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PREVENTION EFFECTS OF METHANOLIC EXTRACT OF *Eurycoma longifolia* ROOTS ON CARBON TETRACHLORIDE-INDUCED HEPATOTOXICY IN RATS

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ABSTRACT

The objective of this study was to investigate preventive effects of methanolic extract of *Eurycoma longifolia* roots (MEEL) against carbon tetrachloride-induced hepatotoxicity in rats. A total of 25 rats were randomly divided into five groups (n=5). Group I was treated with Sodium CMC 1% w/v (peroral, 6 days), and group II was treated with single dose of CCl_4 in olive oil (1:1) 2 mL/kg (intraperitoneal injection). Groups III-V received 75, 150, and 300 mg/kg BW of MEEL (peroral, 6 days), respectively, and administration of CCl_4 in olive oil (1:1) 2 mL/kg (intraperitoneal injection) on the seventh day. The hepatoprotective potential was estimated by measuring serum activity of biochemical parameters. Further, liver weight ratio and histopathological changes were determined. The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were significantly (p<0.05) increased and albumin decreased significantly (p<0.05) in the CCl_4 treated group, but treatment with MEEL 75 mg/kg significantly (p<0.05) prevented the elevation of ALT, AST, and LDH. Histopathological changes also indicated hepatic protection of the MEEL at 75 mg/kg. This finding suggested that methanolic extract of *Eurycoma longifolia* roots at 75 mg/kg was enough to give a protective effect against CCl_4-induced hepatotoxicity.

Keywords: carbon tetrachloride; *Eurycoma longifolia*; hepatotoxicity; protective

INTRODUCTION

The liver is a highly complex organ with regards to various physiological functions in the body (Joshi *et al*., 2015). An endogenous antioxidant system has been developed to maintain redox homeostasis in the liver. Homeostasis will be disturbed because of the excess of free radicals, resulting in oxidative stress, which plays a critical role in liver diseases and other degenerative disorders (Li *et al*., 2015).

Non-alcoholic fatty liver disease (NAFLD) is defined by the presence of liver fat accumulation exceeding 5% of hepatocytes, in the absence of alcohol intake and any other cause of liver injury (Musso *et al*., 2011). NAFLD comprises a wide spectrum of liver damage, ranging from benign hepatocellular steatosis to non-alcoholic steatohepatitis, fibrosis, and cirrhosis (El-Kader and Ashmawy, 2015). Prevalence of NAFLD in Asia is around 25%, like many Western countries (Fan *et al*., 2017). Currently, NAFLD has become a worldwide problem and is associated with significant morbidity and mortality.

Carbon tetrachloride (CCl_4) administration to rodents is a widely used model for hepatotoxicity study (Riordan and Nadeau, 2014). CCl_4 is activated by

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cytochrome P450 2E1 to form trichloromethyl radicals ($CCl_3\cdot$) and trichloromethyl peroxyl radicals ($CCl_3OO\cdot$). Free radical chain reactions lead to lipid peroxidation and finally produce reversible liver damage (Jayesh *et al*., 2017; Weber *et al*., 2003).

Medicinal plants contain a variety of secondary metabolites that can act as antioxidants. Antioxidants are vital substances which can protect the body from damage caused by oxidative stress induced by free radicals (Jothy *et al*., 2011). *Eurycoma longifolia*, which belongs to the Simaroubaceae family, is one of the most well-known herbal medicines in Southeast Asia (Khanijo and Jirangkoorskul, 2016; Rehman *et al.*, 2016). The root possesses strong antioxidant properties (Varghese *et al*., 2013). Previous research has reported anti-hypertriglyceridemia, anti-inflammatory, analgesic, and anti-obesity activities in its methanolic extract (Hendra *et al.*, 2017; Lahrita *et al.*, 2017). Therefore, we aimed to investigate preventive the effects of *Eurycoma longifolia* roots extract against carbon tetrachloride-induced hepatotoxicity in rats.

METHODS

Plant material and chemicals

Eurycoma longifolia roots were collected from Kalimantan, Indonesia and supplied by Merapi Farma Herbal Co. (Yogyakarta, Indonesia). Plant material was identified and authenticated at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. A voucher specimen was deposited in the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Sanata Dharma University, Yogyakarta, Indonesia. Sodium carboxymethyl cellulose (Sodium CMC) was supplied by Brataco Chemika, Indonesia. Olive oil was supplied from Bertolli, Italy. Methanol and carbon tetrachloride as hepatotoxins were from E. Merck (Darmstadt, Germany). Diagnostic kits for the estimation serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin were purchased from Abbott Laboratories, Illinois, USA. Diagnostic kit for estimation of lactate dehydrogenase (LDH) in the serum was purchased from Thermo Fisher Scientific, Massachusetts, USA. All other chemicals were of analytical grade and were purchased from E. Merck, Darmstadt, Germany.

Preparation of plant extract

The dry powder of *Eurycoma longifolia* roots (1.2 kg) was extracted with methanol (95% v/v) in a mechanical shaker for 48 h at room temperature. The mixture was filtered, then concentrated by using a rotary evaporator at 60^{o}C to yield a semi solid residue (1.89% w/w). This methanolic extract of *Eurycoma longifolia* roots (MEEL) was kept in a desiccator and used for further experiments.

Phytochemical screening

The MEEL was screened for various chemical constituents (flavonoids, alkaloids, tannins, phenolic compound, alkaloids and terpenoids) by using established methods (Harborne, 1973; Evans and Trease, 1989).

Experimental animals

Adult male Wistar rats weighing 150-250 g were used for this experiment. The animals were procured from Department of Pharmacology and Therapy, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. Five rats were kept in one cage and acclimatized to the surroundings (Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, Sanata Dharma University, Yogyakarta, Indonesia) for 1 week before starting the experiment. All animals were housed under standard laboratory conditions (temperature 22±3^{o}C and 12 h light/dark cycle), fed with rodent pellet diet and water ad libitum. The study protocol was approved by Medical and Health Research Ethics Committee, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia (KE/FK/0794/EC/2017).

Animal grouping and treatment

The animals were randomly divided into five groups with 5 rats in each group. Group I (normal control): animals were treated with

Sodium CMC 1% w/v p.o., for 6 days. Group II (toxic control): animals were treated with CCl_4 in olive oil (1:1) 2 mL/kg, i.p. Groups III-V (test groups): animals were treated with 75, 150, and 300 mg/kg of MEEL p.o., for 6 days, respectively, and a single dose of CCl_4 in olive oil (1:1) 2 mL/kg, i.p. was given on the seventh day. Blood samples from all groups were obtained directly through retro-orbital plexus venous puncture 24 h after the last administration (Dongare *et al*., 2013; Hendra *et al*., 2017). The blood was allowed to coagulate and centrifuged at 3000 rpm for 10 min. Serum samples were used for determination of some biochemical parameters.

Biochemical determinations

All biochemical parameters were determined in a Cobas C501 analyzer using commercial kits following standard procedures.

Histopathological assessment

Rats were euthanized by cervical dislocation at the end of the experiment. Rats were dissected, livers were collected and weighted. The liver weight ratio is expressed as follows: (liver weight/body weight) x 100% (Li *et al*., 2017; Ekeanyanwu and Njoku, 2014). Then the organs were fixed in neutral buffered formalin. The tissues were embedded in paraffin, cut in 5 μm sections and stained with hematoxylin and eosin for histopathological findings.

Statistical analysis

All the data were expressed as mean±SEM. Groups were compared by Kruskal-Wallis test, followed by Mann-Whitney test. P values less than 0.05 were considered. Statistical analysis was done using IBM SPSS Statistics 22.

RESULTS AND DISCUSSION

This study was designed to investigate the activity of methanolic extract of *Eurycoma longifolia* roots (MEEL) on some biomarker enzymes and histopathological changes of hepatic lesions indicating hepatocellular injury induced by CCl_4 in rats.

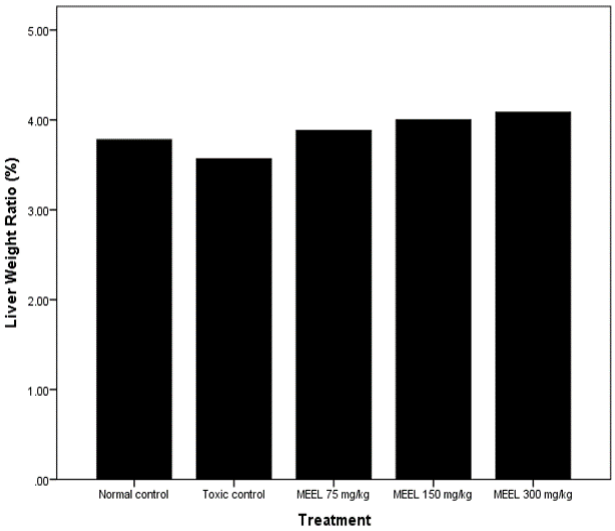

Figure 1. Effect of MEEL on liver weight ratio

Table I. Effect of MEEL on liver biochemical parameters

Treatment	ALT (U/L)	AST (U/L)	LDH (U/L)	Albumin (mg/dL)
Normal control	52.8±2.5[b]	140.0±7.7[b]	529.0±27.1[b]	3.49±0.06[b]
Toxic control	220.5±25.0[a]	839.3±16.5[a]	1511.6±136.1[a]	3.15±0.05[a]
MEEL 75 mg/kg	146.9±18.6[a,b]	566.8±63.6[a,b]	851.0±31.5[a,b]	3.01±0.05[a]
MEEL 150 mg/kg	134.9±11.5[a,b]	581.9±41.3[a,b]	1247.6±165.9[a]	3.05±0.06[a]
MEEL 300 mg/kg	222.2±24.8[a]	743.7±38.8[a,b]	1158.6±102.7[a]	3.12±0.08[a]

All values are expressed as mean±SEM, n=5. [a]p<0.05 when compared with normal control and [b]p<0.05 when compared with toxic control.

Carbon tetrachloride is extensively known as an inducer for hepatotoxicity models. The elevated of serum enzyme ALT and AST as sensitive markers, are indicative of cellular leakage and loss of functional integrity of cell membranes in liver that was initiated by hepatocellular damages caused by CCl_4 (Nirmala *et al*., 2012; Thanh *et al*., 2015). Changes in the activities of serum enzymes (ALT, AST and LDH) and albumin in the serum of CCl_4-induced liver damage as evidence from Table I. The level of serum marker enzymes ALT, AST and LDH were found to be significantly (p<0.05) increased

and albumin decreased significantly ($p<0.05$) in CCl_4 treated group when compared to normal control. In the present study, significant increases in aminotransferase, LDH and decrease of albumin level in the CCl_4 intoxicated rats represent hepatic damage, consistent with previous reports.

Treatment with MEEL 75 mg/kg significantly ($p<0.05$) prevented the elevation of ALT, AST, and LDH. MEEL 150 mg/kg reduced levels of ALT and AST significantly ($p<0.05$). Treatment with MEEL 300 mg/kg significantly ($p<0.05$) decreased levels of AST. However, there were no significant ($p>0.05$) increases in the level of albumin of the rats with all of the doses of MEEL.

Figure 1. shows the average liver weight ratio in the five groups animals. No significant differences in livers among all the treatments were observed.

Histopathological observation of liver tissue of the normal control group showed a normal liver architecture of hepatocytes since they were well arranged without any alteration at the central vein, while CCl_4 intoxicated hepatocytes showed severe multifocal areas of fat degeneration (steatosis). Steatosis is known as a type of liver injury that may manifest as triglyceride accumulation which leads to either a micro vesicular or macro vesicular fatty liver (Sing *et al*., 2014). The MEEL 75 mg/kg treated group's liver sections showed less hydropic degeneration, while MEEL 150 and 300 mg/kg treated groups' liver sections showed hydropic degeneration with multifocal areas of fat degeneration.

In our study, administration of MEEL 75 mg/kg markedly decreased the elevated aminotransaminase and LDH levels induced by CCl_4. A dose-dependent effect was not found in the 150 and 300 mg/kg of MEEL groups when compared to the 75 mg/kg group. These trends were also found in histological observation of liver tissue. Similarly, previous study showed the hepatoprotective effect of water extract of *Eurycoma longifolia* at 1.5 g/kg against CCl_4-induced hepatotoxicity (Al-faqeh *et al*., 2010). An in vivo study reported that the methanolic extract of *Eurycoma longifolia* roots reduced triglyceride levels induced by glucose-fructose enriched diet and shown anti-inflammatory activity (Hendra *et al*., 2017). Another in vitro study confirmed that *Eurycoma longifolia* suppressed lipid accumulation in 3T3-L1 adipocytes (Lahrita *et al*., 2015). Hence, the hepatoprotective effect of methanolic extract of *Eurycoma longifolia* roots 75 mg/kg may be achieved by the anti-inflammatory activity which may be associated with scavenging of free radicals responsible for CCl_4 toxicity. Moreover, flavonoids are known as hepatoprotectives agents, so the mechanism of hepatoprotective activity of MEEL 75 mg/kg may be due to the presence of flavonoids. Preliminary phytochemical screening conducted in this study indicated MEEL contains flavonoids, tannins, phenolic compound, and terpenoids, while alkaloids were absent.

CONCLUSION

In summary, the results of this study showed that CCl_4 caused hepatotoxicity and the administration of methanolic extract of *Eurycoma longifolia* roots 75 mg/kg to rats offered significant protection from the hepatic damage by CCl_4. Evidently, histopathological examination of liver also supported the benefits of the methanolic extract of *Eurycoma longifolia* roots as it helped in improving liver cell architecture damage caused by CCl_4. Further study should be done to characterize the hepatoprotective components of methanolic extract of *Eurycoma longifolia* roots.

Conflict of interest statement

We declare that we have no conflict of interest.

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PHAGOCYTOSIS ACTIVITY OF BINAHONG (*Anredera cordifolia* (Tenore.) Steenis) FROM SECANG, MAGELANG, CENTRAL JAVA, INDONESIA

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ABSTRACT

The use of medicinal plants is increasing due to the search for alternative resources to treat diseases such as hypertensions and infection. Along with the development in science, preventive action should take place to prevent our body from suffering from these diseases. This can be done by increasing the human immune status with immunomodulatory agents. Binahong empirically have benefits for wound healing. The purpose of this research was to investigate the immunomodulatory effect of ethanolic extract of binahong leaves. The non-specific modulatory effects of the ethanolic extracts of binahong leaves on the immune systems were measured based on phagocytosis index and phagocytosis capacity. Tests were conducted on strain Balb/C male mice at the age of 6-8 weeks. Mice were administered orally with the extract of binahong leaves (doses of 25, 50, 75 mg/kgBW) for 14 days. The test results with the index parameters and macrophages phagocytosis capacity at doses of 50 and 75 mg/kgBW did not significantly increased when compared with the controls. From these results, we concluded that the ethanolic extract of binahong leaves with a dose of 25, 50 and 75 mg/kgBW cannot significantly the activity of macrophages by phagocytosis index parameters and phagocytosis capacity significantly.

Keywords: *Anredera cordifolia*; immunomodulatory agent; macrophage; phagocytosis capacity; phagocytosis index

INTRODUCTION

Medicinal plants have been used as an alternative to synthetic drugs, for a long time. Medicinal plants are potential sources of drugs and are widely used to gain health benefit empirically (Rodríguez *et al*., 2018). Medicinal plants are used because they are easier to obtain, cheaper than synthetic drugs and have less side effect (Wardhani and Sulistyani, 2013). Various diseases such as hypertension, diabetes mellitus, headache, inflammation and modulation of the immune system have been widely treated with medicinal plants (Leliqia *et al*., 2017; Putri *et al*., 2017).

Binahong (Anredera cordifolia (Ten.) Steenis) is one of the species from the family of Basellaceae, which has fleshy leaves and thick aerial tubers. It is widely used as a medicinal plant in Indonesia. Binahong leaves are used for treatment of wounds, refreshing the body, headache and lowering blood pressure. Ether fraction of binahong leaves extract exhibited antioxidant activity measured by DPPH (1,1-diphehyl-2-picrylhydrazyl) (Ardianti and Guntarti, 2014). Binahong leaves extract accelerated wound healing infected by Staphylococcus aureus in mice. Binahong leaves extract also inhibited the growth of Staphylococcus aureus and as a result, the healing process the wounds is faster than wound healing without binahong leaves extract (Umar *et al*., 2012). Topical application of binahong leaves extract makes wound healing process faster, IL-6 level higher and increases vascular endothelial growth factor (VEGF) production in burns

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infected by Pseudomonas aeruginosa (Sukrama *et al*., 2017).

We therefore designed this study to determine the immunomodulatory effects of binahong leaves extract by measuring phagocytosis activity and phagocytosis index. The antimicrobial-antioxidant activity was correlated with immunomodulatory effects (Umar *et al*., 2012; Yuniarti and Lukiswanto, 2017). Phagocytosis ratio indicates the percentage of active macrophage to 100 macrophages, and phagocytosis index indicates the number of latex able to be consumed by active macrophages. These number are compared to the controls.

METHODS

Research Materials

CMC-Na, Distilled water, alcohol 70%, ethanol 96% (General Labora), methanol, Giemsa 20%, latex beads (Sigma™), RPMI medium 1640 (Gibco™), FBS (Sigma™), Fungizone (Gibco™), pen-strep (Gibco™), PBS (Sigma™), hepatitis B vaccine (Euvax™).

Preparation of sample

Binahong plant leaves were obtained from the District Secang, Magelang, Central Java. The identify was authenticated by the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada in certificate number UGM/FA/2413/M/03/02. Based on the results, the plants studied is binahong (Anredera cordifolia (Tenore) Steenis).

Preparation of animal test

Male mice, strain Balb/c aged 6-7 weeks weighing 25-35 g were obtained from the Animal Cage Test Faculty of Pharmacy, Universitas Gadjah Mada. The protocol of the study was approved by the Ethics Commission for Preclinical Trials of Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada with certificate number 00123/04/LPPT/X/2017.

Extraction of binahong leaves

Binahong leaves were obtained from Secang, Magelang Regency, Central Java Province, Indonesia. Powdered leaves material of *Anredera cordifolia* was macerated with ethanol 96% (2x) for 24 hours each, filtered and the filtrates obtained were combined and evaporated in vacuum to give thick liquid material of ethanol extract. The ethanol extract with the dose of 25, 50 and 75 mg/kgBW were given orally to assess its immunomodulatory effects by measuring macrophage activity using phagocytosis index and phagocytosis capacity and compared with normal and CMC-Na groups.

Immunomodulatory assay (macrophage activity assay)

Preparation of macrophage cells

Macrophage cells were isolated from BALB/c mice (6–8 weeks old). Mice were euthanized with neck dislocation and 10 mL of cold RPMI medium were then injected inside the stomach. After 3-5 minutes, the RPMI was withdrawn from the stomach using syringe and put into a conical flask, centrifuged at 1,500 rpm (4^{o}C, for 10 minutes). The supernatant was removed and the residue resuspended with RPMI (80% FBS). The numbers of cells was calculated with hemocytometer, diluted with RPMI (80% FBS) till 2.5 x 106/mL cells density was obtained. The cells suspension that had been cultured on 24 wells (200 µL/well, 5x105 cells/well) plate for 24 hours was put in round coverslips, incubated in a CO_2 (5%) incubator, at 37^{o}C for 30 minutes and then complete medium, containing 10% FBS, fungizone 0.5% and penicillin streptomycin 2% (1.0 mL) was added to each well and then incubated for another 2 hours. The cells were washed twice with RPMI, and then complete medium (1.0 mL) was added to each well and incubated for the next 24 hours (Hartini *et al*., 2013).

Macrophages activity measurement

Macrophages activity measurement was done involving latex (2 µm in diameter) as substrates (suspended in PBS, at 2.5 x 107/mL). The cells suspension (200 µL) was

added into each well containing peritoneum macrophages, then incubated for another 60 minutes in a CO_2 incubator. The cells suspension was washed 3 times with PBS in order to remove particles. The cells suspension was dried at room temperature and then fixed with methanol. Coverslips were dyed with Giemsa 20% for 20 minutes, washed with aquadest, lifted up from the wells and re-dried at room temperature. The macrophages activity was calculated as the number (%) of consumed latex (substrates), visualized by light microscope (magnified 400x) as seen as Figure 1. Phagocytosis ratio was indicated by the percentage of active macrophage in 100 macrophages, and phagocytosis index was indicated by the number of latex able to be consumed by active macrophages (Hartini *et al*., 2014). These data were compared to the controls.

RESULTS AND DISCUSSION

The parameters of the observed activity of macrophages were phagocytosis capacity of macrophages and macrophage phagocytosis index. Macrophage phagocytosis capacity was obtained by calculating the percentage of the number of active macrophages phagocyted latex beads per 100 macrophages observed, whereas macrophage phagocytosis index was obtained by counting the number of latex beads phagocyted per 100 macrophages (Jensch-Junior *et al*., 2006). These parameters would be able to show immunostimulatory effects of ethanolic extract of binahong leaves.

Macrophages were isolated from the peritoneal cavity of mice because the number of macrophages in the peritoneal cavity is larger than the other organs and easy to obtain from the peritoneal fluid. The medium used was RPMI because this medium can attract macrophages in the peritoneal cavity and provide nutrition such as vitamins, amino acids, and essential materials required for macrophage cell culture processes. Liquids that have been isolated from the peritoneal cavity contains not only macrophage cells but also granulocytes and lymphocytes. Peritoneal fluid that was place into a conical flasks was centrifuged to separate macrophage cells from other cells such as lymphocytes and granulocytes (Hay and Westwood, 2002).

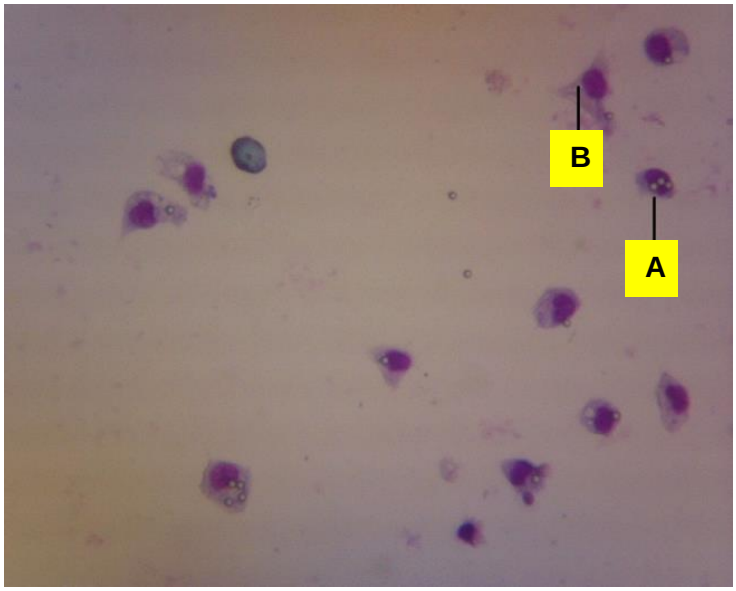

Figure 1. Macrophage observation under microscope magnification 400x. A: active macrophage; B: inactive macrophage.

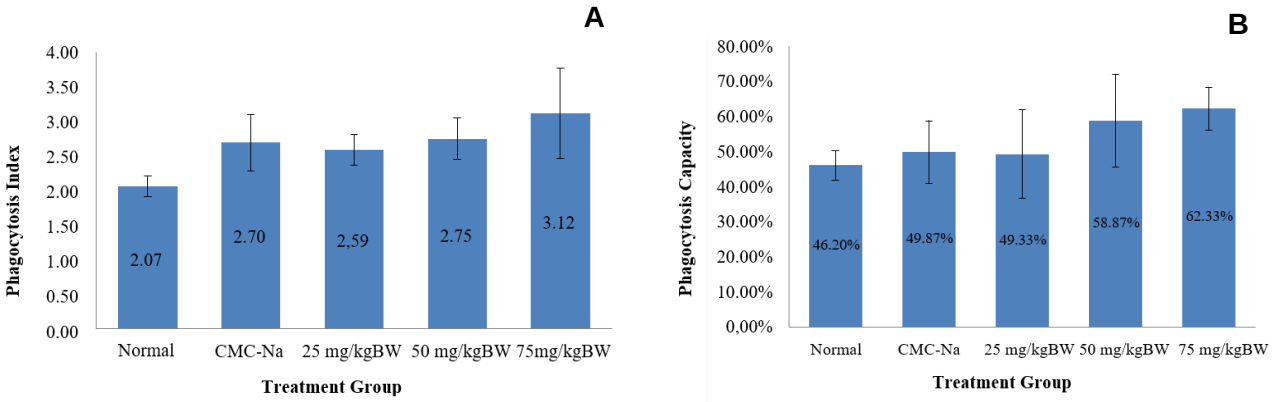

Figure 2. Results of statistical analysis of phagocytosis index (A) and phagocytosis capacity (B).

Table I. Phagocytosis Capacity and Phagocytosis Index of Binahong Leaves Ethanolic Extracts

	Phagocytosis Capacity		Phagocytosis Index	
	Results	Mean ±SD	Results	Mean ±SD
Normal Group	44.00%	46.20% ± 0.0414	1.97	2.07 ± 0.1521
	46.67%		1.92	
	40.33%		2.32	
	50.33%		2.04	
	49.67%		2.08	
CMC-Na + Vaccine Group	48.67%	49.87% ± 0.0899	242	2.70 ± 0.4076
	65.33%		3.02	
	42.00%		3.08	
	47.33%		284	
	46.00%		2.13	
Dose 25 mg/kgBW + Vaccine	63.33%	49.33% ± 0.1269	2.88	2.59 ± 0.2267
	33.67%		2.65	
	54.67%		2.35	
	45.67%		2.48	
Dose 50 mg/kgBW + Vaccine	40.00%	58.86% ± 0.1324	2.81	2.55 ± 0.3002
	59.67%		2.88	
	53.00%		2.76	
	74.33%		2.25	
	67.33%		3.06	
Dose 75 mg/kgBW + Vaccine	61.00%	62.33% ± 0.0610	3.39	3.12 ± 0.6526
	61.67%		2.20	
	56.00%		3.72	
	70.67%		3.17	

Hepatitis B vaccine was administered intraperitoneally on day 5 and 12 as an immune booster. The first vaccination was done to activate the non-specific and specific immune system and the second vaccination was done to increase the expression of the immune system (as a booster) so it is easier to analyze. Surgery was performed on the 15th day because the immune system will be activated optimally up to 3 days after induction of the vaccine and gradually decline thereafter (Abbas *et al*., 2014).

Quantification of macrophages was performed using latex beads with a size of 3 μm which were resuspended in the serum of test animals. The size of the latex beads that resemble the size of bacteria could trigger macrophage phagocytosis by being perceived as foreign particles. The addition of serum of the test animals into the latex suspension would help the macrophage phagocytosis process as serum would facilitate the introduction of the antigen by macrophages (Harvath and Terle, 1999).

Based on our results which was presented in Table I, after examining macrophage phagocytic capacity and macrophage phagocytosis index, the ethanolic extract of binahong leaves at dose of 25, 50 and 75 mg/kgBW did not significantly enhance the ability of macrophage through an increase of both parameters compared with the control group 0.5% CMC-Na. A significant difference was found only in the phagocytosis index data for the dose group 75 mg/kgBW when compared with the normal group, as seen as in Figure 2.

According to previous research, 70% ethanolic extract of binahong leaves dose of 50 mg/ kgBW could raise the profile of leukocytes as an increase in total leukocytes, neutrophils and total monocytes in guinea pigs (Wijayanti *et al*., 2018). In addition, the ethanolic extract of binahong leaves at concentrations of 50% and 100% could increase the phagocytic monocytes with in vitro method (Wahyukundari and Praharani, 2016). Monocytes are produced by the bone marrow which would circulate in the blood before it becomes differentiated in the tissue to macrophages. Other studies found that binahong leaves extract can also increase the production of interleukin-6 in the blood plasma of mice during the healing of burns. Interleukin-6 is a cytokine produced by T-cells and macrophages to stimulate the immune response during an infection or during the healing process (Sukrama *et al*., 2017). From the previous research of Sukrama *et al*., (2017) and Wijayanti *et al*., (2018), ethanolic extract of binahong leaves has the effect to increase the response of the immune system, but in the present study, the increasing phagocytosis capacity and phagocytosis index were not significant when compared to the control group.

Research by Wijayanti *et al*., (2018) was using a guinea pig test animals while this study used Balb/c strain mice. Previous research was conducted to see the effect of binahong leaves extract on blood leukocytes profile and the average number of descendants of the test animals. The increase in the number of monocytes as one of the parameters of blood leukocytes profile in these studies was not always followed by an increase in activity of macrophages in the tissue. Monocytes from the blood can differentiate into multiple cell types of the immune system and in such a network of dendritic cells, osteoclasts and macrophages depends on the existing stimulus inside the body. Monocyte activity in the network tends to be more specific and different in each tissue. The difference is caused by different stimuli derived from the macrophage microenvironment (Hulin *et al*., 1995).

Research conducted by Wahyukundari *et al*. (2016) was performed in vitro by taking blood monocytes. Experiments in vitro have less variables that cannot be controlled as compared to experiments in vivo. In vivo experiments involve factors including pharmacokinetics (absorption, distribution, metabolism and excretion) and the first-past effect that could affect the availability of the extract in the body. In addition, the physiological state of the test animals such as hormones could also influence the effect of the test sample. Absorption through the oral drug

has a lower bioavailability compared with administration by injection and the drug can be metabolized by gastrointestinal fluids or often called first-past experience effect (Atanasov *et al*., 2015).

Another factor that could affect the results when compared with previous studies was the source of the sample. Wahyukundari and Praharani (2016) collected the binahong leaves from Jember, East Java whereas in this study the binahong leaves were collected from Secang, district of Magelang, Central Java. Different areas could cause differences in the content of secondary metabolites in plants because each area has different soil nutrients. Differences in soil nutrients would affect the availability of nutrients and plant precursor to form secondary metabolites (Salim *et al*., 2017).

CONCLUSION

Ethanolic extract of binahong leaves at dose of 25, 50 and 75 mg/kgBW cannot significantly increase the activity of macrophages by phagocytosis index parameters and phagocytic capacity.

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SIMPLE AND RAPID METHOD FOR ISOLATING ANTHOCYANIN FROM WILD MULBERRY (MORUS NIGRA L.)

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ABSTRACT

Wild mulberry (Morus nigra L.) is a kind of berries that has a high content of anthocyanin pigment. Anthocyanin is a natural pigment that has good biological activity so that widely be used as both food and drug ingredients. There are many studies conducted that have isolation anthocyanin from mulberry extract, but most of them used various expensive methods and the process included several steps that make them not cost-effective nor time-efficient. This research was conducted in order to do an isolation of anthocyanin from wild mulberry through a single step. The extraction of compounds was done by maceration and the isolation was done by thin layer chromatography method. The isolation product was identified with reagents, consisting of ferric chloride and sodium hydroxide, and with spectrophotometry methods, consisting of UV-Vis and infrared spectrophotometry. As result, this research was able to isolate anthocyanin from wild mulberry fruit by thin layer chromatography method. The identification with spectrophotometry methods indicated that the isolated compound hypothetically was anthocyanidin-3-O-rutinoside.

Keywords: anthocyanin; isolation; thin layer chromatography; mulberry fruit; *Morus nigra* L.

INTRODUCTION

The increasing of education level in society has led to a higher awareness to a healthy lifestyle. Food, as one of the important lifestyle components, plays a crucial role in health since the body receives nutrients from the food intake. Food contains several chemical constituents including colorant. The use of colorant has shifted from synthetic and insect-base colorant to natural colorant. One natural colorant that promises good use as a safe food colorant and has good antioxidant activity is anthocyanin (Kendrick, 2016).

Anthocyanin is a water-soluble pigment due to the hydroxyl rich chemical structure. It is classified into the flavonoid group and has glycosides attached on the second aromatic ring (Pervaiz *et al.*, 2017). It can be found in various parts of plant which are blue, red, or purple color. A positive charged oxygen that makes it become more reactive in scavenging free electrons in free radical compounds. As a good source of antioxidants, anthocyanin has been used widely as supplements and food additives (Khoo *et al.*, 2017).

Beside its antioxidant activity, anthocyanin has the potential to be used as an active pharmaceutical ingredient. Scientific study showed that anthocyanin has anticancer activities on HepG2 (Yan *et al.*, 2017), SK-Hep1 (Thi and Hwang, 2018), and MCF-7 cells (Roobha *et al.*, 2011). Other researchers found that anthocyanin has a potency as an inhibitor for various enzyme, including monoamine oxidase A, tyrosinase, α-glucosidase and dipeptidyl peptidase-4, as well as enzyme that is responsible for chronic and degenerative diseases (Cásedas *et al.*, 2017). Research has shown that enzymatic

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inhibition activity of anthocyanin acts on cyclooxygenase-2 which is responsible for inflammatory responses in the body (Miguel, 2011).

Berries provide a promising resource for anthocyanin pigment. Mulberry (*Morus nigra* L.) is a kind of berries that grows in tropical regions, including Indonesia. It contains a high amount of anthocyanin. Previously, one anthocyanin characterization study on mulberry showed that the fruit extract of mulberry contained cyanidin-3-O-glycoside and cyanindin-3-O-rutinose. They were identified with spectrometry methods on 527 and 529 nm, respectively (Sitepu *et al.*, 2016). Additionally, anthocyanins can also be prepared using HPLC-MS with UV detectors and ESI interfaces using positive ion models in MS systems (Huang *et al.*, 2012).

The promising use of anthocyanin has led to an increasing demand. Common methods that are used to extract anthocyanin are costly with a great expense in both time and money. The extraction of anthocyanin mostly has used nitrogen to eliminate water from the plant material (Bondre *et al.*, 2012; Huang *et al.*, 2012). Generally, the isolation of anthocyanin is conducted by either HPLC, using the solvents of acetonitrile: water (1:1 v/v) containing 2% formic acid and aqueous 2% formic acid. The gradient was from 6 to 10% B for 4 min, from 10 to 25% B for 8 min, isocratic 25% B for 1 min, from 25 to 40% for 7 min, from 40 to 60% for 15 min, from 60 to 100% for 5 min, from 100 to 6% for 5 min, at a flow rate of 1.0 ml/min. Isolation process also used the multi-step conventional method, including column chromatography and thin layer chromatography (Huang *et al.*, 2012). Thus, a cost-effective and time-efficient method needs to be discovered. This research aimed to isolate anthocyanin from wild mulberry fruit extracts through a single step thin layer chromatography.

METHODS

Material and chemicals

Analytical balance (Ohaus®, 0,001 g), museums jar with glass plate (Duran®, 210x100mm), membrane filter (Sartorius®, 0,22 µm), UV-Vis and Infrared Spectrophotometer (Jasco®, Japan), hand blender (Panasonic®), rotary evaporator (IKA®, RV10), wild mulberries was collected from Karang Besuki District, silica gel 60 GF_{254} TLC plate (Merck ™, Germany), silica gel 60 G for Preparative TLC (Merck ™, Germany), ethyl acetate, ethanol 96%, water, methanol (PT Panadia Corporation Indonesia ™, technical grade), and glacial acetic acid, chloroform (Merck ™, analytical grade).

Extraction of mulberry fruits

The extraction method followed the previous research conducted by Huang *et al.* (2012) with a few modifications. Mulberry fruit was washed and cleaned with water and then sorted to remove the remaining stalks and other impurities. The sorted sample was crushed with hand-blender so that a pulp was formed. The pulp was macerated twice with a solvent (1:3), which consisted of methanol and glacial acetic acid (99:1), for 24 hours. The maceration process was done in an ice bath and with minimum light exposure. The maceration product was filtered to obtain the filtrates. In order to remove smaller particles, the filtrates were centrifuged on 4000 rpm for 5 minutes. An evaporation process was done by rotary evaporator on 60°C under vacuum conditions. The saturated filtrated result went through further evaporation process with a water bath until a thick extract was formed.

Isolation of anthocyanin pigment

Purification of anthocyanin was done through thin layer chromatography methods. The extract was diluted in methanol to get a 10 mg/ml solution, then filtered with a membrane filter to remove insoluble particles. After that, the solution was applied to an activated TLC plate. Activation of TLC plate was done by heating the plate in the oven at 115°C for 15 minutes. TLC was done by eluting the plate with several eluents as shown in Table II. Eluted spots were taken from the plate, then washed with methanol:chloroform (1:1). Those filtrates were evaporated to become dry under room temperature to be identified.

Identification compound

Initial identification of anthocyanin was done by simple qualitative colorimetry methods. An amount of 10 mg extract was diluted with 10 ml methanol. The diluted extract was added by 1.0% $FeCl_3$ and 2.0 N NaOH solution separately. A color change to purple after addition of $FeCl_3$ and blue after addition of NaOH indicated that the solution contains anthocyanin. Isolates of anthocyanin were diluted in methanol:chloroform (1:1). The solution was read with UV-Vis and IR spectrophotometer to obtain the spectra data.

RESULTS AND DISCUSSION

Extraction of mulberry through maceration gave a result of crude extract with dark red color. The addition of glacial acetic acid in the solvent had a functional purpose to maintain the stability of anthocyanin during the extraction and storage. A higher pH of solution can cause a color change of anthocyanin from red, purple, or blue. The mechanism is described in Figure 1 (Wahyuningsih *et al.*, 2017). As the extract was thickening, the weight of the extract was measured and showed a yield of 15, 30 gram or 5.1% relative from the amount of plant material. The initial identification was conducted to ensure that the extraction method gave an extract containing anthocyanin. Table I shows the result of the initial identification using $FeCl_3$ and NaOH as reagents.

Table I. Anthocyanin Initial Identification

Sample	Reagent	Result
Mulberry Fruit Extract	1% $FeCl_3$	Purple
	2,0 N NaOH	Blue

The result of initial identification showed that the mulberry fruit extract contains anthocyanin. A change of color to purple after addition of ferric chloride solution was the expression of ferric-phenol complex from polyphenol groups in anthocyanin (Obouayeba *et al.*, 2015). Figure 3 shows the formation reaction of ferric-phenol complex. Meanwhile, blue color was expressed since the base condition reacts with anthocyanin, causing a form of negatively charged ions. This was the specific color test for anthocyanin.

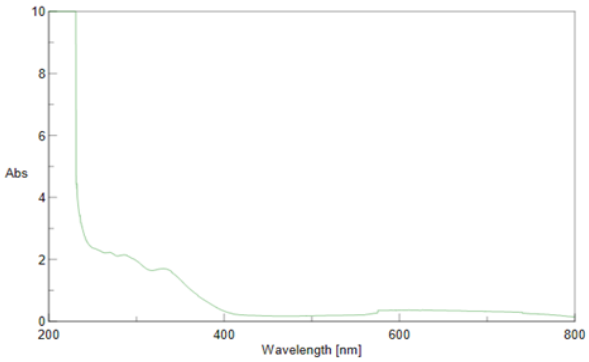

Figure 2. UV-Vis spectrum of second isolate

Since the main purpose of this research was to find cost effective method, the isolation was conducted by thin layer chromatography. The separation of anthocyanin was done on preparative plate which was coated with silica gel 60. Table II shows the eluent used on the TLC method. The two spots that separated shown a difference in color, but had a very close Rf value. Both of the spots appeared on Rf of 0.81 and 0.82, respectively. Both isolates obtained from TLC method were separated from silica gel by diluting them in methanol-chloroform solution. The first isolate's weight was 46 mg and the second was 139 mg. Spectrophotometry was conducted on the second isolate since red color was believed to express the stable anthocyanin (Wahyuningsih *et al.*, 2017). The isolate was diluted with 2 ml methanol-chloroform to be measured by spectrophotometer. Figure 2 shows the UV-Vis spectrum data of the second isolate.

Table II. Separation of Anthocyanin using TLC method

Eluent Composition	Result
Ethanol:Water:Acetic Acid (4:5:1)	2 spots
Ethanol:Water:Acetic Acid (4,5:5:0,5)	-
Ethanol:Water:Acetic Acid (4:2:1)	-
EtOAc: Ethanol:Water (65:25:10)	-

The UV-Vis spectrum shows two peaks around 280 and 560 nm. Based on the previous study conducted by Qin *et al.*, anthocyanidin-3-O-rutinoside had maximum wavelength on 281.1 and 524.7 nm (Qin *et al.*, 2010). Similarity between those spectra

hypothetically indicated that the second isolate was an anthocyanidin-3-O-rutinoside. There was slight differences between the results that could be caused by the effect of different solvents used on each research (Field *et al.*, 2008). This research used methanol-chloroform solvent, while the previous study used acetate buffer (pH 3.6). It means that the solvent from the previous study was more polar, thus causing a detection on shorter wavelength.

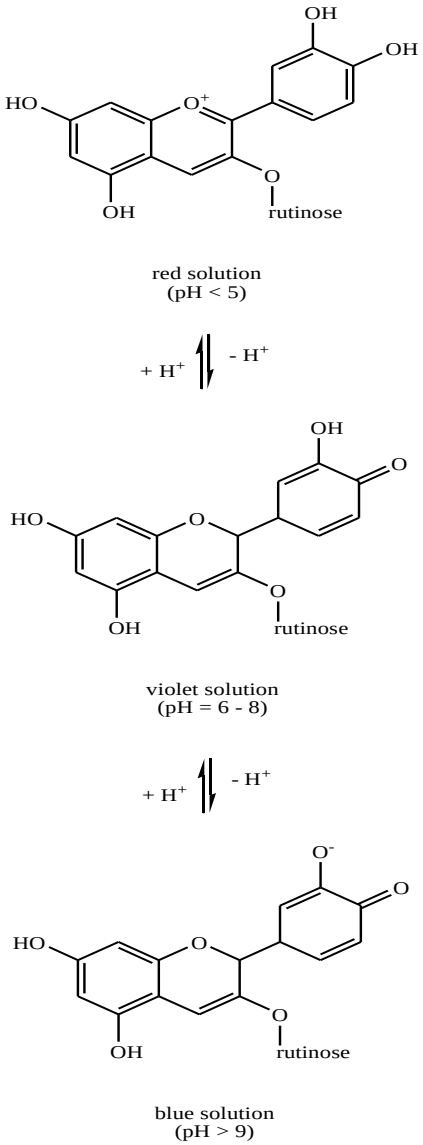

Figure 1. Effect of pH change on anthocyanin solution color

The isolation of anthocyanin compounds was previously done by Huang *et al.* 2012. In his research, anthocyanins were identified and quantified using HPLC/DAD and HPLC/ESI/MS. Cyanidin-3-galactoside was the major compound, making up to 93% of the total anthocyanin content. The content of Cyanidin-3-galactoside reached 23.7 ± 3.2 mg 100 g^{-1} fresh weight. The research used HPLC/DAD and HPLC/ESI/MS which are expensive and complicated. This was different in our study, where the isolation of anthocyanin compounds only used TLC preparative with the mobile phase of ethanol:water:acetic acid (4:5:1). Our study used methods that were relatively cheap and simple with a yield of 32.4 ± 1.3 mg 100 g^{-1} fresh weight.

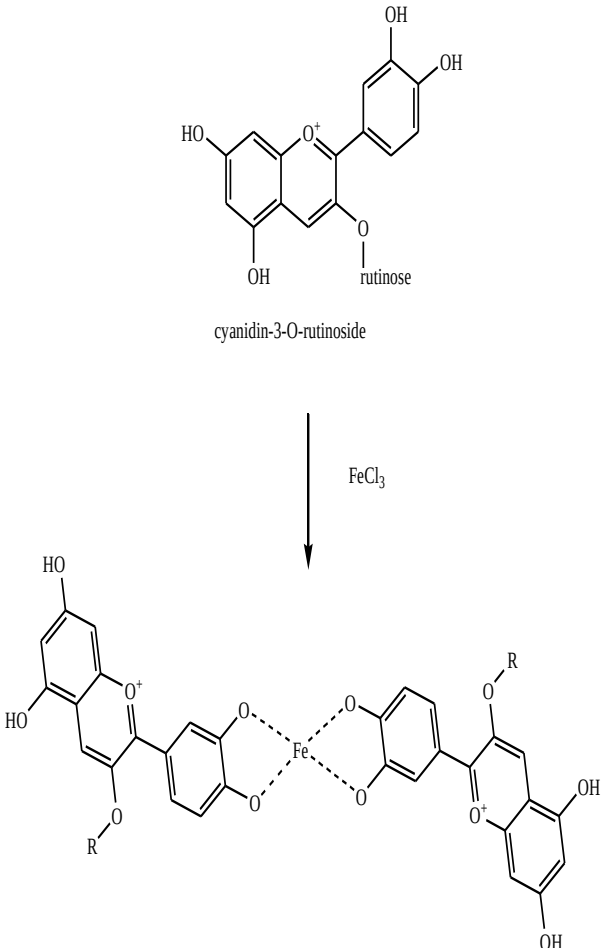

Figure 3. Formation of ferric-phenol complex of cyanidin-3-O-rutinoside

Infrared spectrum shows functional groups in a compound. The diluted second isolate solution was applied to FTIR to be scanned. Figure 4 shows the IR spectrum of second isolate. Infrared spectrum of the second isolate showed peaks on 3310.21; 1640.16; and 1033.66 cm^{-1}. That were similar to the previous study on anthocyanin conducted by Chang *et al.* The characteristic

phenol was shown by the peak on 3310.21 cm^{-1}; alkene groups from the benzene structure were shown by the peak on 1650.16 cm^{-1}; and alkoxy group was shown by the peak on 1033.66 (Chang *et al.*, 2013). The difference between those spectra were the peaks on 665.32 and 420.41 cm^{-1} which were the expression of carbon-halogen bonding, especially carbon-chlorine from the chloroform (Field *et al.*, 2008).

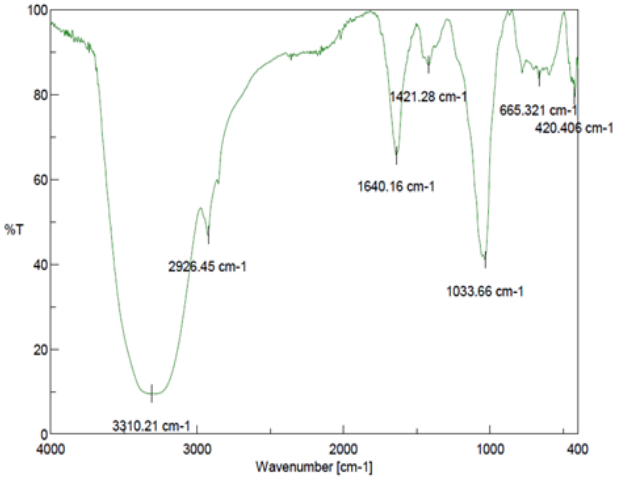

Figure 4. IR spectrum of second isolate

CONCLUSION

Wild mulberry (Morus nigra L.) obtained from the Karang Besuki District was confirmed to positively contain anthocyanin pigment. Based on the spectra study, the anthocyanin isolated from the fruit was hypothetically an anthocyanidin-3-O-rutinoside. This study focused on the isolation of anthocyanin pigment from wild mulberry by using thin layer chromatography method. The result of this study indicates that TLC is a potential method for isolating anthocyanin pigment from wild mulberry fruit.

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APPLICABILITY OF BACTERIAL ENDOTOXIN TEST (BET) FOR SOME RADIOPHARMACEUTICAL STERILE KITS BY THE USE OF TACHYPLEUS AMEBOCYTE LYSATE (TAL)

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ABSTRACT

The application of bacterial endotoxin test (BET) using TAL reagent on radiopharmaceutical kits is very important to conduct. The radiopharmaceutical kits that will be tested are macro aggregated albumin (MAA), tetrofosmin and ethambutol kits. Endotoxin testing stage was TAL 0.25 EU/mL verification test, inhibition/enhancement test, and endotoxin test for sample. Pyrogen testing using rabbits was also performed as a comparison test. The results of the TAL reagent verification test were all samples showed values corresponding to the standards of 2λ = (+), 1λ = (+), 1/2λ = (-), 1/4λ = (-), and negative water control (NWC) = (-). Furthermore, inhibition/enhancement tests for MAA, tetrofosmin, and ethambutol products show non-inhibiting or gel-inducing results, which are in accordance with acceptability standards, so that the samples can be tested using TAL reagents. The pH measurement results in each sample were MAA of 6.0, tetrofosmin of 7.0, and ethambutol of 8.0. The results of MAA, tetrofosmin, and ethambutol product testing were a sample = (-), positive product control (PPC) = (+), positive water control (PWC) = (+), and NWC = (-). In addition, the results of pyrogen testing also showed negative for MAA, tetrofosmin, and ethambutol.

Keywords: Bacterial endotoxin test (BET); Radiopharmaceutical kit; Tachypleus amoebocyte lysate (TAL)

INTRODUCTION

The radiopharmaceutical is a pharmaceutical preparation labeled with radionuclide so that its energy can be used as a diagnosis or therapy in the field of nuclear medicine (Scott and Kilbourn, 2015; Knapp and Dash, 2016). Radiopharmaceutical preparations are generally injected intravenously (Salmanoglu, Kim and Thakur, 2018). Therefore, the classification of the radiopharmaceutical preparation must meet the criteria of a sterile pharmaceutical preparation. One of the requirements of sterile preparations is pyrogen-free or endotoxin-free (Zandieh *et al.*, 2018). Pyrogen is a substance that can cause fever, and generally in pharmaceutical products it comes from gram-negative bacteria (Lopes *et al.*, 2015; Silva *et al.*, 2016) while endotoxin is a complex compound consisting of pyrogenic lipopolysaccharides (Zandieh *et al.*, 2018).

The pyrogen-free test was initially conducted using rabbits. However, since it is known that endotoxins are able to agglomerate limulus blood cells, then in the development to detect endotoxins an alternative pyrogen test was found which is a bacterial endotoxin test using limulus

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amoebocyte lysate (LAL) (Miao *et al.*, 2013). The bacterial endotoxin test was first performed by Frederik B. Bang with the freezing method of LAL cells that are sensitive to endotoxins (Bang, 1971). The application of endotoxin test in various sterile preparations has been done routinely for several decades in a number of countries. In the 1980s the United States approved the endotoxin test as a substitute of pyrogen testing for end-products of parenteral drugs and this change subsequently was followed by other countries (Ochiai *et al.*, 2010).

Many new methods have emerged to replace the role of animal experiments in routine quality control tests such as pyrogenic test transfers to endotoxin test using the monocyte activation test (MAT) (Silva *et al.*, 2016). The need for LAL reagents is very high so that another source of lysate has been required. Therefore, the presence of a very large tachypleus amoebocyte lysate (TAL) horseshoe lysate in Southeast Asia may be an alternative source of lysate for bacterial endotoxin testing (John *et al.*, 2012; Li, Hitchins and Wickramasekara, 2016).

Several radiopharmaceutical kits have been developed in Indonesia, for example, the macro aggregated albumin (MAA) kit used for the diagnosis of pulmonary perfusion (Lestari, 2017), tetrofosmin kit for cardiac diagnosis (Widyastuti, Lestari and Sangaji, 2017) and the ethambutol kit used for the diagnosis of mycobacterium tuberculosis (MBT) in the body (Juwita, 2009). The radiopharmaceutical kits have undergone strict quality control, but the pyrogen test still uses rabbits that require animal maintenance and care which can pose some difficulties, since the sensitivity of the test results is affected by the environment, and the observation time is also so long that the test becomes ineffective. A study of bacterial endotoxin test for 18F-Fludeoxyglucose (FDG) was conducted by Sharma, *et al.* (2011). Therefore, endotoxin tests are routinely performed using TAL for radiopharmaceutical products which are more effective in test time and not influenced by external factors of rabbit test animals. The object of this research was to apply bacterial endotoxin test (BET) using TAL reagent with a sensitivity of 0.25 EU/mL on radiopharmaceutical kit preparation such as MAA, tetrofosmin, and ethambutol.

METHODS

Equipment

Equipment used in this research included syringe 1 mL (BD), micropipette and tips (Eppendorf), digital tele thermometer model 461 (Electronics India), stopwatch, vial, thermo mixer comfort as an incubator (Eppendorf), analog vortex mixer (VWR International)

Materials

Materials used in this research were local rabbit (Oryctolagus cuniculus) identified as the white New Zealand rabbit, which weighs about 2.5-3.5 kg, and initial body temperature of 37.0-39.8ºC, a set of endotoxin detection kits, gel-clots: Tachypleus amoebocyte lysate (TAL), control standard endotoxin (CSE), sterile water free pyrogenic (BET water) (Zhanjiang Bokang Marine Biological Co., Ltd., China), MAA, tetrofosmin, and ethambutol sample kits are manufactured by the Center for Radioisotope and Radiopharmaceutical Technology (PTRR)-National Nuclear Energy Agency (BATAN).

Bacterial Endotoxin Test by using TAL

TAL verification test

Control standard endotoxin (CSE) dilution was done from preparations of 10 EU/mL. CSE was resuspended using 1 mL BET water, then the suspension was homogenized for 15 minutes. The CSE suspension was then diluted into a standard solution used for the confirmation test. Standard solutions were diluted to concentrations 1 EU/mL (4λ), 0.5 EU/mL (2λ), 0.25 EU/mL (1λ), 0.125 EU/mL

(1/2λ), and 0.0625 EU/mL (1/4λ). Then each concentration and negative water control (BET water) was inserted into the TAL reagent. Then it was incubated at 37± 1ºC for 60±2 minutes without vibration. The samples were tested positive for endotoxin (> 0.25 EU/mL) when the gel was formed and negative endotoxin (< 0.25 EU/mL) were not formed after the tube was reversed 180 degrees slowly (USP 35-NF 30 online version, 2012). The dilution scheme for BET verification test is shown in Figure 1.

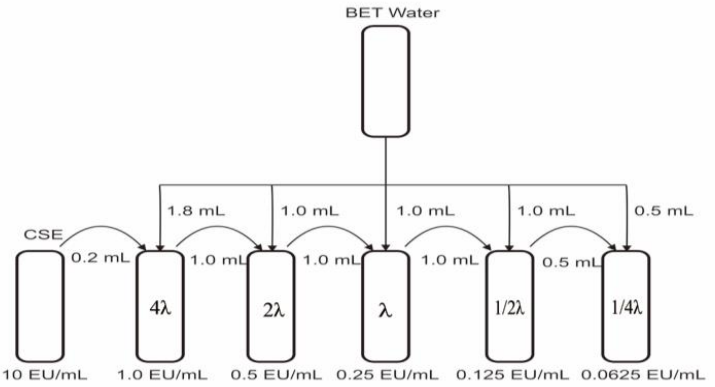

Figure 1. Bacteria endotoxin concentration for the verification test

Test Inhibition / enhancement.

The sample was first tested whether the substance inhibits or induces gel formation in TAL. Each solution contained the composition according to the Table I. Then the sample solutions A, B, C, and D were incubated at 37 ± 1 ºC for 60 ± 2 minutes without vibration. The samples were tested positive when the gel was formed and negative samples were not formed after the tube was reversed 180 degrees slowly (USP 35-NF 30 online version, 2012). Where, solution A is a sample solution of the preparation under test that is free of detectable endotoxins, solution B is test for interference, solution C is control for labeled TAL reagent sensitivity, and solution D is negative control of BET water.

Table I. Preparation of solution for the Inhibition / Enhancement test for gel clot technique

Solution	Endotoxin concentration	Solution to which endotoxin is added	Initial endotoxin concentration	Number of replicates
A	None	0.2 mL Sample solution	-	4
B1	0.1 mL 2λ	0.1 mL Sample solution	2λ	4
B2	0.1 mL 1λ	0.1 mL Sample solution	1λ	4
B3	0.1 mL 1/2λ	0.1 mL Sample solution	1/2λ	4
B4	0.1 mL 1/4λ	0.1 mL Sample solution	1/4λ	4
C1	0.1 mL 2λ	0.1 mL BET water	2λ	2
C2	0.1 mL 1λ	0.1 mL BET water	1λ	2
C3	0.1 mL 1/2λ	0.1 mL BET water	1/2λ	2
C4	0.1 mL 1/4λ	0.1 mL BET water	1/4λ	2
D	None	0.2 mL BET water	-	2

BET for sample

The test was performed using a TAL reagent having a sensitivity of 0.25 EU/mL. Maximum Valid Dilution (MVD) is the maximum allowable dilution of the specimen where the endotoxin limit can be determined. A sample of the diluted solution was taken as much as 0.2 mL and added to the reagent TAL, then incubated at 37±1ºC for 60±2 minutes without vibration. The samples were tested positive for endotoxin (> 0.25 EU/mL) when the gel was formed and negative endotoxin (< 0.25 EU/mL) were not formed after the tube was

reversed 180 degree slowly. The maximum valid dilution in each sample was calculated by Equation 1. Table II shows the MVD values of some radiopharmaceutical kits based on Equation 1 (USP 35-NF 30 online version, 2012). The mechanism of gel formation in bacterial endotoxin test is shown in Figure 2.

$$MVD = \frac{(Endotoxin\ Limit\ x\ Concentration\ of\ sample\ solution)}{\lambda} \quad (1)$$

In general, the radiopharmaceuticals labeled Tc-99m have a standard acceptability of endotoxin 175/V EU/mL (International Pharmacopoeia 8th Ed, online version, 2018). In the hospital, the dose of radiopharmaceutical kit is a single dose, so the sample concentration should be one. In addition, the TAL reagent used has a sensitivity of 0.25 EU/mL, so if included in Equation 1, the MVD value to be obtained is 680 times. This indicates if dilution is more than 680 times, the data to be obtained is invalid.

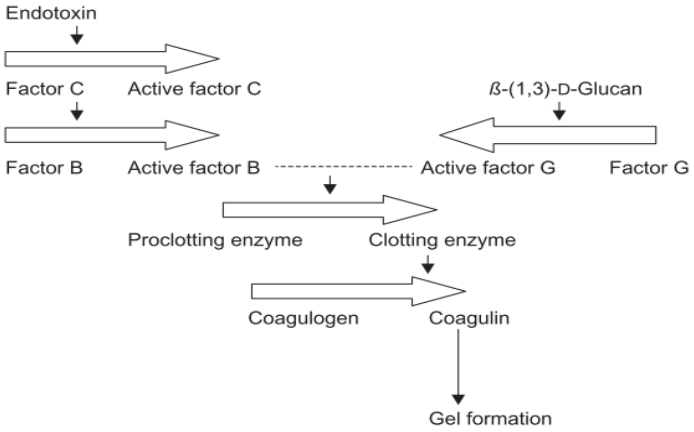

Figure 2. The reaction of gel formation in testing of endotoxin bacteria (Sandle, 2016)

The test sequence on the endotoxin assay should include solution A for the sample, solution B for positive product control (PPC), solution C for positive water control (PWC), and solution D for negative water control (NWC). The compositions of each sample solution are shown in Table III.

Table II. The Maximum Valid Dilution of MAA, tetrofosmin, and ethambutol

Product	Endotoxin limit	λ	MVD
MAA	175/V EU/mL	0.25 EU/mL	680
Tetrofosmin	175/V EU/mL	0.25 EU/mL	680
Ethambutol	175/V EU/mL	0.25 EU/mL	680

Pyrogen test using Rabbit

Pyrogen test using rabbit experimental animals was approved by the ethics commission for the use and maintenance of experimental animals – BATAN with approval number: 001/KEPPHP-BATAN/IV/2016. Test were done in a separate chamber specific to the pyrogen test and with the same environmental conditions as the maintenance room, free of noise that might cause anxiety. In this test, three rabbits were used in one group. Rabbits were not fed during the testing time. Temperature measurement used a calibrated thermometer inserted into the rabbit's rectum. The thermometer probe is kept inside the rabbit's rectum, while holding the rabbit with a neck fitting that allows the rabbit to take a natural resting position. No more than 30 minutes before the injection of the test solution, the "initial temperature" of each rabbit was taken which is the basis for determining the temperature rise. The temperature difference of each rabbit in one group should not exceed 1°C and the initial

temperature of each rabbit should not exceed 39.8°C. Unless otherwise stated in each monograph, the next step involved injecting the solution as much as 1.0 mL, through the ear vein of the rabbit and the injection takes about 5 minutes. The test solution is a preparation which is constituted as shown in each monograph and is injected with the dose as indicated. For the pyrogen test of the injection device or apparatus a washing test solution was used or rinse from the surface of the apparatus which is directly related to the parenteral preparation, site of injection or tissue of the patient. All solutions should be free of contamination. After warming the solution at 37+2°C before injection the temperature was recorded consecutively between 1 and 3 hours after injection with a specific time interval. If no rabbit shows a temperature rise of 0.5°C or more above the temperature of each control, the product meets the requirements for the pyrogen-free (Silva *et al.*, 2016).

Table III. Preparation of solutions for the bacteria endotoxin test

Solution	Endotoxin concentration/ Solution to which endotoxin is added	Number of replicates
A (sample)	None/ 0.2 mL diluted sample solution	2
B (PPC)	0.1 mL 2λ/ 0.1 mL diluted sample solution	2
C (PWC)	0.1 mL 2λ/ 0.1 mL BET water	2
D (NWC)	None/ 0.2 mL BET water	2

RESULTS AND DISCUSSION

Testing of endotoxin bacteria using the tachypleus amoebocyte lysate (TAL) has become one of the alternative methods to quickly identify the number of endotoxin agents in pharmaceutical preparations. Before using a TAL kit for testing, it is important to verify the TAL kit used. This verification test to verify whether the TAL reagent used in accordance with the specification is listed. Generally, in the market there are several concentrations of TAL kits namely reagent TAL 0.25 EU/mL, 0.125 EU/mL, 0.062 EU/mL, 0.031 EU/mL. In this research used TAL kit with sensitivity 0.25 EU/mL. The verification test results of the TAL 0.25 EU/mL kit are shown in Table IV.

Table IV. Results of Verification Test for TAL 0.25 EU/mL

The concentration of Bacteria Endotoxin	Results
2 λ / 0.5 EU/mL	**(+) (+) (+) (+)**
1 λ / 0.25 EU/mL	**(+) (+) (+) (+)**
1/2 λ / 0.125 EU/mL	**(-) (-) (-) (-)**
1/4 λ / 0.062 EU/mL	**(-) (-) (-) (-)**
NWC	**(-) (-)**

The results of the confirmation test in Table IV are shown in accordance with the sensitivity of the TAL used. The endotoxin bacteria concentrations of 0.25 and 0.5 EU/mL showed a positive sensitivity whereas concentrations of 0.125 and 0.062 EU/mL were negative. The negative water control (BET water) showed negative. These results indicate that the sensitivity of TAL reagents tested for verification in accordance with the specification is 0.25 EU/mL. After the verification test was done, the next step was to test the inhibition/enhancement of the product to be tested. This test aimed to determine whether the chemical composition of the product to be measured inhibits or induces gel formation in the TAL reagent. The results of inhibition/enhancement test are shown in Table V.

Table V. Results of the Inhibition / Enhancement test for MAA, tetrofosmin, and ethambutol kits

Solution	MAA					Tetrofosmin					Ethambutol					Requirement
	1	2	3	4	Conc.	1	2	3	4	Conc.	1	2	3	4	Conc.	
A	-	-	-	-	**(-)**	-	-	-	-	**(-)**	-	-	-	-	**(-)**	All negative (-)
B1	+	+	+	+	**(+)**	+	+	+	+	**(+)**	+	+	+	+	**(+)**	All positive (+)
B2	+	+	+	+	**(+)**	+	+	+	+	**(+)**	+	+	+	-	**(+)**	Min. 1 positive (+)
B3	+	-	+	+	**(-)**	+	+	-	+	**(-)**	-	-	-	-	**(-)**	Min. 1 negative (-)
B4	-	-	-	-	**(-)**	-	-	-	-	**(-)**	-	-	-	-	**(-)**	All negative (-)
C1	+	+			**(+)**	+	+			**(+)**	+	+			**(+)**	All positive (+)
C2	+	+			**(+)**	+	+			**(+)**	+	+			**(+)**	Min. 1 positive (+)
C3	-	-			**(-)**	-	-			**(-)**	-	-			**(-)**	Min. 1 negative (-)
C4	-	-			**(-)**	-	-			**(-)**	-	-			**(-)**	All negative (-)
D	-	-			**(-)**	-	-			**(-)**	-	-			**(-)**	All negative (-)

The A solution containing samples of MAA, tetrofosmin, and ethambutol and TAL reagents showed negative results. The B1 and B2 solutions contain samples and BE 2λ and 1λ served to see if the sample can inhibit gel formation. The results in B1 and B2 solutions were positive. Although there is a negative on one replica in sample B2 ethambutol, to get the conclusion at least one positive is needed so it remains concluded positive in solution B2. This result was confirmed by endotoxin concentration 2λ and 1λ plus BET water solution of C1 and C2 which also showed a positive. The D solution containing only BET water showed a negative.

In B3 and B4 solutions the result should show a negative because it contains samples plus endotoxin concentration 1/2λ and 1/4λ. In addition to the results of the B3 and B4 solutions they serve to show whether the sample can induce gel formation in the TAL reagent. The results of MAA, tetrofosmin, and ethambutol samples showed negative. Although in MAA and tetrofosmin samples, three replicas in B3 showed positive but to get a conclusion at B3 that at least one replica is needed to show negative result hence it can be concluded the solution is negative. In comparison, C3 and C4 solutions containing endotoxin concentration 1/2λ and 1/4λ plus BET water showed negative.

The TAL verification test according to specification and inhibition/enhancement tests did not show result contrary to the requirements. Once the entire above test was done and the solution was qualified for acceptance then the next step is to test the sample in accordance with the MVD of each sample. In general, the endotoxin limit of radiopharmaceutical preparations is 175 EU/V. If the value is inserted in Equation 1 then the MVD obtained from the sample of MAA, tetrofosmin, and ethambutol is 680 times. This MVD is the maximum suggested dilution but dilution below this value does not cause problems. However, if dilution was done above of the MVD value then a possible wrong result could be found.

The pyrogen test was performed as a comparison in the sample determination. Although the pyrogen test results can show whether the samples cause heat, it still has some disadvantages. However, pyrogen tests using rabbits are difficult due to their size, and also the test results are affected by the environment or weather. Since the testing time is about 3 hours long, it requires a qualified analyst to make the injections and accurately measure the temperature. On the other side, the BET using TAL has several advantages because it is efficient and can be done in about one-hour testing time, while the test results are easy to interpret, and the tool or testing process is easy to do.

The sample of MAA, tetrofosmin, ethambutol kits were randomized on three batches. One of the requirements of the gel clot reaction is the range pH around 6.0 – 8.0. The BET consists of three batch samples, from PPC, PWC, and NWC solutions. The results of MAA, tetrofosmin, ethambutol using BET and pyrogen test are shown in Table VI.

Table VI. Comparison bacteria endotoxin test using TAL reagent between pyrogenicity test using rabbit for radiopharmaceutical kits (MAA, tetrofosmin, and ethambutol)

Radiopharmaceutical	BET test					Pyrogenicity test
	pH	Sample	PPC	PWC	NWC	
Kit MAA						
Batch 1	6.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Batch 2	6.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Batch 3	6.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Conc.		**(-)**	**(+)**	**(+)**	**(-)**	**(-)**
Kit Tetrofosmin						
Batch 1	7.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Batch 2	7.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Batch 3	7.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Conc.		**(-)**	**(+)**	**(+)**	**(-)**	**(-)**
Kit Ethambutol						
Batch 1	8.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Batch 2	8.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Batch 3	8.0	(-) (-)	(+) (+)	(+) (+)	(-) (-)	(-) (-) (-)
Conc.		**(-)**	**(+)**	**(+)**	**(-)**	**(-)**

Table VI shows the endotoxin test results on MAA, tetrofosmin, and ethambutol kits of three batches. The effective pH required for endotoxin testing using TAL is in the range 6.0 - 8.0 [15]. MAA samples have a pH of 6.0, tetrofosmin of 7.0, while the highest ethambutol of 8.0. The endotoxin test results for MAA, tetrofosmin, and ethambutol samples showed negative, while PPC containing samples plus endotoxin concentration 2λ showed positive, PWC containing endotoxin concentration 2λ plus BET water showed positive and NWC containing BET water showed negative. The results of endotoxin testing using TAL reagents 0.25 EU/mL showed that samples of MAA, tetrofosmin, and ethambutol contained no endotoxin over 0.25 EU/mL (< 0.25 EU/mL).

The MAA, tetrofosmin and ethambutol samples were tested using the rabbit pyrogen test. Table VI shows the results of the pyrogen test in each replica of the test in MAA, tetrofosmin, and ethambutol samples were negative. This result can be inferred negative because there is no single rabbit that indicated a temperature increase of 0.5 oC or more. The result of endotoxin testing showed that the value of less than 0.25 EU/mL was in accordance with the pyrogen test results showing that all MAA, tetrofosmin and ethambutol products did not cause fever in rabbits.

CONCLUSION

Based on the results of the TAL reagent verification tests, all samples showed values corresponding to the standards of 2λ = (+), 1λ = (+), 1/2λ = (-), 1/4λ = (-), and NWC = (-). Furthermore, inhibition/enhancement tests for MAA, tetrofosmin, and ethambutol products demonstrated to be non-inhibiting or gel-inducing, and these results are in accordance with acceptability standards so that the samples can be tested using TAL reagents. The results of MAA, tetrofosmin, and ethambutol product testing on three batches with pH according to test range (6.0 - 8.0) were a sample = (-), PPC = (+), PWC = (+), and NWC = (-). The same results were obtained from the pyrogen test using rabbits.

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FORMULATION OF CHLORPHENIRAMINE MALEATE TABLETS USING CO-PROCESSED EXCIPIENT AS A FILLER AND BINDER

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ABSTRACT

Co-Processed Excipient (CPE) is technological innovation for tablet preparation through the direct compression method with a quick and straight forward manufacturing process because it improves the compressibility and flowability. This research aimed to formulate and evaluate of chlorpheniramine maleate tablets using spray dried CPE as filler and binder. The spray dried CPE containing MCC PH 101, and Kollidon® K30 was made into tablets through a direct compression method. Meanwhile, Ludipress® and Avicel® PH 102 were used as filler-binder comparators. All the prepared tablet formulations were then evaluated for weight variation, hardness, friability, disintegration time, content uniformity of active ingredient, and dissolution test. The physical properties of tablets with CPE as a filler and binder produced an average weight of 151.65 ± 1.53 mg, 5.92 ± 0.38 kg of hardness, 0.06 ± 0.051% friability, 520.00 ± 2.00 seconds of disintegration time, and 99.24 ± 0.15% content uniformity of active ingredient. The comparators indicated better disintegration time than CPE ($p<0.05$), while the dissolution test showed that more than 80% (Q) of the amount of active ingredient was dissolved in 30 minutes. CPE could be successfully used to prepare tablet dosage form, and the tablets had fulfilled the standards of pharmacopoeia.

Keywords: Avicel® PH 102; chlorpheniramine maleate; co-processed excipient; Kollidon® K30

INTRODUCTION

As the most widely used dosage form, oral administration, including tablets, in particular, accounts for 70-80% of any pharmaceutical preparations mainly because of its manufacturing simplicity, dose accuracy, and high level of patient compliance (Syukri *et al*., 2015). Meanwhile, despite the well-known significance of excipients for the success of pharmaceutical products, there has been only a minor development of new excipients. They are rarely introduced to the market probably because discovering novel excipients is challenging or due to the modest profit. Novel excipients can be produced from one of the following routes: new chemical entities developed as further excipients, new grades of existing excipients, or new combinations of existing excipients (Wang *et al*., 2015).

The poor mechanical properties of active ingredients in a high dose make tabletting difficulty in tablet production, mostly forcing formulators to apply granulation techniques that provide appropriate compression properties of drug-excipient agglomerates. Direct compression (DC), however, is a preferable tablet manufacturing method due to its simplicity, rapidity, and cost-effectiveness (Al-Zoubi, Odeh and Nikolakakis, 2017). A DC process requires appropriate diluents with good flowability and compaction properties, and formerly, single-component excipients were physically mixed to obtain such diluents. Today, the basic DC process has even been successfully simplified by the innovative development of co-processed excipients (Aljaberi *et al*., 2013).

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The new technology of co-processed excipients can meet the ever-increasing demand for excipients with multiple functions in DC tabletting obtained by including one excipient in the particle structure of another employing hot-melt extrusion, co-drying, co-precipitation, or freeze-thawing. In co-processing, the interaction between excipients occurs at a sub-particle level to maintain or develop desirable properties, improve functionality, and conceal undesired properties of each component. The characteristics and tabletting properties of co-processed excipients with multiple functions are superior to those of a single substance or physically-mixed excipients concerning compatibility, intrinsic flow, lubricating efficiency, binding properties, and blending properties (Rojas and Kumar, 2011). Meanwhile, as an alternative to preparations of co-processed compressible powder mixture, the spray drying method is reportedly able to improve the compression properties of hypromellose and α-lactose monohydrate as a binder (Mužíková *et al*., 2014), with erythritol, mannitol, various maltodextrins, crospovidone, colloidal silicon dioxide, polyoxyethylene 20 sorbitan monooleate (Gonnissen *et al*., 2008), HPMC, lactose, and PVPP (Wang *et al*., 2015).

Prior to this current research, a study of MCC PH 101, lactose, and Kollidon® K30 as co-processed excipients (CPE) in spray drying was conducted to examine their compressibility as a filler and binder. The findings showed that spray-dried MCC PH 101, lactose, and Kollidon® K30 could serve as an alternative filler and binder in a direct compression process, but the best CPE consisted of MCC PH 101 and Kollidon® K30 only. The study showed that the optimum CPE had lower tapping index and higher hardness than the physical mixture. It indicates that the optimum CPE had good flowability and compatibility characters. Then, the optimum CPE also reported no chemical changes following the characterization through infrared spectrophotometer (IR), scanning electron microscope (SEM), differential scanning calorimetry (DSC), and X-ray diffraction (XRD) (Kusuma *et al*., 2017).

This research involved chlorpheniramine maleate as the drug model, the first generation of alkylamine antihistamines commonly used to treat symptoms of allergies, such as rhinitis and urticaria(Lashkarbolooki *et al*., 2013). The appropriate method for the preparation of low-dose chlorpheniramine maleate is the direct compression. While pharmacists and pharmaceutical companies are seeking new approaches to improve drug products, they also focus more on exploring and enhancing excipients with better physicochemical properties (Eraga *et al*., 2015). Therefore, excipient compressibility, flowability, and carrying capacity in tabletting should be taken into consideration (Dave *et al*., 2017).

This study aimed to formulate tablets of chlorpheniramine maleate through direct compression with CPE as the filler-binder. The tablets would then be evaluated for the fulfilment of requirements from the Indonesian Pharmacopeia to control the quality of pharmaceutical preparations.

METHODS

Materials

Chlorpheniramine maleate was manufactured by Brataco Ltd. Company Indonesia, Kollidon® K30 was the product of Hangzhou Nanhang, and microcrystalline cellulose (MCC PH 101) and Avicel® PH 102 came from Asahi Kasei Chemicals. Ludipress® was obtained from BASF Indonesia, and primogel, magnesium stearate, as well as aerosil were produced by Brataco Ltd. Company Indonesia. All the materials were of pharmacopeial grades, and other solvents were of analytical grades.

CPE Preparation

A 100-g mixture of MCC PH 101 (79.63%) and Kollidon® K30 (20.37%) was suspended in 1000 ml of water to obtain a suspension of 10% w/v co-processed excipient. A spray dryer (The BUCHI Mini Spray Dryer B-290) with 1-mm nozzle was used to suck the suspension. The constant spray drying parameters consisted of 120°C inlet temperature, 4 ml/min suction speed, and 3 Bars of pump pressure. The obtained powder

was then dried in an oven at 50°C for 24 hours. Physical properties evaluation and characterization of CPE optimum proportions have been reported previously (Jacob *et al*., 2007; Kusuma *et al*., 2017).

Tablet Preparation

Tablets were prepared from 4 mg of chlorpheniramine maleate with the various proportions of each filler and binder (CPE, Avicel® PH 102 and Ludipress®), disintegrant (Primogel), lubricant (magnesium stearate) and glidant (Aerosil) to attain 150 mg of the total quantity. All of the materials were homogeneously mixed, then followed by compression into a tablet with direct compression method by using the single-punch tablet compression machine (Korsh EK 0). The machine was set to produce diameters, thickness, hardness and weight in the same conditions. Table I shows the composition of three different tablet formulations.

Table I. Formulations of chlorpheniramine maleate tablets with CPE (quantity for a single tablet)

Ingredient	Amount (mg)		
	F1	F2	F3
Chlorpheniramine maleate	4	4	4
CPE	129.5	-	-
Avicel® PH 102	-	129.5	-
Ludipress®	-	-	129.5
Primogel	12	12	12
Magnesium stearate	3	3	3
Aerosil	1.5	1.5	1.5
Total quantity	**150**	**150**	**150**

Tablet Evaluation

The parameters of tablet evaluation included hardness, friability, weight variation, and disintegration time. Weight average was tested according to the Indonesian Pharmacopeia from the measurement of the weight of each tablet (20 tablets in total) (Anonim, 1995), followed by determining the weight variation with the formula: the standard deviation is divided by the average weight and multiplied by 100. Ten tablets were tested for their hardness using a hardness tester (Erweka TBH 125). To determine the percentage of friability, ten tablets were weighed and then rotated at 25 rpm for 4 minutes in a friability tester followed by the calculation of the total remaining weight. A disintegration tester (Erweka ZT 502) was used to individually determine the disintegration time of six tablets per formulation with aquadest at 37±0.5oC followed by the calculation of the mean disintegration time. The United States Pharmacopeia was referred for the content uniformity assessment. Ten tablets were tested to determine whether the concentration of active ingredient in each tablet ranged between 93% and 107% of the label claim; if so, the USP requirement would be claimed fulfilled by the batch (Anonim, 2014; United States Pharmacopeial Convention, 2014).

In-vitro Tablet Dissolution

The paddle stirrer of dissolution equipment (Erweka DT 708) was set in a dissolution medium (900 ml of pH 7.4 phosphate buffer) rotating at 75 rpm and a maintained temperature of 37±0.5^{o}C. Then, using a fitted pre-filter syringe, 5 ml dissolution medium sample was taken at specific time intervals, and the drug release was analyzed based on the absorbance measured at 262 nm. The current quantity of dissolution medium was used to replace the volume taken at each interval of time, and then the calculation and time plotting of the

percentage of released chlorpheniramine maleate were performed (Anonim, 2014; United States Pharmacopeial Convention, 2014).

RESULTS AND DISCUSSION

Tablet Evaluation

Table II shows the evaluation results for chlorpheniramine maleate tablets with different fillers-binders. The parameters include hardness, weight variation, disintegration time, active-ingredient content uniformity, and friability in formulations containing CPE (F1), Avicel® PH 102 (F2), and Ludipress® (F3).

Table II. Results of chlorpheniramine-maleate tablet evaluation with various fillers and binders

Evaluation	F1	F2	F3
Weight variation (mg)	151.65 ± 1.53	151.55 ± 0.6	150.05 ±0.6
Weight variation, CV (%)	0.4	1.01	0.4
Diameter (mm)	7.04 ± 0,00	7.09 ± 0.00	7.07 ± 0.00
Thickness (mm)	3.48 ± 0.02	3.77 ± 0.01	2.93 ± 0.04
Hardness (kg)	5.92 ± 0.38	6.57 ± 0.35	4.73 ± 1.11
Friability (%)	0.06 ± 0.051	0.09 ± 0,033	0.09 ± 0.07
Disintegration time (sec)	520.00 ± 2.00	15.67 ± 2.08	131.33 ± 6.43
Content uniformity (%)	99.24 ± 0.15	97.88 ± 0.10	98.87 ± 0.17

Weight variation is evaluated to guarantee the right amount of active ingredient in each of the tablets. The weights variations of each formulation with optimum CPE (F1), Avicel® PH 102 (F2), and Ludipress® (F3) were 0.4%; 1.01%; and 0.4%, respectively, indicating a small variation. All of the formulations had <1% friability with the longest disintegration time in the formulation containing CPE. As the time remained below 900 seconds, the formulations were considered meeting the criteria determined in the Indonesian Pharmacopeia (Anonim, 1995).

Weight variation is affected by flow properties and equipment conditions during the study. In addition, SEM analysis showed that Kollidon® K30 was invisible as it had enveloped Avicel® PH 102 (Kusuma *et al*., 2017), thereby enhancing the flow properties of the excipients. In evaluating the uniformity of tablet size, the parameters evaluated were tablet diameter and thickness. The results of the uniformity evaluation of the size were F1 with a diameter of 7.04 mm and 3.48 mm thick; F2 with a diameter of 7.07 mm and a thickness of 3.77 mm and F3 with a diameter of 7.09 mm and thickness of 2.93 mm. Factors that can influence uniformity in size are flow velocity, mixture homogeneity, and punch press stability. This uniformity of size is related to the die diameter used and the amount of powder entering the die. A good size criterion is: if a tablet has a diameter of not less than 1 1/3 and is not more than 3 times the thickness of the tablet (Anonim, 1979).

In addition, tablets should be strong enough to withstand mechanical shocks while they are manufactured, packaged, shipped, and dispensed, which can be fulfilled when the hardness falls between 4 and 8 kg with <1% friability (United States Pharmacopeial Convention, 2014). Table II indicates that the hardness and friability of all of the formulated tablets have met the mechanical property criteria. The investigation could be clarified that MCC showed low elastic recovery and good compressibility. This condition will reduce tablet failures, include sticking, capping, lamination and binding (Osamura *et al*., 2016).

According to the Pharmacopeia, 1% maximum mass loss or 0.8% - 1.0% weight loss should be fulfilled by tablets tested for

crushing strength without laminating, capping, or breaking during the test. Tablet crushing strength can greatly influence the rate of drug release. The increased crushing strength of a tablet generally means the decreased rate of drug release because of reduced porosity of the tablet (Eraga *et al*., 2015). Differences in the profiles of drug release are likely caused by the changes in crushing strength experienced by the tablet batches studied (Komersová *et al*., 2016).

Evaluation should also be done for the disintegration time, which is the time required by a tablet to completely break down and penetrate the basket mesh in a disintegration test. Such tests represent the process of a tablet breaking down into particles in the gastrointestinal tract, which, according to the Indonesian Pharmacopeia, should last no longer than 15 minutes for an uncoated tablet (Anonim, 2014), and the tablet disintegration time in this research has fulfilled the requirement.

Tablets containing optimum CPE as the filler-binder are influenced by their hardness and friability as well as the nature of Kollidon® K30 as the CPE component. Such component will turn into gel when it interacts with water, thus trapping other components and preventing them from dissolving, which consequently results in longer disintegration time. Meanwhile, tablets with Avicel® PH 102 as the filler-binder have shorter disintegration time because such component has multiple functions as a filler, binder, and disintegrant.

Content uniformity test is conducted to ensure the fulfilment of the standard for active ingredient concentration in a tablet, which will influence the results of a therapy. For chlorpheniramine maleate tablets, the concentration of active ingredient should range from 93% to 107% (Anonim, 2014). Both powder homogeneity and weight uniformity affect the concentration of active ingredient in tablets. This study found that the concentrations of chlorpheniramine maleate in Formulation 1, 2, and 3 reached 97.88%, 99%, and 98.87%, respectively.

In-vitro Tablet Dissolution

To determine the concentration of active ingredient dissolved in a medium, a dissolution test is performed in vitro. Tablet dissolution is strongly influenced by the disintegration time, which will also affect the bioavailability. Figure 1 shows the dissolution profile of chlorpheniramine-maleate tablets with CPE (F1), Avicel® PH 102 (F2), and Ludipress® (F3) as the fillers-binders.

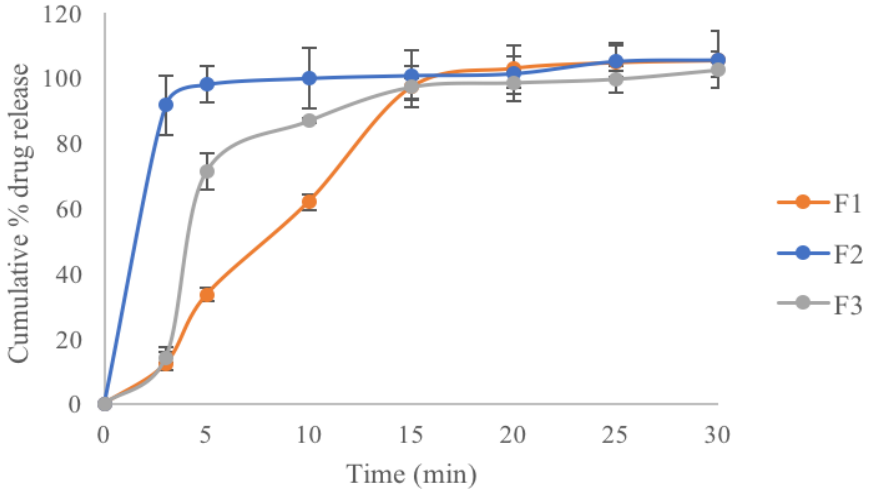

Figure 1. Dissolution characteristics of chlorpheniramine maleate tablets containing different fillers and binders (n = 6)

The formulation with CPE as the filler-binder has 80% dissolved active ingredient with longer disintegration time compared to the other formulations. The use of Kollidon® K30 as a component of CPE in tablets affects the disintegration time since such component naturally forms a gel mass that obstructs the release of the active ingredient. However, all

of the formulations in this research had met the dissolution criteria defined by the Indonesia Pharmacopoeia (Anonim, 2014).

This finding is also supported by previous research, which showed that HPMC co-processed filler prepared by fluid bed coating and spray drying has the potential to be developed as a filler and binder in direct compression method (Dong *et al*., 2018). Co-processed excipients also facilitate direct compression of orally disintegrating tablets. Multifunctional excipients exhibited more dominant impact on the investigated tablet properties, especially for tablet disintegration properties (Drašković *et al*., 2018).

CONCLUSION

The CPE comprising MCC PH 101 and Kollidon® K30 as a filler and binder in tablet formulation can improve the compressibility of chlorpheniramine maleate tablets prepared using the direct compression method. The physical properties of a tablet with CPE as the filler-binder were 0.4 weight variation, 5.92±0.38 kg hardness, 0.06±0.051% friability, 520.00±2.00 seconds of disintegration time, and 99.24±0.15% content uniformity of active ingredient. Therefore, the tablets had fulfilled the standards of pharmacopoeia.

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PROFILE OF LYCOPENE UV-SPECTRA OF LACTIC ACID BACTERIA FERMENTED MILK-TOMATO PASTE

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ABSTRACT

Lycopene is one of the famous ingredients in tomatoes, because of its important role as a potent anti-oxidant. Besides being consumed as fresh fruit, tomato paste can be combined with probiotic fermented milk to increase its health benefits. Probiotics are lactic acid bacteria (LAB), which when consumed in sufficient quantities will benefit health. Probiotic fermented milk is reported to have various properties including anti-microbial, antioxidant, immunomodulatory and anti-cancer, so the combination with tomato paste is expected to increase its benefits for health. This research aimed to study the effect of probiotic fermented milk on the content of tomato paste lycopene. Lactobacillus acidophillus, Lactobacillus bulgaricus, and Lactobacillus casei were used as mixed probiotic cultures. It was found that at the ratio of 2:8 for the LAB fermented milk and tomato paste, the highest lycopene drive was obtained by spectrophotometric measurement at 663, 645, 505, and 453 nm. Determination of the lycopene content was performed according to the Nagata and Yamashita simple methods. In addition, the lycopene content in the LAB fermented milk-tomato paste was greater than the content in the tomato paste without fermented milk addition and was tested stable after storage for 14 days.

Keywords: antioxidant; lycopene; probiotic fermented milk; tomato paste

INTRODUCTION

Yogurt is a product that is obtained from pasteurized milk and then fermented with bacteria to obtain a characteristic acidity, odor and taste with or without the addition of other permissible ingredients (Anonim, 2009). *Lactobacillus spp.* are the most predominate probiotic bacteria, and some of them are commonly used in making yogurt such as *Lactobacillus bulgaricus*, *Lactobacillus acidophillus*, *Lactobacills casei*, and *Streptococcus thermophillus*. Lactic acid bacteria (LAB) are the most important probiotics because of its beneficial effects on the human digestive tract or other activities, such as immunomodulatory (Isnaeni, 2016). Rajarajan *et al.* (2018) evaluated antimicrobial activity and probiotic potential of *Lactobacillus* strains against some human pathogens. Isnaeni and Mertaniasih (2015) reported the growth inhibitory activities of mixed culture LAB against MRSA and ESBL. Inhibitory activity of LAB fermented milk and tomato juice combination against *Escherichia coli* and *Staphylococcus aureus* has been reported (Hidayah, 2014). Wang *et al.* (2017) reviewed antioxidant activity of the probiotic, although its mechnisms of action have not been completely understood.

Various food matrices have been developed with probiotics and were reported. With different technologies such as microencapsulation, nanoparticle (Sugiyartono, 2014; Sholeha *et al.*, 2014) and continuous fermentation, the probiotic will become an important ingredient in the functional foods (Soccol *et al.*, 2010). Yogurt product is one of the popular probiotic fermentation foods, found on the market in various forms including food, drinks and ice cream. The yogurt products are very popular in countries with four seasons, but in

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Indonesia the fermented milk containing LAB has also been popular, especially in cold climate cities with surplus milk dairy products. Yogurt preparations are often combined with fruits such as soursop, mangoes, avocados, durians, oranges, and others. Various tomato-yogurt combinations include yogurt curry tomatoes, tomato yogurt sauce, yogurt smoothie tomatoes, and salad tomatoes with chili yogurt and even for cosmetic preparations as a tomato face mask. Kaur *et al.* (2016) studied fermentation of tomato juice by probiotic LAB. They demonstrated that *Lactobacillus acidophylus*, *Lactobacillus plantarum*, and *Lactobacillus casei* probiotics showing the ability to use the tomato juice as raw material to increse viability count.

Tomatoes (*Lycopersicon esculentum*) and its products contain lycopene, predominant carotenoid (5.7-26.3mg/kg) and an antioxidant which plays an important role in health and disease reduction (Erge and Karadeniz, 2011). The bioactive compounds in tomato fruit are carotenoid, phenolic and L-ascorbic acid; which act as antioxidants, which has a protective effect against various forms of cancer and cardiovascular diseases (Kopsel *et al.*, 2006). There are various manners of tomato processing to gain several kinds of tomato products, such as tomato juice, tomato paste, tomato boiled sauce, tomato ketchup with different specifications and lycopene content (Alda *et al.*, 2009). The lycopene content in tomato paste is higher than fresh tomatoes. Lycopene contents of fresh, sun-dried and canned tomatoes were found to be 1.74, 5.51 and 3.55 mg/100g, showing different antioxidant activities (Karakaya and Yilmaz, 2007).

This research was a breakthrough to make Nutraceutical food from tomatoes products combined with LAB fermented milk derived from LAB probiotics mixed culture (*Lactobacillus acidophilus, Lactobacillus bulgaricus,* and *Lactobacillus casei*). A nutraceutical is a food or part of food that provides health benefits including prevention and treatment of diseases. From this definition, nutraceuticals can be in the form of isolated nutrients, food supplements, herbal products or genetic engineering, or processed foods such as cereals, soups and drinks (Kaira, 2003).

Tomato-yogurt neutraceutical products were made by optimization of LAB probiotic fermented milk and tomato paste ratio to find the optimal composition with the highest lycopene content. The optimization was done by trying several compositions of fermented milk and tomato paste, which were then analyzed for their lycopene content using spectrophotometry method according to Nagata and Yamashita (1992).

METHODS

Material and chemicals

The tomatoes used were Lycopersicum esculentum or tomato fruit, obtained from the Karang Menjangan Market in Surabaya in one-time purchase to obtain a uniformity of the tomatoes condition. Based on the results of determination mophologically by Herbarium Malangiensis, tomatoes fruit used in this study (Figure 1) included the family Solanaceae, genus Lycopersicum, species of *Lycopersicon esculentum* Mill (Pratiwi, 2014). Dairy milk was obtained from the Jemursari dairy cow, Surabaya, Indonesia. The probiotic fermented milk was obtained from plain yogurt containing LAB *Lactobacillus Acidophillus, Lactobacillus bulgaricus*, and *Lactobacillus casei* production of Microbiology Practical Room Faculty of Pharmacy, Universitas Airlangga. N-hexane and acetone p.a (Merck) were used as solvents.

Figure 1. Tomato fruits

Preparation of probiotic fermented milk-tomato paste

The tomatoes fruits used in this research were specifically selected to be red both in outside and inside color, round shape with diameter of 7-10 cm, juicy texture and sour-sweet taste. As much as 2 kg of tomato fruits

were washed, set aside to dry, and steamed for 10 minutes at ± 110^{o}C. The tomato rind was removed and the fruit was crushed with a blender. Tomato juice was made by the same manner without steaming. Yogurt or probiotic fermented milk was prepared by mixing 1 liter of pasteurized fresh cow's milk (90^{o}C, 15 minutes) with 25 mL plain yogurt containing a combination of LAB *Lactobacillus bulgaricus*, *Lactobacillus casei*, and *Lactobacillus acidophilus* 106-107 CFU/mL at 43^{o}C. The mixture was left to stand for 24 hours at room temperature (30^{o}C). Characteristics of the probiotic fermented milk were observed based on the acid pH and viscous texture. A mixture of the fermented milk-tomato pasta was made in 9 compositions (g/g), 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9. Each combination was blended for homogenization.

Extraction of lycopene

Approximately 1 gram of each tomato juice, tomato paste, and fermented milk-tomato pasta at all composition were extracted with 8 mL acetone:n-hexane (4:6), then centrifuged 3000 x g for 10 minutes at 4^{o}C. The organic phase was separated after standing for 10 minutes and adding by 1 mL water. The organic phase was added by n-hexane up to 10 mL and then dilution was done twice, before measurement by a spectrophotometer.

Qualitatively and quantitatively analysis of lycopene

The dilution results were measured quantitatively by spectrophotometer Agilent Technologies Cary 60 UV-Vis at wavelengths of 300-700 nm. Qualitative analysis of lycopene content was carried out by determination of the UV-Vis spectra profile of lycopene in a sample of tomato paste and probiotic fermented milk-tomato paste extract at 300-700 nm. Furthermore, quantitative analysis was done by measuring the absorbance at 663, 645, 505, and 453 nm using acetone-n-hexane (4:6) blank. The probiotic fermented milk extract in acetone-n-hexane (4:6) was used as a blank for probiotic fermented milk-tomato paste analysis, in order to evaluate the effect of the probiotic fermented milk addition to lycopene in pasta or tomato juice. The lycopene levels were calculated using the formula proposed by Nagata and Yamashita (1992) as follows:

$$[\text{lycopene}](\mu g/mL) = -0.0458 \times A663 + 0.2040 \times A645 + 0.3720 \times A505 - 0.0806 \times A453$$

A663, A645, A505, A453, were absorbance at 663, 645, 505, and 453 nm, respectively.

RESULTS AND DISCUSSION

The studies by Bunghez *et al.* (2011) and Alda *et al.* (2009) on the determination of lycopene in tomato and tomato products reported spectra profile of the lycopene extract from tomatoes samples in hexane indicated that profile of the spectra consisted of three peaks at 340, 245, and 506 nm respectively. Based on the spectrophotometric data analysis, the profiles of tomato paste and probiotic fermented milk-tomato paste were obtained as shown in Figures 3a and 3b. Tomato paste and yogurt-tomato paste showed the same spectra profile measured at wavelengths between 300-700 nm. There were three peaks detected and the maximum wavelength was obtained at 472 nm. This phenomenon indicated that the spectra profile of the tomato pastes was similar to probiotic fermented milk-tomato paste qualitatively. The spectra profile of lycopene extract of Bunghez's and Alda's research's showed the similar profile as spectra extract of tomato paste and probiotic fermented milk-tomato paste. Alpha-carotene and lutein had maximum wavelengths at 420 nm and 444 nm (Bunghez, 2011), while the lycopene has a maximum wavelength of 470 and 500 nm. Comparing with the profile of the probiotic fermented milk-tomato paste spectra, Bunghez's spectrum depicted peaks at 444 nm, 470 nm, and 502 nm with acetone:n-hexane (1:1, v/v) solvents. On the other hands, Suwanaruang (2016) used hexane:ethanol: acetone (2:1:1, v/v/v) as solvent and observed the lycopene extract at 503 nm. In this study, we found three peaks at 446 nm, 472 nm, and 502 nm (Figure 2). Alda *et al.* (2009) measured absorbance of the lycopene in n-

hexane at 472 and 502 nm. It can be seen that there was a shift in the wavelength of 2 nm might be due to differences in solvent composition. Djenni *et. al.* (2010) found that dichloromethane lycopene extract from tomato fruit showed three peaks at 457, 483, and 516 nm in hexane solvent. Absorbance at 483 was selected for determination of the lycopene.

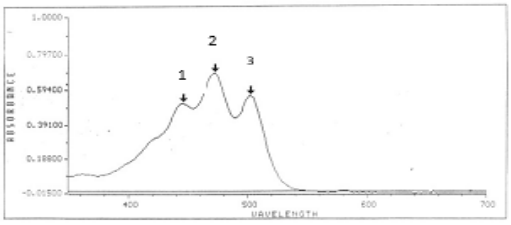

Figure 2. Spectra of yogurt-tomato paste scanned at λ 400-700 nm. (1) 446 nm, (2) 472 nm, (3)502 nm

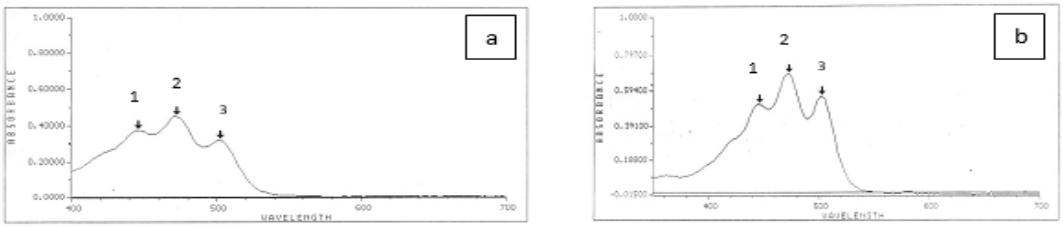

Figure 3. UV-Vis spectra of tomato paste (a) and tomato juice (b) scanned at λ 400-700 nm. (1) 446 nm, (2) 472 nm, (3) 502 nm

Calculations of lycopene levels in tomato juice, tomato paste, and probiotic fermented milk-tomato paste were based on the absorbance values at 663 nm, 645 nm, 505 nm, and 453 nm (Table I and Table II) following the method of Nagata and Yamashita (1992). All the composition of fermented milk and yogurt produced maximum absorption at 505 nm, both on day 1 and 2 weeks after storage. Optimum composition was obtained on day 1 and 2 weeks after storage of 2:8 each then followed by 3:7. Profile of the lycopene levels for day 1 was different compared to spectra profile 2 weeks after storage. Decreased uptake on days 1 and 2 weeks after storage occurred in the ratio of 5:5 and 4:6, respectively, for a combination of fermented milk and tomato paste (Table III, Figure 4).

Table I. Absorbance of Sample Extract (first day)

No.	Sample	Fn1=663nm	Fn2=645nm	Fn3=505nm	Fn4=453nm
1	BLANK	-7.2E-04	-0.00122	-0.00110	-0.00108
2	Y:TP = 9:1	0.06888	0.06761	0.17310	0.15422
3	Y:TP = 8:2	0.08623	0.08516	0.27588	0.24934
4	Y:TP = 7:3	0.12714	0.12656	0.39714	0.35194
5	Y:TP = 6:4	0.16049	0.16457	0.53644	0.50517
6	Y:TP = 5:5	0.06808	0.06873	0.53687	0.46980
7	Y:TP = 4:6	0.09492	0.09697	0.56096	0.50552
8	Y:TP = 3:7	0.15346	0.15494	0.81306	0,71440
9	Y:TP = 2:8	0.15221	0.15500	1.1161	0.98357
10	Y:TP = 1:9	0.11249	0.11507	0.80241	0.76291
11	Tomato juice	-0.02861	-0.03575	0.54292	0.47690
12	Tomato paste	0.00862	0.00345	0.44748	0.48274

Y: TP was Yogurt: tomato paste

It was found that the highest lycopene content was found in probiotic fermented milk-tomato pasta with a composition of 2:8. The second highest content was found in the composition of 3:7. When converted, with the same number of samples, lycopene in probiotic fermented milk-tomato pasta with a ratio of 3:7 and 2:8 is higher than both tomato paste and tomato juice. It was estimated that there was interaction between yogurt and tomato paste; which causes lycopene levels to increase. The LAB might be contributed for

the interaction, although the action mechanism of the interaction could not be understood. The effect of acid (CO_2) on the presence of lycopene has been reported by Helyes *et al.* (2011). The higher the CO_2 concentration the lower the level of lycopene. In this research, the longer storage time, the higher the number of LAB cells, the more acidic the pH, might increase the influence on lycopene content. Combination of LAB fermented milk - tomato paste 3:7 and 2:8 was the optimal composition, resulting in higher lycopene. Further investigation needs to be done to examine the mechanism of the LAB to increase the lycopene levels.

Table II. Absorbance of Sample (after two weeks)

No.	Sample	Fn1=663nm	Fn2=645nm	Fn3=505nm	Fn4=453nm
1	BLANK	7.2E-04	4.3E-04	9.2E-04	8.5E-04
2	Y:TP = 9:1	-0.01102	-0.01251	0.02681	0.02177
3	Y:TP = 8:2	0.06377	0.06558	0.38768	0.36409
4	Y:TP = 7:3	0.11874	0.12413	0.41507	0.40907
5	Y:TP = 6:4	0.02751	0.02632	0.42239	0.36458
6	Y:TP = 5:5	-0.01244	-0.01270	0.33188	0.28586
7	Y:TP = 4:6	0.01268	0.00880	0.18983	0.16942
8	Y:TP = 3:7	0.05418	0.05327	0.45279	0.41934
9	Y:TP = 2:8	0.10034	0.09859	0.63196	0.56865
10	Y:TP = 1:9	-0.04057	-0.04414	0.31941	0.27258

Y:TP was Yogurt: tomato paste

The same results were shown in the observation of LAB fermented milk-tomato paste after storage for 14 days. However, there was a decrease of lycopene content in several compositions after storage. Acidity (pH 4-5.5) and concentration of the LAB fermented milk were critical factors that affected the lycopene concentration in the tomato-paste. At a ratio of LAB fermented milk and tomato pasta 9:1, the lycopene level decreased dramatically after 14 days storage. Alda *et al.* (2009) reported that lycopene content of tomatoes remained high during the multistep processing operations for the production of juice and paste. Kaur *et al.* (2016) found that the fermented tomato juice could be used as a raw material for LAB fermentation. Stability of lycopene against degradation is affected by long heating time or at temperature above 50°C (Lambelet *et al.*, 2009). The lycopene will undergo isomerization under several conditions, such as heat, acids and light (Xianquan *et al.*, 2005). Degradation proceeds faster than isomerization at temperature above 50°C. This phenomenon is very interesting for future investigation to obtain an ideal formula with high lycopene content and antioxidant activities.

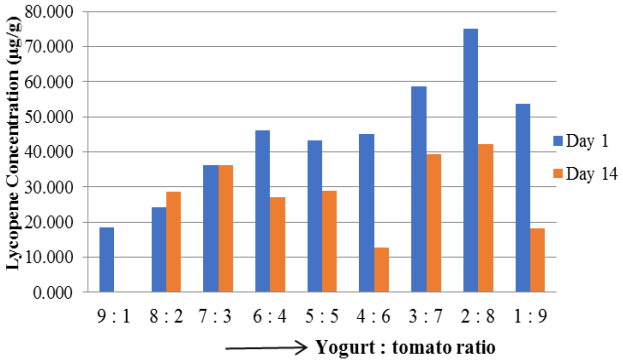

Figure 4. Lycopene content in yogurt-tomato paste

Lycopene content in tomato paste was higher than lycopene content in tomato fruit (Alda *et al.*, 2009), but in this study, lycopene content in tomato juice was higher than tomato paste. Lycopene is known to exist in a variety of isomeric forms, including the all-trans, mono-cis, and poly-cis forms. The all-trans isomer is the predominant geometrical isomer

in fresh tomatoes. All-trans-isomer lycopene ($C_{40}HS_6$) is an acyclic, open chain polyene hydrocarbon with 13 double bonds, of which 11 are conjugated in a linear array, but 7 bonds can isomerize from the trans-form to the mono or poly-cis form under the influence of heat, light, and certain chemical reactions (Su *et al.* 2002).

Table III. Lycopene Content in Yogurt-Tomato

Ratio of Yogurt-Tomato paste	Lycopene content	
	Day-1 (µg/g)	Day-14 (µg/g)
9:1	1.8491	0.1383
8:2	2.4232	2.8751
7:3	3.6174	3.6213
6:4	4.6011	2.7003
5:5	4.3300	2.8937
4:6	4.5090	1.2614
3:7	5.8652	3.9372
2:8	7.4993	4.2116
1:9	5.3730	1.8299

Fresh tomato = 3.7568 µg/g; Tomato paste = 2.8194 µg/g

Takeoka *et al.* (2001) studied processing effects of lycopene content in tomato and found that sample of raw tomatoes and tomato juice after adding hot scalding water and final paste indicated that the lycopene losses (9-28%) during processing into final paste. Isomerization and oxidation would degrade lycopene; by which its bioactivity is reduced. The lycopene oxidation and isomerization of all-trans form to cis form are induced by heat and light. Su *et al.*, (2002) have investigated the effects of thermal treatment and light irradiation on the stability of the lycopene. It has been found that lycopene stability depends on the extent of oxidation and isomerization. Cis-isomers are less stable than trans-isomers, conformed to studies results reported by Gupta *et al.* (2010). The major effect of thermal treatment and light irradiation was a significant decrease in the total lycopene content (Su *et al.* 2002). Honda *et al.* (2017) reported that Z-isomers of lycopene increased by thermal treatment at 120^{o}C and 150^{o}C for 1 hour to 10.0% and 56.2%, respectively.

This research was a preliminary study to obtain the tomato- yogurt paste composition with the most maximal lycopene content. Further investigations are needed to determine the probiotic fermented milk-tomato formula with the most optimal antioxidant activity with lycopene and other component content of the formula. Wang *et al.* (2017) reviewed antioxidant activities showing some LAB, such as *Bifidobacterium animalis*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus*, although the mechanisms have not been completely understood. It was also found that there was a decrease in the amount of lycopene in the probiotic fermented milk-tomato paste after storage for two weeks, therefore in the next study it is necessary to investigate the effect of storage time on the lycopene levels and antioxidant activity of probiotic fermented milk-tomatoes products.

CONCLUSION

The highest content of lycopene was found in probiotic or LAB fermented milk-tomato paste 2:8. The lycopene content was higher than lycopene in tomato paste and tomato juice without addition of the probiotic fermented milk.

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EFFECTS OF PARTICLE SIZE, EXTRACTION TIME, AND SOLVENT ON DAIDZEIN YIELD EXTRACTED FROM TEMPEH

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ABSTRACT

Daidzein, one of the isoflavone aglycones contained in tempeh, has several biological activities such as anti-inflammatory, anti-oxidant, anti-breast cancer, and suppression of expression of matrix metalloprotease-9. As a fermented product from soybeans, daidzein content in tempeh was found in higher concentration compared to the soybean raw material. It was important to optimize several factors affecting extraction process such as particle size of tempeh simplicia, extraction time, and solvent in order to develop an effective method for the daidzein isolation from the tempeh or other natural products. Evaluation of extraction factors was conducted by applying variations for each factor followed by quantitative analysis using HPLC methods. The optimization condition was performed by daidzein standard and achieved with the particle size of tempeh simplicia of 1.2 mm, extraction time of 360 minutes, and 70% ethanol was used as solvent. Furthermore, the optimized condition was applied for the daidzein isolation from tempeh, a soybean fermented product.

Keywords: daidzein; extraction time; particle size; solvent; soybean fermented product

INTRODUCTION

Daidzein, an isoflavone aglycone, was reported as the major compound contained in soybeans (Kuligowski *et al.* 2016). In recent years, pharmacological activities of daidzein were reported such as anti-breast cancer agent (Liu *et al.* 2012, Yuliani *et al.* 2016), cell growth inhibitor (Takaoka *et al.* 2018), anti-diabetic agent, (Park *et al.* 2013), anti-inflammatory agent, anti-oxidant agent (Peng *et al.* 2017), and matrix metalloproteinase-9 suppressor (Oh *et al.* 2013). Tempeh is a fermented product of soybean and commonly used as Indonesian traditional food with high isoflavones content (Yuliani *et al*, 2018). Fermentation process increases the isoflavone aglycone content in tempeh including daidzein due to the hydrolysis reaction that occurs on the glycoside bond of acetylglycoside and malonilglycoside (Hong *et al.* 2012).

Extraction can be a challenging step in the extraction of active compounds from natural products. There are several factors that can affect extraction process such as particle size, extraction time, solvent used, and temperature condition (Hernández *et al.* 2009, Zhu *et al.* 2011). Previous study reported that the longer the extraction time, the higher level of isoflavone aglycones extracted from simplicia (Jyoti *et al.* 2015). The particle size factor is related to the contact area between simplicia and solvent used in the extraction process. The smaller particle size, the bigger the contact area with solvent, and the higher the concentration of daidzein achieved from simplicia (Sapri *et al.* 2014). It is important to use appropriate solvents considering the physicochemical properties of target analyte. Ethanol was commonly used in isoflavone aglycones extraction due to the analyte solubility consideration (Lakshmi *et al.* 2013).

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Another study on isoflavones extraction reported that the addition of water up to 30% in the ethanol solvent will increase the concentration of genistein extracted from soybeans (Rostagno *et al.* 2009).

The aim of this study was to evaluate the effect of three factors on the extraction of daidzein i.e. particle size, extraction time, and solvent used toward daidzein yield from tempeh, a fermented product of soybeans.

METHODS

Materials

Reference standard daidzein (Sigma-Aldrich, Singapore) were used as external standard for this study. Other materials used were ethyl acetate, petroleum ether, methanol for liquid chromatography grade (E. Merck), and redistilled water from Organic Chemistry Laboratory, Faculty Pharmacy, Universitas Sanata Dharma, Yogyakarta. The tempeh used in this study was obtained from the traditional market in Yogyakarta. The selected tempeh "M" was controlled with the fermentation time of three days.

Instrumentation

The instrumentation used in this study were including: Shimadzu® LC-2010HT system with UV/Vis detector, Retsch® T460 ultrasonicator, ultramicro analytical balance RADWAG® UYA 2.3Y (max: 2.1 g, min 0.8 mg), membrane filter holder of Whatman®, organic solvent membrane filter of Whatman®; inorganic solvent membrane filter of Whatman®, Millipore syringe filter, and Socorex® micropipettes. Design Expert™ 10.0.6.0 software.

HPLC system

The HPLC used in this study was developed according to Yuliani *et al.* (2018). The Shimadzu LC-2010 CHT with Lab-Solution software and UV-VIS detector was used in quantitative analysis of daidzein. Analytical column used in this study was Luna Phenomenex® C_{18} column (250x4.6 mm, 5 µm). The mobile phase mixture containing methanol-water (70:30) and the flow rate of 0.6 ml/minute was applied in the isocratic reverse-phase HPLC system. The daidzein was measured at UV 261 nm.

Simplicia preparation

The tempeh "M" used in this study was obtained from the traditional market in Yogyakarta. The fresh tempeh was cut and dried in an oven at 50˚C for 24 hours. The small pieces of dried tempeh were ground into rough powder and then macerated using petroleum ether. About 100 ml petroleum ether were added into the 50 g rough powder tempeh in an erlenmeyer. The maceration process was conducted by shaking the mixture at 150 rpm for 24 hours. The suspended solid was separated from the solvent by centrifugation and dried in the oven at 50˚C for 24 hours. The dried mass obtained was called the tempeh simplicia.

Evaluation of Extraction Factors

Observations of the factors that affected the extraction were done to evaluate how the particle size, extraction time, and ethanol solvent affected the extraction process of the daidzein from tempeh.

Particle size

The tempeh simplicia was ground into four types of powder size i.e. 0.6; 0.85; 1.2; and 1.7 mm^2. Fifty-grams of the tempeh simplicia of each particle size group were mixed with 150 ml of 70% ethanol and macerated by shaking at 150 rpm for 270 minutes. After the filtering process, the yellow filtrate was separated from the suspended solid, then concentrated using rotary evaporator until 10% of initial volume was achieved.

Extraction time

Tempeh simplicia with certain particle size of 1.2 mm was used to evaluate the effect of extraction time. Fifty-grams of tempeh simplicia were mixed with 150 ml of 70% ethanol and macerated by shaking at 150 rpm for four extraction time i.e. 90, 180, 270, and 360 minutes, respectively. The yellow filtrate was separated from suspended solid then

concentrated using rotary evaporator until 10% of initial volume was achieved.

Solvent

The ethanol solvent concentrations used for optimization of extraction process were 50%, 60%, 70%, 80%, 90% and 96%. Fifty-grams of tempeh simplicia with the particle size of 1.2 mm were mixed with 150 ml of 70% ethanol and macerated by shaking at 150 rpm for 270 minutes. The yellow filtrate was separated from suspended solid and then concentrated using rotary evaporator until 10% of the initial volume obtained.

Sample Preparation

The liquid-liquid extraction method was performed to extract daidzein from the concentrated samples. One gram of concentrated sample was weighed and extracted by 30 ml ethyl acetate and water (50:50 v/v). The extraction process was repeated three times. The ethyl acetate fraction was collected and dried. A constant weight of the dried extract was transferred into a 10 ml volumetric flask followed by dilution to volume with methanol. Fifty microliters of the solution were transferred into a micro tube for dilution to 1.0 ml with methanol. All sample solutions were sonicated for 10 minutes, filtered using Millipore syringe filter, and transferred into HPLC vial before injection.

RESULTS AND DISCUSSION

Extraction could be one of the crucial steps in developing research on natural products. Several factors may contribute to the extraction efficiency of active compounds from the natural products (Rostagno *et al.* 2003; Zhang *et al.* 2007; Yoshiara *et al.* 2012). This study was conducted to evaluate the effect of three factors on extraction such as particle size, extraction time, and solvent used on the concentration of daidzein, one of an isoflavone aglycone extracted from tempeh, a fermented product of soybean. The highest daidzein concentration obtained among different variations for each factor was stated as the parameter for determining optimum condition.

Daidzein concentration was determined by HPLC from the previous study (Yuliani *et al*., 2018). The HPLC method obtained daidzein calibration curve equation y = 127910x – 179548 with r = 0.999 at the range of 5.07 – 17.75 µg/ml. The accuracy was within the required range of 80-115% (AOAC, 2012). Percentage of RSD for intraday and inter day as the precision parameter was 6%, lower than the maximum limit of AOAC requirements for RSD% (AOAC, 2012). The LOD and LOQ of the method were 0.796 and 2.653 µg/ml, respectively. This method indicated the successful result by a good separation of daidzein peak for further quantitative analysis achieved at retention time of 7.831 minutes (Figure 1).

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The effect of particle size of tempeh simplicia

Contact area between tempeh simplicia and the solvent used in extraction process was affected by the pacticle size factor. The higher the concentration of daidzein achieved from simplicia was related to the smaller particle size which leads to the bigger the contact area with solvent (Sapri *et al.* 2014). On the other hand, too small of particle size can cause disadvantages in the extraction process, such as the forming of particle aggregation which leads to the contact blocking between tempeh simplicia and the solvent (List and Schmidt, 1989).

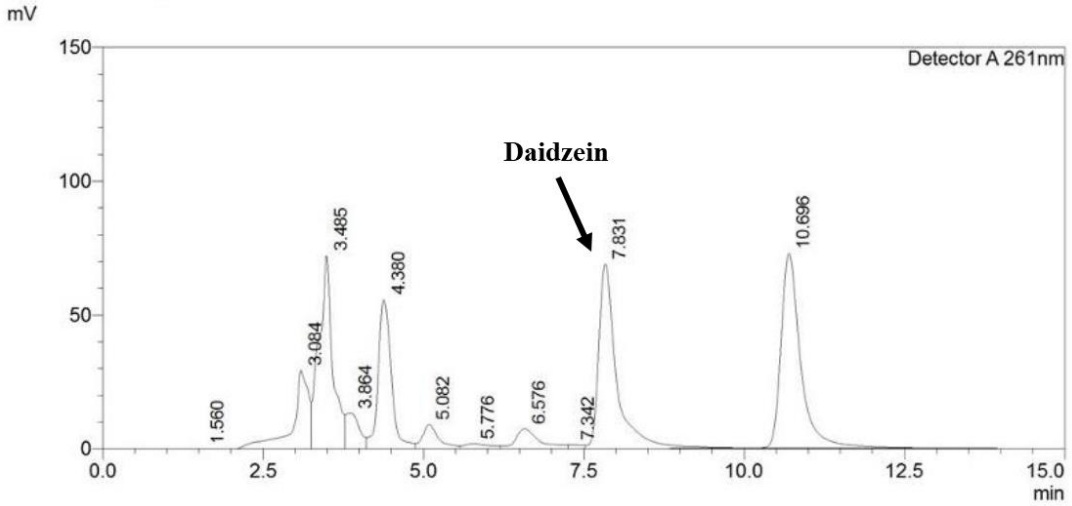

Figure 1. Representative chromatogram of tempeh simplicia containing daidzein. Column: Luna Phenomenex® C_{18} (250 x 4.6 mm, 5 µm). Mobile phase: methanol-water (70:30). Flow rate: 0.6 ml/min. Detection at 261 nm.

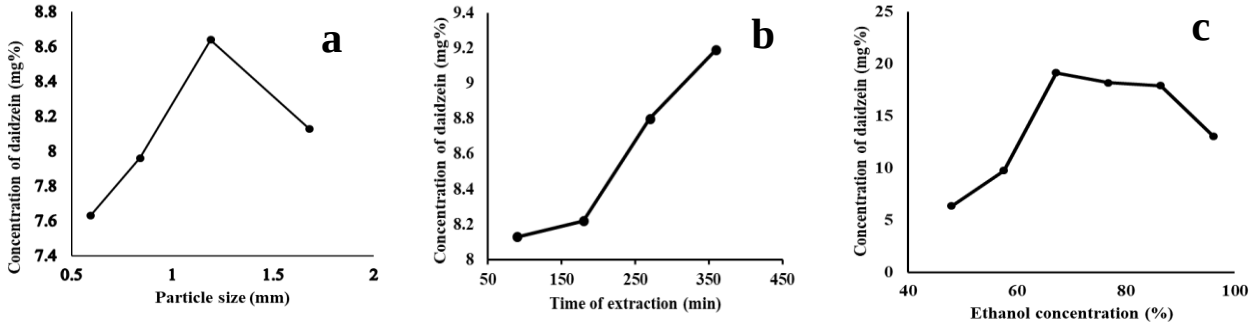

Figure 2. Graphic of effects from three factors on tempeh simplicia extraction such as particle size (a), extraction time (b), and ethanol concentration (c).

Selected particle sizes used in this study were 0.6; 0.85; 1.2; and 1.7 mm. The daidzein obtained from the tempeh simplicia for each particle size group were 7.63±1.1; 7.96±0.47; 8.64±0.85; and 8.13±0.38 mg%, respectively (Figure 2a). The particle size of 1.2 mm was the optimum for gaining the highest concentration there was 8.64 mg daidzein extracted from 100 g tempeh simplicia. The lower daidzein concentration extracted from tempeh simplicia with the particle size types smaller than 1.2 mm indicated that the particle aggregation may be formed in the particle size group of 0.6 and 0.85 mm.

The effect of extraction time

In general, longer extraction time in the extraction process lead to increases in the concentration of daidzein extracted from simplicia. It was related to the daidzein diffusion time into the surface of the simplicia before dissolving in the solven. Hence, increasing extraction time will enhance the extraction capacity. Nevertheless, too long extraction time could be ineffective for the extraction process because it is dependent on the equilibrium between the solute inside and outside the solid material (Zhang *et al*. 2018). As a result, it was important to optimize the extraction time in order to develop an effective extraction method with the highest analyte extracted from the simplicia.

Extraction time variations were 90, 180, 270, and 360 minutes. The daidzein concentration extracted from the tempeh simplicia for each extraction time were

8.13±1.00; 8.22±1.67; 8.80±0.81; and 9.19±0.50 mg%, respectively (Figure 2b). It could be concluded that the longer extraction time leads to the higher daidzein yield. The extraction time of 360 minutes has been recommended as the optimum extraction time.

The effect of solvent composition

In this research the effect of solvent composition was investigated. The solvent was prepared in the concentration series of 50%, 60%, 70%, 80%, 90% and 96% ethanol in water. The daidzein concentrations extracted from the tempeh simplicia by each ethanol concentration were 6.34±0.40; 9.73±0.57; 19.14±2.30; 18.19±4.90; 17.91±1,89 and 13.04±2.17 mg%, respectively (Figure 2c). The highest daidzein concentration was obtained by using 70% ethanol solvent since the molar solubility of daidzein in ethanol (3.2375 mol/L) was higher than its molar solubility in water (0.1695 mol/L) (Yang *et al.* 2013).

Based on its molar solubility, the highest concentration of daidzein should be obtained with the highest concentration of ethanol. However, tempeh simplicia used in this research were found in the form of dry powder. It was assumed that soybean cells underwent shrinkage during the drying process. The presence of water in the extraction steps enhanced the resizing cells into the normal size and resulted in a porous surface of simplicia. In addition, daidzein diffusion process was facilitated by the presence of pores on the surface of simplicia. Therefore solvent of 70% ethanol has been recommended as the optimum condition.

CONCLUSION

In summary, the results of this study showed the effect of particle size, extraction time, and solvent composition on the concentration of daidzein extracted from tempeh which was successfully evaluated in this study. The optimized condition was achieved with the particle size of 1.2 mm, extraction time of 360 minutes, and 70% ethanol used as solvent for maceration process.

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