/g).

References

Adefegha. S.A., Oboh, G. (2012), "In vitro inhibition activity of polyphenol-rich extracts from *Syzygium aromaticum* (L.) Merr. & Perry (clove) buds against carbohydrate hydrolyzing enzymes linke to type 2 diabetes and Fe-induced lipid peroxidation in rat pancreas", *Asian Pacific Journal of Tropical Biomedicine*, Vol. 2 No. 10, pp. 774-781.

Aditama, T.Y. (2015), "Jamu dan Kesehatan Edisi II", Badan Penelitian dan Pengembangan Kesehatan, available at: http://terbitan.litbang.depkes.go.id/penerbitan/index.php/blp/catalog/download/160/179/372-1 (accessed 9 June 2016)

Apea-Bah, F.B., Hanafi, M., Dewi, R., Fajriah, S., Darmawan, A., Artanti, N., *et al.* (2009), "Assessment of the DPPH and a-glucosidase inhibitory potential of gambier and qualitative identification of major bioactive compound", *Journal of Medicinal Plants Research*, Vol. 3 No. 10, pp. 736-757.

Braichu, C., Ladomery, M.R>, Chedea, V.S. (2013). "The relationship between the structure and biological actions of green tea catechins", *Food Chemistry*, Vol. 141 No. 3, pp. 3282-3289.

Braun, C., Brayer, G.D., Withers, S.G. (1995), "Mechanism-based inhibition of yeast □-glucosidase and human pancreatic □-amylase by a new class of inhibitors. 2-Deoxy-2,2-difluoro-□-glycosides", *The Journal of Biological Chemistry*, Vol. 270, pp. 26778-26781.
Campbell, L.K., White, J.R., Campbell, R.K. (1996), "Acarbose: Its role in the treatment of diabetes mellitus", *The Annals of Pharmacotherapy*, Vol.30, pp. 1255-1262.

Dewoto, H.R. (2007), "Pengembangan obat tradisional Indonesia menjadi fitofarmaka", *Majalah Kedokteran Indonesia*, Vol. 57.

Dwek, R.A., Butters, T.D., Platt, F.M., Zitzmann, N. (2002), "Targeting glycosylation as a therapeutic approach", *Nature Reviews Drug Discovery*, Vol. 1, pp. 65-75.

Gunawan-Puteri, M.D.P.T., Kato, E., Kawabata, J. (2012), "□-Amylase inhibitors from an Indonesian medicinal herb, *Phyllanthus urinaria*", *J. Sci. Food. Agric.*, Vol. 92 No. 3, pp. 606-609.

International Diabetes Federation (2015), "IDF Diabetes Atlas 7th Edition", International Diabetes Federation, available at: http://www.diabetesatlas.org/ (accessed 9 June 2016)

Jong-anurakkun, N., Bhandari, M.R., Kawabata, J. (2007), "□-Glucosidase inhibitors from Devil tree (*Alstonia scholaris*)", *Food Chemistry* Vol. 103, pp. 1319-1323.

Kassim, M., Hussin, M., Achmad, A., Dahon, H., Suan, K., Hamdan, H. (2011), "Determination of total phenol, consensed tannin and flavonoid contents and antioxidant activity of *Uncaria gambier* extracts", *Majalah Farmasi Indonesia*, Vol. 22, pp. 50-59

Kumar, S., Narwal, S., Kumar, V., Prakash, O. (2011), "□-Glucosidase inhibitors from plants: A natural approach to treat diabetes", *Pharmacogn. Rev.* Vol. 5, pp. 19-29.

Laar, F.A. van de, Lucassen, P.L., Akkermans, L.P., Lisdonk, E.H. van de, Rutte, G.E., Weel, C. (2005), "□-Glucosidase inhibitors for patients with type 2 diabetes", *Diabetes Care*, Vol. 28 No. 1, pp. 154-163.

Manaharan, T., Palanisamy, U.D., Ming, C.H. (2012), "Tropical plant extracts as potential antihyperglycemic agents", *Molecules*, Vol. 17, pp. 5915-5923.

Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N., Matsumoto, K. (2001), "□-Glucosidase inhibitory action of natural acylated anthocyanins. 1. Survey of natural pigments with potent inhibitory activity", *Journal of Agricultural and Food Chemistry*, Vol. 49, pp. 1948-1951.

Matsuur, H., Asakawa, C., Kurimoto, M., Mizutani, J. (2002), "Alpha-glucosidase inhibitor from the seeds of balsam pear (Momordica charantia) and the fruit bodies of Grifola frondosa", *Bioscience Biotechnology and Biochemistry*, Vol. 66 No. 7, pp 1576-1578.

Robinson, K.M., Begovic, M.E., Rhinehart B.L., Heineke, E.W., Ducep, J.B., Kastner, P.R., *et al.* (1991), "New potent □-glucohydrolase inhibitor MDL 73945 with long duration of action in rats", *Diabetes*, Vol. 40, pp. 825-830.

Sugiwati, St., Setiasih, S., Afifah, E., (2009), "Antihyperglycemic activity of the mahkota dewa leaf extracts as an alpha-glucosidase inhibitor", *Makara Kesehatan*, Vol. 13 No. 2, pp. 74-78

Tundis, R., Loizzo, M.R., Menichini, F. (2010), "Natural products as □-amylase and □-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: an update", *Mini Rev. Med. Chem.*, Vol. 10 No. 4, pp. 315—331

Wild, S., Roglic, G., Green, A., Sicree, R., King, H. (2004), "Global prevalence of diabetes", *Diabetes Care*, Vol. 27 No. 5, pp. 1047-1053.

World Health Organization (2016), "Global report on diabetes", World Health Organization, available at: http://apps.who.int/iris/bitstream/10665/204871/1/9789241565257_eng.pdf (accessed 8 June 2016).

Yonemoto, R., Shimada, M., Gunawan-Puteri, M.D.P.T., Kato, E., Kawabata, J. (2014), "□-Amylase inhibitory triterpene from *Abrus precatorius* leaves", *Journal of Agricultural and Food Chemistry*, Vol. 62, No. 33, pp. 8411-8414.

Potency of high protein *oyek* for decreasing cholesterol of diabetic rats

Bayu Kanetro[1], Sri Luwihana[2], Dwiyati Pujimulyani[3]

[1]Food Technology Department Mercu Buana Yogyakarta University (bayu_kanetro@yahoo.co.id)
[2]Food Technology Department Mercu Buana Yogyakarta University (sri_luwihana@yahoo.co.id) *[3]Food Technology Department Mercu Buana Yogyakarta University (dwiyati2002@yahoo.com)*

ABSTRACT

High protein oyek is traditional food from Kulon Progo Special Region of Yogykarta that was made of cassava and developed by addition of cowpeas sprout for increasing the protein of oyek This research was aimed to determine the potency of hypocholesterolemic of oyek through in vivo bioassay by using Sprague Dawley male rats. There were two treatments of the research, the first treatment were normal rats and diabetic rats which was induced by aloxan injection, and the second treatment were standard feed and oyek feed. The blood triglyceride, cholesterol total, High Density Lipoprotein (HDL), Low Density and Lipoprotein (LDL) were analysed on 3th, and 18 th days for the treatment and before the treatment as control (0th). The result of this research showed that The total cholesterol of normal and diabetic rats increased during the feed treatment but the increase of cholesterol of diabetic rats were higher than normal rats. The potency of hypocholesterolemic were shown by decreasing of blood triglyceride, cholesterol total, LDL, and increasing of blood HDL in diabetics rats with oyek feed treatment.

Keywords: oyek, protein, cassava, cowpeas, hypocholesterolemic

Introduction

Oyek is traditional food from Kulonprogo Yogyakarta Indonesia. Oyek is staple food providing energy especially in dried climate condition that is high price of rice. Oyek is produced by spontaneous fermentation of cassava in water for 5 days, and then the fermented cassava are pressed to remove water, formed, steamed, and dried (Kanetro and Luwihana, 2015). The same product as oyek in the other country is *gari* or *rale* that is fermented cassava using lactic acid bacteria (Eduardo et al., 2013). Oyek had been developed into artificial rice that its glycemic index was lower than original rice, and the artificial rice was more preferably than oyek according to the sensory testing by Kalirejo community (Kanetro et al., 2015)

The protein of oyek was lower than rice, so the addition of legumes flour into oyek is important to increase protein. There are many local legumes in Indonesia, such as cowpeas. Cowpeas may be potential for increashing protein of food, such as oyek. In the preliminary research was known that oyek with addition of cowpeas sprout flour 30% could increased protein, that was the same as rice (Kanetro and Luwihana, 2015). The potency of legumes as functional food could be increased by germination. Germination of soybean increased arginine (Kanetro etal, 2008) that was known as hypocholesterolemic amino acid (Damasceno etal, 2000).The protein of cowpeas sprout contained high of arginine (Arg) that was the same as protein soybean (Kanetro and Dewi, 2013).In the preliminary research also showed that the arginine /lisine ratio of cowpeas sprout protein was no significant different with protein of soybean (Kanetro and Dewi, 2013). The ratio of arginine/lysine was important to controle cholesterol level (Damasceno *et al*.,2000). Protein isolate of soybean had been known as functional food due to hypocholesterolemic effect (Damasceno et al., 2000). Protein isolate of cowpeas sprout had hypocholesterolemic properties based on in vivo bioassay by using Sprague Dawley male (Kanetro, 2015). Therefore oyek with addition of cowpeas sprout might has hypocholesterolemic effect, so it was potential as functional food. This research was aimed to determine hypocholesterolemic properties of the oyek in normal and diabetic rats through in vivo bioassay..

Materials and Method

Material

The cowpea (*Vigna unguiculata*) were obtained from Beringharjo market in Yogyakarta. Chemical agents, such as aloxan, corn starch, casein, vitamin mix, mineral mix, sucrose, choline bitartat, soy oil, and kholesterol kit (*DiaSys Diagnostic System GmBH & Co*), were purchased from Sigma Chemical Co. The other material was rats that were obtained from Animal Experiment Development Unit, Gadjah Mada University, Yogyakarta.The methods of experimental activities were performed as follows:

Preparation of Cowpeas Sprout Flour and Oyek

Cowpea seeds were soaked for 8h, and then germinated for 36h. The germinated cowpeas were dried and milled to make flour (Kanetro and Dewi, 2013). The cowpeas sprout flour were prepared for increashing protein of oyek. Oyek was made of cassava through many step process according to Kanetro and Luwihana (2015). The first step, cassava was dikupas, washed, and size reduced. After that cassava was fermented by soaking in water for 5 days. Then the fermented cassava are pressed to remove water, mixed with 30% cowpeas sprout

flour, formed, steamed, and dried. The dried product was called oyek that was prepared to in vivo bioassay.

In Vivo Bioassay

The *in vivo* bioassay was done to determine the potency of hypocholesterolemic of oyek by using 24 *Sprague Dawley* male rats. The experiment sequences of the steps were adaptation of rats for 3 days, diveded rats into 4 groups, treated rats for 18 days with the condition of rat and feed treatments, and analysed the blood triglyceride, cholesterol total, High Density Lipoprotein (HDL) cholesterol , and Low Density Lipoprotein (LDL) cholesterol for the treatment of rats on 3^{th}, 18^{th} days and before treatment as control (0^{th}). The experimental design of this research was randomized complete design with 2 factors. The first factors were rat condition treatments, that were normal rats and diabetic rats which was induced by aloxan injection. The second factors were feed treatments, that were standart feed according to AIN–93 (Reeves *etal,* 1993) and oyek feed which was prepared by subtitution of corn starch in standard feed with the oyek. The data of this experiments was statistical analysed by Anova (analysis of varian) and DMRT (Duncan Multiple Range Test). The in vivo bioassay of this research had passed ethical clearance that was approved by Center Research Laboratorium of Gadjah Mada University, Yogyakarta, Indonesia.

Results and Discussion

Trygliceride

Table 1 showed that the trygliceride of normal rats treatment were no significant differences between standart feed and oyek feed treatment. While the trygliseride of diabetic rats teratment showed significantly differences, especially for the treatment of rats on 18^{th}. The trygliseride of diabetic rats with protein isolate feed treatment increased after injection of aloxan at 3^{th} days of the tratment, and then decreased until below the standart of normal for the treatment of rats on 18^{th}.

The normal trygliceride of human according to *US National Cholesterol EducationProgram* (NCEP) was < 150 mg/dl (Anonim, 2007). While the normal trygliceride of rat was < 120 mg/dl (Herlina *et al.*, 2013). Based on this data indicated that oyek could inhibit increashing trygliceride that was ussual happen in complication of diabetic.

Table 1. The effect of oyek feed treatment on blood tryglyceride of normal and diabetic rats (mg/dL) *)

Rats condition	Feed treatment	0^{th} days	3^{th} days	18^{th} days
Normal	Standard	72,79a	72,92a	79,33a
	Oyek	67,15a	69,07a	75,82a
Diabetic	Standard	69,52a	117,45b	120,67c
	Oyek	69,33a	113,60b	90,30b

*) The same notation of statistic in the table showed not significantly differences at the same column

Total cholesterol

The total cholesterol of normal and diabetic rats increased during the feed trealment but the increase of cholesterol of diabetic rats were higher than normal rats, that was seen at Table 2.

Table 2. The effect of oyek feed treatment on blood total cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0^{th} days	3^{th} days	18^{th} days
Normal	Standard	106,28a	112,15a	112,59a
	Oyek	106,46a	108,66a	112,53a
Diabetic	Standard	104,11a	152,49b	154,31c
	Oyek	103,40a	156,54b	133,45b

*) The same notation of statistic in the table showed not significantly differences at the same column

Data of the table showed that the increase of cholesterol was inhibited by oyek feed treatment. The cholesterol of diabetic rats reduced 20.5% after oyek feed treatment for 3^{th} days until 18^{th} days. The data indicated that protein isolate of oyek was potential to controle cholesterol of diabetic patient. However the cholesterol level of all rats were still normal, that were < 200mg/dl (Anonim, 2007; Herlina *et al.*, 2013).

HDL cholesterol

Decreashing HDL of diabetic rats could be avoided by oyek treatment, that was seen at Table 3.

Table 3. The effect of oyek feed treatment on blood HDL cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0^{th} days	3^{th} days	18^{th} days
Normal	Standard	44,16b	45,08b	40,12c
	Oyek	41,69b	40,84b	38,50bc
Diabetic	Standard	35,91a	16,16a	14,76a
	Oyek	39,34ab	19,49a	30,86b

*) The same notation of statistic in the table showed not significantly differences at the same column

The HDL of diabetic rats after oyek treatment for18^{th} days increased significantly. Although the HDL of all rat was abnormal. The normal HDL cholesterol of human according to *US National Cholesterol Education Program* (NCEP) was > 60mg/dl (Anonim, 2007). While the normal HDL cholesterol of rat was > 45mg/dl (Herlina *et al.*, 2013). This fact indicated that oyek with additionof cowpeas sprout could induce the formation of HDL, so the complication of diabetic could be prevented. The result conformed with Airliss and Biermann (2002) who showed that protein isolate of soybean feed treatment could increase 50% HDL and decrease 30-40% total cholesterol. Kanetro (2014) also showed that protein isolate of cowpeas sprout could increase HDL through in vivo bioassay.

LDL cholesterol

The LDL of all rats were normal that was seen at Table 4, The normal LDL cholesterol of human according to *US National Cholesterol Education Program* (NCEP) was < 100mg/dl

(Anonim, 2007). While the normal LDL cholesterol of rat was < 135mg/dl (Herlina *et al.*, 2013). The increase of LDL after injection of aloxan at 3th, conformed with the increase of total cholesterol (Table 2).

The trend was same as the diabetic rats after oyek feed treatment. The LDL of this treatment increased at 3th days and then decreased until treatment for 18th days. The LDL of diabetic rats after standard feed treatment for 18th days increased 84,4% compared with control treatment for 0th days. While The LDL of diabetics rats after oyek feed treatment increased 36,0% compared with control treatment for 0th days. The result indicated that oyek with addition of cowpeas sprout could inhibit the increase of LDL cholesterol.

Table 4. The effect of oyek feed treatment on blood LDL cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0th days	3th days	18th days
Normal	Standard	57,54b	62,91a	59,59a
	Oyek	58,09b	58,19a	61,59a
Diabetic	Standard	46,75a	86,20b	87,18
	Oyek	49,01a	82,78b	66,67ab

*) The same notation of statistic in the table showed not significantly differences at the same column

The Ratio of Total Cholesterol/HDL and LDL/HDL

The ratio was computed from the data of Table 2 and 3 for total cholesterol/HDL ratio and the data of Table 3 and 4 for LDL/HDL ratio, that was seen at Table 5 and 6 respectively. The ratio indicated the risk of coronary heart disease (Fernandez and Webb. 2008). The normal level of the ratio of total cholesterol/HDL was < 5 and LDL/HDL was <3.2 for women and < 3.5 for men (Chandler and Zamora, 2011). Based on the ratio of cholesterol total/HDL and LDL/HDL were known that the ratio of all the rats including diabetic rats with oyek feed treatment for 18 days were normal, exception diabetic rats with standard feed treatment

Table 5. The effect of protein isolate from cowpea sprout feed treatment for 0thand 18th days on the ratio of total cholesterol/HDL *)

Rats condition	Feed treatment	0th days	18th days
Normal	Standard	2.41	2.81
	Oyek	2.55	2.92
Diabetic	Standard	2.90	10.45
	Oyek	2.63	4.32

*computed according to Table 2 and 3.

Table 6. The effect of protein isolate from cowpea sprout feed treatment for 0^{th} and 18^{th} days on the ratio of LDL/HDL*)

Rats condition	Feed treatment	0^{th} days	18^{th} days
Normal	Standard	1.30	1.49
	Oyek	1.39	1.60
Diabetic	Standard	1.30	5.91
	Oyek	1.25	2.16

*computed according to Table 3 and 4.

Conclusions

The potency of hypocholesterolemic of oyek were shown by decreasing of blood triglyceride, cholesterol total, LDL, and increasing of blood HDL in all rats treatments, especially diabetics rats with oyek feed treatment. Based on the ratio of cholesterol total/HDL and LDL/HDL showed that the ratio of all the rats including diabetic rats with oyek feed treatment for18 days were normal. This result indicated that oyek with addition of cowpeas sprout protein had the potency of hypocholesterolemic and might be used to prevent diabetic complication. Oyek with addition of cowpeas sprout could be potential as functional food.

Acknowledgements

The Authors are grateful to Directorate of Higher Education, Republic of Indonesia that has given financial support for this research.

References

Anonim, (2007), "Normal and high cholesterol value", available at: http://www.prolipid.com/cholesterol-reference-levels/normal-and-high-cholesterol-values.html/ (accessed 25 September 2012)

Arliss**,** R.M. and Biermann, C.A**. (**2002), "Do soy isoflavon lower cholesterol inhibit atherosclerosis and play a role in cancer prevention", Holistic Nursing Practice, Vol. 16 No. 5, pp. 40-48.

Chandler ,S. and Zamora, A. 2011, "Cholesterol-LDL-HDL-Ratio". available at: http://www.scientificpsychic.com/health/cholesterol-LDL-HDL.html/ (accessed 25 september 2012)

Damasceno, N.R., Goto, H., Fernada, M.D.R., Dias, C.T.S., Okawabata, F.S., Dulcinela, S.P., Abdalia, D.S. and Gidlund, M.A. (2000), "Soy protein isolate reduces the oxidizability of ldl and generation of oxidized ldl autoantibodies in rabbit with diet-induced atherosclerosis", Journal of Nutrition, Vol. 130 No. 11, pp. 2641-2647.

Eduardo,M., Svanberg,U., Oliveira,J., dan Ahrné,L. (2013), "Effect of cassava flour characteristics on properties of cassava-wheat-maize composite bread types". available at: http://www.hindawi.com/journals/ijfs/2013/305407/ (accessed 16 May 2016)

Fernandez,M.L. and Webb, D. (2008), "The LDL to HDL cholesterol ratio as a valuable toolto evaluate coronary heart disease risk", Journal of the American College of Nutrition Vo 27 No.1, pp.1–5.

Garrison R, 2013. Normal Rat Blood Glucose Level. http://www.ehow.com/facts_5990203_normal-rat-blood-glucose-level.html 25/9/2012

Herlina, H., Subagio A., and Estiasih T. (2013), "Hipolipidemic Potential of Water Soluble Polysaccharides from Gembili Tuber (*Dioscoreaesculenta* L.) in Hiperlipidemia Rats", Agritech J Teknol Pertanian Vol.33 No. 1, pp.8-15

Kanetro, B., Noor, Z., Sutardi, and Indrati, R. (2008), "Potency of soybean sprout protein to stimulate insulin secretin of pancrease in normal and diabetic rat", Agritech J Teknologi Pertanian Vol 28 No. 2, pp. 50-57

Kanetro, B. and Dewi,S.H.C. (2013), "Effect of various local legume sprouts as raw materials of meat analog on the physical (texture), preference and arginine/lysine ratio characteristics", Agritech J Teknologi Pertanian 33:1-7.

Kanetro, B. (2015), "Hypocholesterolemic properties of protein isolate from cowpeas (*vigna unguiculata*) sprout in normal and diabetic rats", Procedia Food Science Vol 3 No.1, pp.112 – 118.

Kanetro, B., Pujimulyani, D., Sahrah, A., and Luwihana, S. (2015), "Community empowerment of kalirejo kulonprogo special region of yogyakarta for developing oyek into artificial rice as staple food", proceedings of the international conference in University of Mercu Buana Yogyakarta, 2015, Directorate of Development and Cooperation, Yogyakarta, pp....

Reeves, P.G., Nielsen, F.H., and Fahey, G.C. (1993), "AIN-93 Purified diets for laboratory rodents: final report of the american institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet", Journal of Nutrition Vol. 123 No.11, pp.1939-1951.

Attachment :

Table 1. The effect of oyek feed treatment on blood tryglyceride of normal and diabetic rats (mg/dL) *)

Rats condition	Feed treatment	0th days	3th days	18th days
Normal	Standard	72,79a	72,92a	79,33a
	Oyek	67,15a	69,07a	75,82a
Diabetic	Standard	69,52a	117,45b	120,67c
	Oyek	69,33a	113,60b	90,30b

*) The same notation of statistic in the table showed not significantly differences at the same column

Table 2. The effect of oyek feed treatment on blood total cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0th days	3th days	18th days
Normal	Standard	106,28a	112,15a	112,59a
	Oyek	106,46a	108,66a	112,53a
Diabetic	Standard	104,11a	152,49b	154,31c
	Oyek	103,40a	156,54b	133,45b

*) The same notation of statistic in the table showed not significantly differences at the same column

Table 3. The effect of oyek feed treatment on blood HDL cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0th days	3th days	18th days
Normal	Standard	44,16b	45,08b	40,12c
	Oyek	41,69b	40,84b	38,50bc
Diabetic	Standard	35,91a	16,16a	14,76a
	Oyek	39,34ab	19,49a	30,86b

*) The same notation of statistic in the table showed not significantly differences at the same column

Table 4. The effect of oyek feed treatment on blood LDL cholesterol of normal and diabetic rats (mg/dL)*)

Rats condition	Feed treatment	0th days	3th days	18th days
Normal	Standard	57,54b	62,91a	59,59a
	Oyek	58,09b	58,19a	61,59a
Diabetic	Standard	46,75a	86,20b	87,18
	Oyek	49,01a	82,78b	66,67ab

*) The same notation of statistic in the table showed not significantly differences at the same column

Table 5. The effect of protein isolate from cowpea sprout feed treatment for 0thand 18th days on the ratio of total cholesterol/HDL *)

Rats condition	Feed treatment	0th days	18th days
Normal	Standard	2.41	2.81
	Oyek	2.55	2.92
Diabetic	Standard	2.90	10.45
	Oyek	2.63	4.32

*computed according to Table 2 and 3.

Table 6. The effect of protein isolate from cowpea sprout feed treatment for 0^{th} and 18^{th} days on the ratio of LDL/HDL*)

Rats condition	Feed treatment	0^{th} days	18^{th} days
Normal	Standard	1.30	1.49
	Oyek	1.39	1.60
Diabetic	Standard	1.30	5.91
	Oyek	1.25	2.16

*computed according to Table 3 and 4.

POSTER PRESENTATION

Phenolic contents and antioxidant activities of *Sonneratia caseolaris*

Johnny Tang Jock Lee[1], Angelie Dely[2], Madushika Ramanayake[3], Isuriy Adasuriyya[4], Irine Runnie Ginjom[5]

1 Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 Kuching, Sarawak, Malaysia, (johnnytang714@gmail.com)

2 Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 Kuching, Sarawak, Malaysia, (7440359@students.swinburne.edu.my)

3 Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 0 Kuching, Sarawak, Malaysia, (4315065@students.swinburne.edu.my)

4 Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 Kuching, Sarawak, Malaysia, (iadasuriyya@swinburne.edu.my)

Corresponding author:

5 Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Jalan Simpang Tiga, 93350 Kuching, Sarawak, Malaysia, (ihenry@swinburne.edu.my)

Abstract

Plant-based antioxidants are playing emerging role in the prevention of chronic degenerative diseases such as cancers, cardiovascular diseases and neurodegenerative conditions. Borneo Island is home to many exotic tropical fruits, where most remain underutilised. As with most fruits and vegetables, these tropical fruits are good source of nutrients and bioactive compounds. In this paper, three edible local fruits, namely, Sonneratia caseolaris (mangrove apple or `pedada'), Lepisanthes alata (`perupok' or `engkilili'), and Baccaurea angulata (wild star fruit or `gerumin') were evaluated for their total phenolic content (TPC) and total flavonoid content (TFC). Their antioxidant activities were measured using DPPH and ABTS assays. Different sections of these fruits (pericarp and seed or aril) were lyophilised, ground into powder, and subjected to 80% methanol extraction. All results were calculated in terms of 1 g dry weight (DW) sample. The results demonstrated that S. caseolaris seed consistently demonstrated the highest phenolic contents (TPC: 104 ± 0.8 mg gallic acid eq./g; TFC: 5.6 ± 0.3 mg quercetin eq./g), and antioxidant activities (DPPH EC_{50}: 16 ± 3 mg/L; ABTS: 502 ± 22 mg Trolox eq./g), while B. angulata aril sample consistently elicited the lowest values for all assays (TPC: 2.1 ± 0.2 mg gallic acid eq./g; TFC: 1.3 ± 0.3 mg quercetin eq./g; DPPH EC_{50}: 3,338 ± 42 mg/L; and ABTS: 32 ± 3 mg Trolox eq./g). The antioxidant activity (ABTS) values are well-correlated with the total phenolic contents (TPC) for all samples. It is thus recommended that S. caseolaris should be further studied to evaluate its potential as a dietary source of antioxidant.

Keywords: Phenolic, Flavonoid, Antioxidant activity, DPPH, ABTS, Sonneratia caseolaris, Lepisanthes alata, Baccaurea angulata.

Introduction

Fruits and vegetables are notably known for their health promoting properties specifically due to their micronutrient, fibre and antioxidant contents. Dietary antioxidants are the exogenous antioxidants that protect the body against the damaging effects of free radicals on biological cells. As free radicals are unstable, they react readily with other molecules to achieve stability. This leads to tissue damages that can contribute toward the aetiology of several chronic diseases such as cardiovascular and inflammatory diseases, cataracts, and cancer. Antioxidant is a substance that has ability to prevent or slow down the oxidation process when present in minute amounts (Fusco *et al*., 2007). While the evidence supporting the benefit of antioxidant supplement on human health is still inconclusive, antioxidants is widely used in the food industry as a preservative (e.g. BHT and BHA) to protect food ingredients against oxidative damages. BHT and BHA are both synthetic antioxidants. While extremely effective, there are concerns over their toxicity effects upon their long term consumptions.

Plants naturally produce antioxidants, mostly in the form of polyphenols, vitamin E and C as part of their defence mechanism against UV radiation and pathogens (Kasote *et al*., 2015). These secondary metabolites are synthesized for the normal growth, development and defence of plants (Kasote *et al*., 2015). Both *in vitro* and *in vivo* studies suggest that phenolic compounds are significantly promising antioxidants (Kasote *et al*., 2015). These are generally classified into phenolic acids, flavonoids, lignans, stilbenes and tannins with flavonoids and phenolic acids making up the largest group (Scalbert and Williamson, 2000). Based on their structures, flavonoids can be further classified as anthocyanidins (e.g. cyanidin, delphinidin), flavan-3-ols (e.g. catechin, epicatechin), flavonols (e.g. quercetin, kaempferol, myricetin), flavones (e.g. apigenin, luteolin), flavanones (e.g. hesperetin, naringenin) and isoflavones (e.g. genistein, daidzein). They are excellent antioxidants through their synergistic effects with endogenous antioxidants, electron donation, reducing power and metal ion chelating abilities (Kasote *et al*., 2015).

Skin and seed of fruits are rich source of phenolic compounds (Duda-Chodak and Tarko, 2007, Xu *et al*., 2010). The skin of Black Pearl grapes can contain up to 40 mg GAE/g of total phenolic contents while the seeds of Cabernet Sauvignon grapes can have up to 99 mg GAE/g (Xu *et al*., 2010). Most local edible plants are naturally nutritious, and some could be excellent source of antioxidants. Tropical berries such as Surinam (40 mg GAE/g) and Acerola (290 mg GAE/g) cherries are also excellent source of phenolic compounds and antioxidants (da Silva *et al*., 2014). Commonly found tropical fruits that are good source of phenolic compounds are cashew apple, soursop, guava, and papaya (53 mg GAE/g, 29 mg GAE/g, 17 mg GAE/g, 13 mg GAE/g, respectively) (da Silva *et al*., 2014). With so much variety of less commercialised fruits remains underutilised in Sarawak, there is so much opportunities to discover the next super-antioxidant fruit.

Three underutilized local fruits, namely *Baccaurea angulata, Lepisanthes alata* and *Sonneratia caseolaris* were selected in this study. To date, there is very limited information on the phytochemical information on all fruits. *Baccaurea angulata* or wild star fruit is widely distributed in Borneo. The fruit is covered in red angular pericarp with white tangy aril. The fruit contains about 5.2 mg GAE/g of total phenolic contents (Ahmed *et al*., 2015).

Its ethanolic extract was effective against Gram negative bacteria *K. pneumoniae* (Momand *et al*., 2014) while its juice was reported to reduce lipid peroxidation and increase antioxidant enzyme activities in rabbits fed with high-cholesterol diet (Mikail *et al*., 2016). *Lepisanthes alata* is indigenous to Southeast Asia, commonly grown as ornamental tree in Malaysia, Indonesia and the Phillipines. Locally known as `perupok' or `engkilli', the fruit has dark reddish purple pericarp with edible white pulp. To date, there is no reported medicinal property on this fruit (Lim, 2013). Mangrove apple or `pedada' (*Sonneratia caseolaris*) has persimmon-like fruit with sepals and usually grows in deep muddy soils of mangrove in tropical and sub-tropical areas worldwide. The leaves and fruits of *S. caseolaris* contain luteolin and its glycosides (Sadhu *et al*., 2006; Wu *et al*., 2009). These phenolic compounds are known for their antioxidant, anti-inflammatory, anticancer, neuroprotective, and cardioprotective effects (Nabavi *et al*., 2015). These underutilized fruits may contain a significant amount of phytochemicals which might have high antioxidant capacities, and thus, health properties. This paper discusses the results from the study on the phenolic contents and antioxidant activities of *B. angulata, L. alata* and *S. caseolaris* fruits.

Materials and methods

A. Sample collection and preparation

Mature fruits of *L. alata and B. angulata* were collected from their natural habitats in Bau, Sarawak, while *S. caseolaris* fruit was collected from Sarikei, Sarawak. The collected fruits were immediately rinsed with clean water and air-dried. The fruits were then manually separated into sections: (i) *B. angulata* – pericarp (BAP) and aril, including seed (BAA); (ii) *L. alata* - pericarp (LAP) and seed (LAS); and (iii) *S. caseolaris* - pericarp (SCP) and seed, including placenta (SCS); cut into smaller pieces and air-dried for another 1.5 hours. The samples were weighed and stored in tight containers at -22℃ overnight. The frozen samples were lyophilised for 48 hours and the final (dry) weights of the samples were recorded to determine their moisture contents. These were then ground into powder and stored in air-tight containers, in the dark at -22℃ until analysis.

B. Chemicals

Gallic acid, quercetin, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All the other chemicals used were of analytical grade.

C. Sample extraction

On the day of analysis, 10 mL of 80% aqueous methanol was added to 0.2 g of dry sample powder. The mixture was swirled briefly, sonicated for 30 minutes at room temperature, centrifuged for 10 minutes at 2500 rpm (Eppendorf centrifuge 5702, Hamburg, Germany) and finally filtered with Whatman No.1 filter paper. The extract volume was accurately adjusted to 10 mL with distilled water to make a 20,000 mg/L extract solution. All of the phenolic and antioxidant assay results were based on the dry weight mass of the powder used in the extract preparation. When not immediately used, the extract solution was stored in tight container, in the dark at -22°C, and analysed within one week. On the day of analysis, the frozen extracts were thawed and diluted (as necessary) with distilled water. Triplicate extractions were prepared for each sample (on separate weeks), and subsequently, each was separately analysed for the TPC, TFC and antioxidant activities. A batch of the extracts was dried to completeness in a refrigerated CentriVap centrifugal vacuum concentrator

(Labconco, Kansas City, Mo) to determine the crude phenolic extract yield estimation (dry weight basis). The % of extraction yield was calculated as below:

$$\text{Yield (\%)} = \frac{\text{Weight of dry extract (g)}}{\text{Weight of dry sample (g)}} \times 100$$

D. Determination of total phenolic content (TPC)

The TPC of the extracts was determined using Folin-Ciocalteu assay as described by Amado *et al*. (2014) with slight modification. Briefly, on the day of analysis, the frozen extract solution was thawed and subsequently diluted (0-1000 mg/L) with distilled water. In duplicates, exactly 1 mL of the diluted extract was mixed with 100µl of Folin-Ciocalteu reagent and mixed. After 5 min, 1 ml of 7% of sodium carbonate (Na_2CO_3) solution was added and mixed. After incubation for 1 h at room temperature in the dark, the absorbance read at 760 nm using Genesys 20 Visible Spectrophotometer (ThermoFisher Scientific, Germany) against a blank (0 mg/L). This was repeated for gallic acid standard solution (0-25mg/L) which was used to construct the reference calibration curve. The TPC was expressed as mg of gallic acid equivalent per g of lyophilised sample (mg GAE/g).

E. *Determination of total flavonoid content (TFC)*

The TFC of the extracts was estimated using aluminium-complexation reaction assay as described by Amado *et al*. (2014) with slight modification. Firstly, each of the frozen sample extracts was thawed and subsequently diluted (0-8000 mg/L) with distilled water. In duplicates, an aliquot of 1 mL of the diluted extract solution was accurately transferred into a disposable cuvette. Then, 1mL of 2% $AlCl_3$ was added into all cuvettes and vortexed 5s. The mixture was allowed to stand in the dark for 10 mins, and absorbance was then read at 415 nm against a blank (0 mg/L). The steps were repeated for quercetin solution (0-60 mg/L) which is the reference standard. The TFC was expressed as mg of quercetin equivalent per g of dry sample (mg QE/g).

F. Determination of antioxidant activity: DPPH assay

The antioxidant activity of the extracts was determined by using DPPH assay described by Miladi and Damak (2008) with slight modification. Briefly, a 0-10,000mg/L of extract dilutions were prepared from the 20,000 mg/L extract solution with distilled water as a diluent. Exactly 1 mL of DPPH solution (80 mg/L or 2 mM, in methanol) was incubated with 1 mL of the diluted samples. The mixture was vortexed, and kept in the dark at room temperature for 30 minutes. Then, the absorbance was measured at 517 nm against a blank (distilled water). Control sample was the sample containing 0 mg/L sample dilution. For positive comparisons, the steps were repeated for gallic acid solution (0-10 mg/L) and quercetin solution (0-15 mg/L). The radical scavenging effect (RSE) was measured as a decrease in the absorbance of DPPH after 30 min incubation. RSE was calculated using the following equation:

$$\text{RSE (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

The extract concentration providing 50% RSE (effective concentration, EC_{50}) was calculated from the graph of RSE against the extract concentration. The EC_{50} was expressed as the extract concentration at 50% RSE (mg of dry sample per L of solution or mg/L).

G. Determination of antioxidant activity: ABTS assay

The antioxidant activity of the extracts was determined by using ABTS assay as described by De Beer *et al*. (2003) and Re *et al*. (1999) with slight modification. Each of the sample extracts was thawed and diluted (0-100mg/L) with distilled water. To 0.5 ml of the diluted sample, 1 mL of working ABTS radical solution (~7.73 x 10^{-2} mM) was added. After mixing and 30 mins incubation in the dark at room temperature, absorbance was measured at 734 nm against a blank (distilled water). The ABTS value was determined using standard curve prepared with Trolox (0-10 mg/L) and was expressed as mg of Trolox equivalent per g of dry sample (mg TE/g).

H. Statistics

Except for the moisture contents and extraction yield, all data collected are means of three replicates along with standard deviations. Student's T-test was performed on the means to determine whether they differed significantly ($p<0.05$). As the data was not normally distributed, Spearman's correlation coefficients were calculated in order to characterize the relationship between antioxidant capacities detected by different assays, phenolics and flavonoids content. All statistical analysis was conducted using Excel 2010 (Microsoft Office Professional Plus 2010).

Results and Discussion

Moisture content and extraction yield

The moisture content of the fruit samples ranged from 66% to 92% (Table 1). For *B. angulata* and *L. alata*, the moisture contents were higher in the pericarps (BAP, LAP) and lower in the sections containing the seeds (BAA, LAS). It was the opposite for *S. caseolaris*. The SCS section has more moisture content possibly due to the presence of the moisture-rich placenta and the relatively smaller seed sizes. The difference in moisture content or solid content led to the usage of dry weights basis in comparing the results of each chemical analysis.

Table 1. Moisture contents and extraction yields of different sections of *L. alata*, *B. angulata* and *S. caseolaris*.

Sample	Moisture content, (%)	Extraction yield, (%, d.w.b)
B. angulata pericarp (BAP)	92	38
B. angulata aril and seed (BAA)	70	28
L. alata pericarp (LAP)	79	32
L. alata seed (LAS)	66	25
S. caseolaris seed and placenta (SCS)	80	26
S. caseolaris pericarp (SCP)	75	17

Values were determined from a single determination.

From these dry samples, crude phenolic compounds were extracted using 80% aqueous methanol. The extraction yield ranged from 17% to 38% (Table 1), with the highest recorded in BAP and the lowest in SCP. Overall, the extraction yield for *B. angulata* in this study was slightly lower than those reported by Ahmed *et al*., (2015) who recorded 31% to 61% yield

from different fruit sections. The main differences were in the use of 100% methanol and 24 h incubation at 37°C by Ahmed *et al*., (2015).
While it is generally beneficial to have greater extraction yield, the potency of those extracts in terms of phenolic contents and antioxidant activities vary, as shown in the following analytical results.

I. Total phenolic contents (TPC) and total flavonoid contents (TFC)

Table 2 shows the TPC and TFC values of the six samples. There were significant (p<0.05) differences between the TPC and TFC in the extracts of each sample. The ranking of the sample was quite consistent with SCS possessing the highest TPC and TFC, while BAA eliciting the lowest TPC and TFC (Table 2).

Table 21 Total phenolic contents (TPC) and total flavonoid contents (TFC) of different sections of *L. alata*, *B. angulata* and *S. caseolaris*.

Sample	TPC (mg GAE/g dw)	TFC (mg QE/g dw)
S. caseolaris seed and placenta (SCS)	103.9 ± 0.8^{a}	$5.6 \pm 0.3^{a,c}$
L. alata pericarp (LAP)	62.7 ± 2.5^{b}	3.4 ± 0.1^{b}
L. alata seed (LAS)	38.1 ± 0.8^{c}	1.9 ± 0.1^{d}
S. caseolaris pericarp (SCP)	20.4 ± 0.2^{d}	4.4 ± 0.1^{e}
B. angulata pericarp (BAP)	6.30 ± 0.3^{e}	3.3 ± 0.1^{f}
B. angulata aril and seed (BAA)	2.10 ± 0.2^{f}	1.3 ± 0.3^{g}

Values are expressed as mean ± standard deviation (n=3). Means in a column followed by different letters differ significantly (P<0.05). All results are per g of dry sample weight.

The results suggest that the seeds and placenta of *S. caseolaris*, a `mangrove apple' are rich in phenolic compounds. Its TPC and TFC values (104 mg GAE/g and 5.6 mg QE/g, respectively) are almost two times more that the TPC and TFC values of the second ranking sample, LAP (63 mg GAE/g and 3.4 mg QE/g, respectively). This value is comparable to those found in the seed of Cabernet Sauvignon seeds (99 mg GAE/g) and higher than those found in cashew apple (53 mg GAE/g) (Xu *et al*., 2010; da Silva *et al*., 2014). *S. caseolaris* is a mangrove plant. Banerjee *et al*. (2008) stated that mangroves plants have unique adaptations to overcome the environmental stress conditions such as high salinity, high temperature, low nutrients and excessive radiation. Therefore, it is possible that the high phenolic content of *S. caseolaris* fruit is an adaptation to the extreme environment.

Based on the TFC results, the flavonoids make up 5% to 62% of the total phenolics. The different in flavonoid fraction is most likely due to the different distribution and types of phenolic compounds in different fruit sections and in different plant species (Xu *et al*., 2010; da Silva *et al*., 2014). It is also possible that the assay did not evaluate all of the flavonoids present in the sample. According to Pekal and Pyrzynska (2014), the method employed in this study was selective only for flavonols and flavones luteolin. Thus, to imply that the assay determines the total flavonoid content is actually misleading. Other flavonoids that may be present but not determined in this study include anthocyanidins, flavan-3-ols (e.g. catechins), flavanones, and isoflavones. Phenolic compounds are also made up of non-

flavonoids which were not specifically measured in this study such as phenolic acids (e.g. gallic acid), and stilbenes (e.g. resveratrol).

For *B. angulata*, the recorded values of TPC and TFC in this study were relatively lower than those reported in a similar study (Ahmed *et al*., 2015), possibly due to the variances in the fruit samples. Difference in agriculture practices, soil nutrients, weather, fruit maturity level, biotic and abiotic factors will affect the phenolic contents the fruits (Briskin and Gawienowski, 2001; Huang *et al*., 2014; Jaafar *et al*., 2014, Mokhtar *et al*., 2014).

J. Antioxidant activity

For the DPPH assay, lower EC_{50} values signify higher antioxidant activities, whereas for the ABTS assay, higher values mean higher antioxidant activities. Table 3 summarizes the results obtained from the antioxidant assays. Based on the results, the ranking order of antioxidant activities of the samples closely resembles those obtained for the phenolic content assays. For both DPH and ABTS antioxidant assays, SCS topped the antioxidant values of both assays (16 mg/L, 501 mg TE/g, respectively), whilst BAA ranked the lowest (3,338 mg/L, 32 mg TE/g, respectively).

Table 3 Antioxidant activity as determined using DPPH and ABTS assays of different sections of *L. alata*, *B. angulata* and *S. caseolaris*.

Sample	DPPH assay	ABTS assay
	EC_{50} value (mg/L)	**(mg TE/g dw)**
S. caseolaris seed and placenta (SCS)	16 ± 3^{a}	501.5 ± 21.9^{a}
L. alata seed (LAS)	$76 \pm 6^{c,d}$	178.8 ± 17.8^{c}
L. alata pericarp (LAP)	113 ± 24^{d}	$220.0 \pm 19.6^{c,d}$
S. caseolaris pericarp (SCP)	117 ± 11^{e}	143.6 ± 11.0^{e}
B. angulata pericarp (BAP)	$1{,}615 \pm 75^{f}$	41.8 ± 3.2^{f}
B. angulata aril and seed (BAA)	$3{,}338 \pm 42^{g}$	31.7 ± 2.5^{g}
Gallic acid	2.3 ± 0.3^{h}	-
Quercetin	5.4 ± 1.4^{i}	-

Values are expressed as mean ± standard deviation (n=3). Means in a column followed by different letters differ significantly ($P<0.05$).

By ranking all the data for Spearman's correlation calculation, all variables showed positive correlations ($r_s \geq 0.600$) (Table 4). However, only TPC and ABTS values showed significant correlation at $p=0.01$. At $p=0.05$, only DPPH and TPC, and DPPH and ABTS showed significant correlations. The data strongly suggested that the four variables, especially the TPC and the ABTS, were significantly and positively correlated to each other. Similar findings was reported by Floegel et al (2011), who recommended that ABTS assay is a more useful antioxidant assay than DPPH assay in assessing antioxidant capacity in a variety of foods. This is encouraging as TPC and ABTS assays are simple to conduct and can be modified into a high throughput assay using microplate readers. Rapid preliminary screening allows for more new potential antioxidant sources to be discovered and investigated.

Table 4 Spearman's correlation coefficients (r_s) for the association between antioxidant activities as measured by DPPH and ABTS assays, total phenolic contents (TPC) and total flavonoids (TFC) of different sections of *L. alata*, *B. angulata* and *S. caseolaris.*

	DPPH ($1/EC_{50}$)	ABTS	TPC	TFC
DPPH ($1/EC_{50}$)	1.000	0.943	0.943	0.600
ABTS	0.943	1.000	1.000	0.714
TPC	0.943	1.000	1.000	0.714
TFC	0.600	0.714	0.714	1.000

Conclusions

With total phenolic contents equivalent to Cabernet Sauvignon grape seeds, the seed and placenta section (SCS) of *S. caseolaris* fruit can be a rich source of antioxidants. Other fruits (*L. alata* and *B. angulata*) and *S. caseolaris* pericarp also possessed phenolic compounds and antioxidant activities, but at much lower levels. While all variables show correlations with each other, it was the TPC and ABTS that showed the strongest and most significant positive correlations. This led to the conclusion that the TPC and ABTS assays are sufficient for the preliminary screening of antioxidant capacity in food samples.

This study also showed that local fruits have equivalent, if not higher contents of phenolic compounds and antioxidants than those widely reported fruits. As these fruits are readily available at much cheaper price, they should be promoted as an alternative source of antioxidant instead of the more expensive imported fruits or supplements. As they are more widely utilised, more studies can be done on their cultivation and sustainability. This, ultimately provide a positive economic outcome for the local community and the environment.

Acknowledgements

The authors would like to express their gratitude to Swinburne University of Technology Sarawak Campus for providing the funds (SSRG 2014), materials and facilities to complete this study. The authors would also like to thank Sarawak Biodiversity Centre for the permits (SBC/RES/I/0003) for plant samples collections.

References

Ahmed, I.A., Mikail, M.A., Ibrahim, M., Hazali, N., Abdul Rasad, M.S.B., Abdul Ghani, R., Abdul Wahab, R., Arief S.J., and Adros Yahya, M.N. (2015), "Antioxidant activity and phenolic profile of various morphological parts of underutilised *Baccaurea angulata* fruit", *Food Chemistry*, Vol.172, pp. 778-787.

Amado, I.R., Franco, D., Sanchez, M., Zapata C, and Vazquez JA (2014), "Optimisation of antioxidant extraction from *Solanum tuberosum* potato peel waste by surface response methodology", *Food Chemistry*, Vol. 165, pp.290-299.

Benerjee, D., Chakrabarti, S., Harza, A.K., Banerjee, S., Ray, J., and Mukherjee, B. (2008), "Antioxidant activity and total phenolic of some mangroves in Sundarbans", *African Journal of Biotechnology*, Vol. 7 No. 6, pp.805-810.
Briskin, D.P. and Gawienowski, M.C. (2001), "Differential effects of light and nitrogen on the production of hypericins and leaf glands in *Hypericum perforatum*", *Plant Physiology*, Vol. 39, pp.1075-1081.

da Silva, L.M.R, de Figueiredo, E.A.T., Ricardo, N.M.P.S., Vieira, I.G.P., de Figueiredo, R.W., Brasil, I.M., and Gome, C.L. (2014), "Quantification of bioactive compounds in pulps and by-products of tropical fruits from Brazil", *Food Chemistry*, Vol.143, pp. 398-404.

De-Beer, D., Joubert E., Gelderblom, W.C.A., and Manley, M. (2003), "Antioxidant activity of South African red and white cultivar wines: free radical scavenging", *Journal of Agricultural and Food Chemistry*, Vol. 51, pp. 902-909.

Duda-Chodak, A. and Tarko, T. (2007), "Antioxidant properties of different fruit seeds and peels", *ACTA Scientiarum Polonorum Technologia Alimentaria*, Vol. 6 No. 3, pp. 29-36.

Floegel, A., Dae-Ok, K., Sang-Jin, C., Sung-I, K.. and Ock-K, C. (2011), "Comparison of ABTS/ DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods", *Journal of Food Composition and Analysis*, Vol. 24 No. 7, pp. 1043-1048.

Fusco, D., Colloca, G., Monaco, L.M.R. and Cesari M (2007), "Effects of antioxidant supplementation on the aging process", *Clinical Interventions in Aging*, Vol. 2 No. 3, pp. 377-387.

Huang, H., Sun, Y., Lou, S., Li, H. and Ye, X. (2014), "*In vitro* digestion combined with cellular assay to determine the antioxidant activity in Chinese bayberry (*Myrica rubra* Sieb. Et Zucc.) fruits: A comparison with traditional methods", *Food Chemistry*, Vol. 146, pp. 363-370.

Jaafar, H.Z.E., Mohamed, H.N.B. and Rahmat, A. (2008), "Accumulation and partitioning of total phenols in two varieties of *Labisia pumila* Benth. under manipulation of greenhouse irradiance", *Acta Horticulturae*, Vol. 797, pp. 387-392.

Kasote, D.M., Katyare, S.S., Hegde, M.V., Bae, H. (2015), "Significance of antioxidant potential of plants and its relevance to therapeutic applications", *International Journal of Biological Sciences*, Vol. 11 No. 8, pp. 982-991.

Lim, T.K. (2013), "*Lepisanthes alata*", in Lim, T.K. (Ed.), Edible medicinal and non-medicinal plants, Springer, Dordrecht Heidelberg New York London, pp. 39-41.

Mikail, M.A., Ahmed, I.A., Ibrahim, M., Hazali, N., Rasad, M.S.B.A.R., Ghani, R.A., Wahab, R.A., Arief, S.J., Isa, M.L.M., Draman, S., Ishola, A.A. and Yahya, M.N.A. (2014), "Changes in the markers of atherosclerosis following administration of Belimbing Dayak

(*Baccaurea Angulata)* fruit juice in experimental rabbits fed with cholesterol diet", *Journal of Advances in Agricultural and& Environmental Engineering*, Vol. 1 No. 1, pp. 151-154.
Miladi, S. and Damak, M. (2008), "*In vitro* antioxidant activities of Aloe vera leaf skin extracts", *Journal de la Societe Chimique de Tunisie*, Vol. 10, pp. 101-109.

Mokhtar, S.I., Leong, P.C., Ven, L.E. and Aziz, N.A.A. (2014), "Total phenolic contents, antioxidant activities and organic acids composition of three selected fruit extracts at different maturity stages", *Journal of Tropical Resources and Sustainable Science*, Vol. 2, pp. 40-46.

Momand, L., Zakaria, R., Mikail M., Jalal, T., Ibrahim, M. and Wahab, R.A. (2014), "Antimicrobial effect of *Baccaurea angulata* fruit extracts against human pathogenic microorganisms", *Merit Research Journal of Medicine and Medical Sciences*, Vol. 2 No. 10, pp. 229-237.

Nabavi, S.F., Braidy, N., Gortzi, O., Sobarzo-Sanchez, E., Daglia, M., Skalicka-Woźniak, K. and Nabavi, S.M. (2015), "Luteolin as an anti-inflammatory and neuroprotective agent", *Brain Research Bulletin*, Vol. 119 Part A, pp. 1-11.

Pekal, A. and Pyrzynska, K. (2014), "Evaluation of aluminium complexation reaction for flavonoid content assay", *Food Analytical Methods*, Vol. 7, pp. 1776-1782.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999), "Antioxidant activity applying an improved ABTS radical cation decolorization assay", *Free Radical Biology and Medicine*, Vol. 26, pp.1231-1237.

Sadhu, S.K., Ahmed, F., Ohtsuki, T. and Ishibashi, M. (2006), "Flavonoids from *Sonneratia caseolaris*", *Journal of Natural Medicine*, Vol. 60, pp. 264-265.

Scalbert, A. and Williamson, G. (2000), "Dietary intake and bioavailability of polyphenols", *The Journal of Nutrition*, Vol. 130 No. 8, pp.2073-2085.

Wu, S., Wen, Y., Li, X., Zhao, Y., Zhao, Z , Hu, J. (2009), "Chemical constituents from the fruits of *Sonneratia caseolaris* and *Sonneratia ovata* (Sonneratiaceae)", *Biochemical Systematics and Ecology*, Vol. 37 No 1, pp. 1-5.

Xu, C., Zhang, Y., Cao, L., Lu, J. (2010), "Phenolic compounds and antioxidant properties of different grape cultivars grown in China", *Food Chemistry*, Vol. 119, pp. 1557-1565.