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ANTI-DIARRHEAL ACTIVITY OF AQUEOUS EXTRACT OF NAGASARI FLOWERS (*Mesua ferrea* L.) IN BALB/c MICE INDUCED BY *Escherichia coli*

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ABSTRACT

Nagasari (*Mesua ferrea* L.) is empirically used for management for diarrhea in Nusa Tenggara Barat Province. However, the safety and efficacy of the nagasari have not been scientifically validated in an animal model. This study evaluated the activity of aqueous extract of the nagasari flowers in BALB/c mice induced by Escherichia coli to determine the effective dosage for anti-diarrheal effect. The aqueous extract of nagasari flowers (0,4% (w/v); 0,8% (w/v); and 1,6% (w/v)) was administered orally to 3 groups of mice, whereas negative and positive control received aquadestilate and ciprofloxacin 130 mg/kgBW respectively orally once daily for 3 days. The frequency and consistency of feces were observed then the total colony of E. coli in feces was calculated using colony counter. Aqueous extract of nagasari flowers at concentration 1,6% showed significant inhibitory activity against Escherichia coli comparable with positive controls (diarrhea index 0,425; $p>0,05$ and total colony of E. coli in feces 100,2; $p>0,05$). This study provides scientific support for the traditional use of aqueous extract of nagasari flowers for the treatment of diarrheal diseases.

Keywords: anti-diarrheal; Aqueous extract; diarrhea index; *Escherichia coli*; nagasari.

INTRODUCTION

Diarrhea is an abnormal event that characterized by an increased in the frequency of defecate more than 3 times in 24 hours accompanied by the consistency of liquid stool (Kotloff *et al*., 2013).

Based on the Health Profile in Nusa Tenggara Barat (NTB) Province, the number of diarrhea patients had not experienced a significant decrease that is 158.993 and 175.361 in the year of 2015 and 2017 (Dinkes RI, 2015; 2018). Diarrhea can be caused by infectious agents such as bacteria, viruses and parasites, including *Escherichia coli* which includes Gram-negative bacteria.

Ciprofloxacin as the first-line in the treatment of diarrhea but has limitations such as side effects and resistance if used over a long period of time. In addition, this drug is a second-line treatment for tuberculosis XDR/MDR (Extensively Drug Resistance/Multi-Drug Resistance) so it must be used wisely (Raini, 2016). Herbal medicine is an option to be developed as anti-diarrhea.

Nagasari (*Mesua ferrea* L.) is one of the plant used traditionally as anti-diarrhea. In Dharma Usada Kuranta Bolong, states that part of the nagasari plant can be used as a diarrhea medicine (Listiawati *et al*., 2016). *Mesua ferrea* L. flowers is illustrated in Figure 1.

In vitro, the methanol extract of all parts of the nagasari flowers at concentration of 10 µg/mL, 50 µg/mL and >200 µg/mL showed the minimum inhibitory concentration values of *E. coli* of 2, 6 and 14 strains. Flavonoids mesuaferrone-A and mesuaferrone-B are through to acts in the antibacterial effect (Mazumder *et al*., 2004).

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In vivo scientific studies of nagasari flower extract as anti-diarrhea are still limited. Therefore, this study evaluated the activity of aqueous extract of the nagasari flowers in Balb/c mice induced diarrhea by *E. coli* in decreasing diarrhea index and total of *E. coli* colony in feces.

METHODS

Collection and Identification of Flowers

The fresh flowers of *Mesua ferrea* were collected from Lingsar, West Lombok in January 2018 and identified by Herbarium Bandungense SITH, Bandung Institute of Technology, Bandung No. 1493/11.CO2.2/PL/2018.

Drying and Grinding

The whole parts of flowers were washed, dried under shade for five days. The powdered of dry flowers were prepared with a mechanical grinder and passed through a 35-mesh sieves. The powder was stored in a well-closed container until the analysis was commenced.

Extraction

The powder of *M. ferrea* (nagasari) flowers were boiled in aquadestilate (0,8% $^{w}/_{v}$) during 15 minutes. The aqueous extract was stored properly (Hamad *et al*, 2017).

Bacterial Preparation

Escherichia coli ATCC 25922 strain was isolated from urine samples. This strain is part of the pathogen from the Yogyakarta Hall Health's Laboratory. The bacteria were grown on Nutrient Agar (NA) (Oxoid) (14 gram dehydrated NA in 500 mL aquadest) then transferred to the *Eosin Methylene Blue Agar* (EMBA) (Oxoid) (18,75 gram dehydrated EMBA in 500 mL aquadest (from Health Research and Development Installation of NTB Provincial Hospital) as selective media and Gram staining was carried out for the identification process accordance with the Bergey's Manual of Systematic Bacteriology (Whitman, 2010).

Figure 1. *Mesua ferrea* L. flowers

The bacteria were suspended in sterile physiological saline (0,9 gram NaCl (Merck) in 100 mL aquadest) until a concentration of approximately 10^6 CFU/mL (Arief *et al*, 2010). The concentration of bacteria was calculated using Neubauer methods.

Experimental Animals

All procedures involving animals were approved by the Ethics Committee Faculty of Medicine of The Mataram University No. 91/UN18.8/ETIK/2018.

A total of 30 BALB/c mice weight between 25-40 g obtained from the Immunology Laboratory of Mataram University were used in this study. The mice were acclimatized under the standard environmental condition for 7 days. All mice had free access to food and destilate water *adlibitum* throughout the experimental period. The mice were divided into 6 groups randomly with 6 mice per group as follow: normal group (normal mice received destilate water 0,5 mL), negative control group (diarrhea mice received destilate water 0,5 mL), positive control group (diarrhea mice received ciprofloxacin 130 mg/kg BW (13 mg ciprofloxacin (Hexpharm Jaya) powder in 5 mL aquadest)), and three group of treatment (0,4% $^{w}/_{v}$; 0,8% $^{w}/_{v}$; 1,6% $^{w}/_{v}$).

Assessment of In-vivo Anti-diarrheal Activity *Escherichia coli* induced diarrhea

Each mice was administrated orally with 100 µL of 10^6 CFU of *E. coli* except the normal group to induced diarrhea (Astawan *et*

al, 2009). After 30 minutes, each mouse is treated with the extract and ciprofloxacin.

All mice were treated every morning for 3 days. After the treatment, all mice were placed in individual cages, the floor of which was covered with filter paper. The number and consistency of stool were recorded for 6 hours. The severity quantitatively of diarrhea was defined using three indexes (1) *Loose Stool Incidence Rate* / LSIR, (2) *Average Loose Stool Grade* / ALSG, (3) *Diarrhea Index* / DI and reduction in the total of colony of *E. coli* in feces (Hui *et al*, 2012; Han *et al*, 2014; Yu *et al*, 2017).

Table I. Effect of aqueous extract of *M. ferrea* (nagasari) flowers in decreasing total colony of *Escherichia coli* in feces

	Group	Before treatment (CFU)	After treatment (CFU)
I	*E. coli* + AEONF 0,4%	142,2 ± 97,9	155,6 ± 96,9
II	*E. coli* + AEONF 0,8%	97,6 ± 42,4	41,4 ± 31,2
III	*E. coli* + AEONF 1,6%	122,2 ± 51,5	22 [a)] ± 15,9
IV	*E. coli* + Cip	149 ± 34,7	29 ± 8,8
V	*E. coli* + Destilated water	128,2 ± 51,4	136,4 ± 48,6
VI	Destilated water	0 ± 0	0 [a)] ± 0

Note. AEONF = Aqueous extract of Nagasari flowers, Cip = Ciprofloxacin, [a)] $p > 0,05$ comparable with positive controls.

Table II. Effect of aqueous extract of *M. ferrea* (nagasari) flowers in decreasing LSIR

	Group	LSIR day 1	LSIR day 3
I	*E. coli* + AEONF 0,4%	0,161 ± 0,06	0,144 ± 0,033
II	*E. coli* + AEONF 0,8%	0,273 ± 0,202	0,036 [a)] ± 0,05
III	*E. coli* + AEONF 1,6%	0,355 ± 0,25	0 [a)] ± 0
IV	*E. coli* + Cip	0,194 ± 0,169	0,040 ± 0,089
V	*E. coli* + Destilate water	0,367 ± 0,031	0,315 ± 0,159
VI	Destilate water	0 ± 0	0 [a)] ± 0

Note. AEONF = Aqueous extract of Nagasari flowers, Cip = Ciprofloxacin, [a)] $p > 0,05$ comparable with positive controls.

Table III. Effect of aqueous extract of *M. ferrea* (nagasari) flowers in decreasing ALSG

	Group	ALSG day 1	ALSG day 3
I	*E. coli* + AEONF 0,4%	1,04 ± 0,089	1,2 ± 0,447
II	*E. coli* + AEONF 0,8%	1 ± 1	0,8 [a)] ± 1,095
III	*E. coli* + AEONF 1,6%	1,131 ± 0,144	0 [a)] ± 0
IV	*E. coli* + Cip	1,167 ± 0,745	0,267 ± 0,596
V	*E. coli* + Destilate water	1,218 ± 0,287	1,506 ± 0,389
VI	Destilate water	0 ± 0	0 [a)] ± 0

Note. AEONF = Aqueous extract of Nagasari flowers, Cip = Ciprofloxacin, [a)] $p > 0,05$ comparable with positive controls.

Table IV. Effect of aqueous extract of *M. ferrea* (nagasari) flowers in decreasing DI

	Group	DI day 1	DI day 3
I	*E. coli* + AEONF 0,4%	0,170 ± 0,074	0,164 ± 0,03
II	*E. coli* + AEONF 0,8%	0,273 ± 0,202	0,362 ± 0,099
III	*E. coli* + AEONF 1,6%	0,425 ± 0,33	0,000 [a)] ± 0
IV	*E. coli* + Cip	0,306 ± 0,251	0,053 ± 0
V	*E. coli* + Destilate water	0,458 ± 0,108	0,638 ± 0,713
VI	Destilate water	0,000 ± 0	0,000 [a)] ± 0

Note. AEONF = Aqueous extract of Nagasari flowers, Cip = Ciprofloxacin, [a)] p > 0,05 comparable with positive controls.

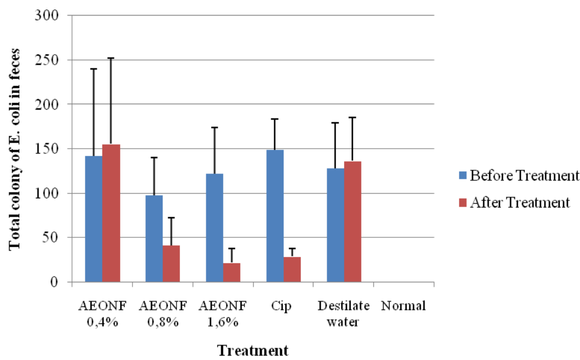

Figure 2: Effect of aqueous extract of *Mesua ferrea* in decreasing total colony of *Escherichia coli* in feces. AEONF = Aqueous extract of Nagasari flowers, Cip = Ciprofloxacin, [a)] p > 0,05 comparable with positive controls.

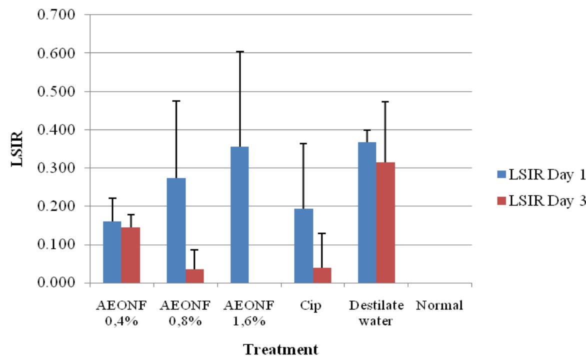

Figure 3: Effect of aqueous extract of *Mesua ferrea* in decreasing LSIR. AEONF = Aqueous extract of Nagasari flowers, Cip = Ciprofloxacin, [a)] p > 0,05 comparable with positive controls.

Data Collection and Analysis Samples

Total colony of *Escherichia coli* in feces

The fecal sample as much 50 mg was placed in 2 mL Eppendorf tube, to which distilled water was added. The sample was diluted 4 times dilution. The fourth dilution (100 µL) was fertilized by spread plate method on EMBA, then incubated at 37°C for 24 hours. Metallic green color on media showed a positive result of *Escherichia coli* in feces (Zhang *et al*, 2010; Analytic Jena, 2011).

Calculation of the total colony of *Escherichia coli* was carried out using a colony counter (Stuart™ Scientific colony counter Model SC5, AC/DC input 220 V AC). Mice infected if the total colony of bacteria in feces are 10^5 CFU (Newsome *et al*, 1987). Calculation formula for the total colony of bacteria in feces:

$$N = \frac{\Sigma C}{((1 \times n) \times d)} \quad \text{.................(1)}$$

N : total of colonies (mL/g)
n : total of plates in the first dilution;
d : the level of dilution obtained from the first cup calculated.

Three indices of severity diarrhea

The LSIR the ratio of the number of loose stools to the total stools within an animal. Loose stool grade describes the degree of loose stools, based on the diameter of the stool on the filter papers. The LSG was classified into four grades according to the diameter of loose stools; Grade 1 (< 1 cm), Grade 2 (1~< 2 cm), Grade 3 (2~3 cm) and Grade 4 (>3cm). The ALSG is the ratio of the sum of the LSG of each loose stool to the total number of loose stools within each mice. The DI is the result of LSR multiplying ALSG (Hui *et al*, 2012; Han *et al*, 2014; Yu *et al*, 2017).

Statistical analysis

The data were analyzed by using Kruskal Wallis procedures, followed by Mann Whitney. Values of p < 0,05 were showed a significant difference between the effect of aqueous extract with positive controls statistically using SPSS 16 software.

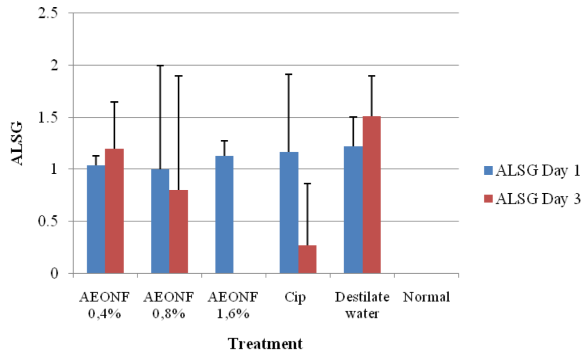

Figure 4: Effect of aqueous extract of *Mesua ferrea* in decreasing ALSG. AEONF = Aqueous extract of Nagasari flowers, Cip = Ciprofloxacin, [a)] p > 0,05 comparable with positive controls.

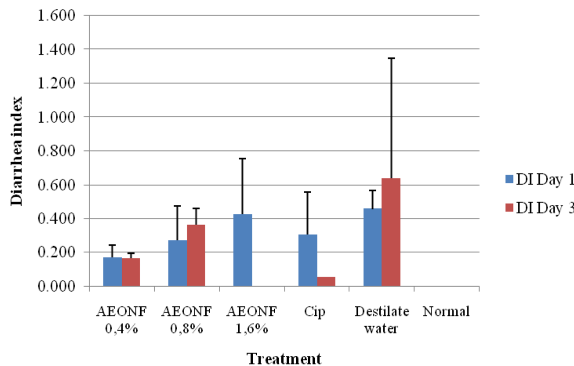

Figure 5: Effect of aqueous extract of *Mesua ferrea* in decreasing diarrhea index. AEONF = Aqueous extract of Nagasari flowers, Cip = Ciprofloxacin, [a)] p > 0,05 comparable with positive controls.

RESULTS AND DISCUSSION

The AEONF (Table I and Figure 2) at concentration 1,6% showed the highest decreasing in the total colony of *Escherichia coli* compared to other concentration and the antibacterial effect compared to positive controls (p>0,05).

The LSIR (Table II and Figure 3) describes the level of frequency of defecation. The LSIR did not differ between positive control and treatment group. The AEONF at concentration 1,6% showed the highest decreasing in the frequency of defecation compared to other concentration and the effect comparable with positive controls (p>0,05).

The ALSG (Table III and Figure 4) describes the level of consistency of feces, which is viewed from the size of the feces diameter on filter paper. The ALSG did not differ between positive control and treatment group (AEONF at concentration 0,8% and 1,6%). AEONF at concentration 1,6% showed the highest decreasing in the consistency of feces compared to AEONF at concentration 0,8% and the effect comparable with positive controls (p>0,05).

Diarrhea index (Table IV and Figure 5) is an accumulation of LSIR and ALSG. The DI did not differ between positive control and treatment group. The AEONF at concentration 1,6% the highest decreasing in the values of DI significantly different with negative control. The AEONF at concentration 1,6% showed the highest decreasing in the DI compared to other concentration and the effect comparable with positive controls (p>0,05).

In this study, the AEONF showed anti-diarrhea activity as evident from the inhibition of the increased diarrhea index induced by *Escherichia coli*.

Based on these four parameters, the AEONF at concentration 1,6% showed the highest effectiveness as specific anti-diarrheals. Mazumder *et al* (2004) have reported, nagasari flowers contain several flavonoid compounds. Allegedly, the content of flavonoids and tannins causes a decreasing in all four parameters.

Flavonoids can inhibit Gram-negative or Gram-positive bacteria. Alcohol groups in flavonoids can bind to peptidoglycan and lipopolysaccharide on the cell wall so that it will damage bacteria cell membranes. The Flavonoids have mechanisms such as ciprofloxacin by inhibiting the function of gyrase DNA by which interferes with the process of bacterial replication obtained (Jawetz *et al*, 2013; Mufti *et al*, 2017).

The role of tannin in adhesion cell and enzyme inactivating and disturb with protein transport in the inner layer of the cell. In addition, tannin bind to cell wall polypeptides which cause the formation of cell walls to be less than perfect. This cause osmotic pressure in cells so that bacterial cells die and lysis (Okuda, 2005; Smullen *et al*, 2007).

In this study, no signs of toxicity and mortality were found in mice so it was safe to use. Toxicity data on ethanol extracts of nagasari flowers doses of 500-2000 mg/kgBW also did not cause signs of acute toxicity in

mice (Tiwari *et al*, 2012). However, further toxicity tests on the AEONF need to be carried out.

CONCLUSION

Aqueous extract of nagasari flowers at concentration 1,6% has the most effective specific anti-diarrheal activity indicated by the highest decreasing in the total colony of *Escherichia coli* and diarrhea index.

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LIPID AND SILVER NANOPARTICLES GELS FORMULATION OF TEMPEH EXTRACT

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ABSTRACT

Tempeh extract is used in this study as an active ingredient in lipid nanoparticles and reductant in silver nanoparticles because tempeh is an authentic Indonesian food ingredient and is known to have the main content of isoflavones. Gel preparations were chosen to increase the acceptability and stability of lipid and silver nanoparticles. This research aim is to formulate lipid nanoparticle gel formulations with tempeh extract as active substances and silver nanoparticle gel formulations with tempeh extract as bioreduction. Lipid nanoparticles were made from soy lecithin phospholipids by heating at 60˚C and sonication method for 30 minutes then the tempeh extract was added just before sonication. Silver nanoparticles were made by adding tempeh extract to $AgNO_3$ solution at 90˚C for 30 minutes. The average particle size of tempeh extract lipid nanoparticles was 130.03 nm and silver nanoparticle was 94.76 nm. The average viscosity of tempeh extract lipid nanoparticles gel was 4.02 d.Pa.s and silver nanoparticles was 4.22 d.Pa.s. The average spreadability of tempeh extract lipid nanoparticles gel was 4.37 cm and silver nanoparticles is 4.05 cm. The average pH value of tempeh extract lipid nanoparticles was 7.70 and silver nanoparticles was 7.33.

Keywords: gel; lipid nanoparticles; particle size; silver nanoparticles; tempeh extract

INTRODUCTION

Nanoparticles are one of the technologies developed to increase the effectiveness of drug delivery (Latarissa 2017). Nanoparticles have the advantage to penetrate the space between cells and it able to increase the surface area contact. Nano-sized particles have unique physical properties because they can be combined with a variety of technologies. They are expected to produce a more effective drug delivery system (Martien *et al.* 2012). Nanoparticles can be made with specific colloidal formation systems, and one example is liposomes that are made using soy lecithin (Dwiastuti, Noegrohati, and Istyastono 2016). Another method for preparation of nanoparticles is to use metals then reduced with specific materials to form nanoparticles, one example is silver nanoparticles using $AgNO_3$ solution added with specific reducing agents (Sileikaite *et al.* 2006).

Lipid nanoparticles are made through the formation of soy lecithin phospholipid nano-liposomes by heating and sonication methods (Dwiastuti, Noegrohati, Istyastono, *et al.* 2016). Soy lecithin contains unsaturated fatty acids. It has excellent penetration in the skin and high compatibility in the body (Dwiastuti, Noegrohati, Istyastono, *et al.* 2016). Lipid nanoparticles can combine lipophilic and hydrophilic properties in preparations (Dwinna 2010). Silver nanoparticles are produced through a method of mixing $AgNO_3$ solution (Tatang Wahyuni, Doni Sugiyama 2011) and specific bioreduction (Muliadi *et al.* 2015). Bioreduction are extracts of natural substances that can act as reductant (Jain D *et al.* 2009). The success of silver nanoparticle formation can be known shortly after manufacture by measuring the maximum wavelength using UV-Vis spectrophotometry (Jain, Arora, *et al.* 2009).

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Tempeh extract on lipid nanoparticles is used as an active substance, while tempeh extract on silver nanoparticles is used as a bioreduction. Tempeh extract is known to have the main content of isoflavones derived from flavonoid compounds that function as wound healers (Park *et al.* 2011). In this research, lipid nanoparticles and silver nanoparticles were formulated to review the physical properties between the two preparations. Lipid nanoparticles were developed as topical preparations because they have good penetration ability (zur Mühlen *et al.* 1998) (Jafar *et al.* 2015). Silver nanoparticles were developed as topical preparations because they have the anti-bacterial ability (Ariyanta 2014). It can be developed in preparations for wound healing preparations (Ariyanta 2014) and anti-acne (Septyarin 2017). The development of these two preparations needs to be reviewed for particle size and physical properties as seen from the parameters of viscosity, dispersion, and pH. This preparation is expected to be a choice of drug dosage forms, especially topical preparations for various expected pharmacological effects, for example: wound healing and anti-acne preparations. Therefore, this study aims to formulate lipid nanoparticle gel formulations with tempeh extract as active substances and silver nanoparticle gel formulations with tempeh extract as bioreduction with a review of physical properties and particle size.

METHODS

Materials

The material used in this study were: soybean lecithin (Sigma-Aldrich), distilled water, tempeh with three days fermentation under the brand name "Muchlar", $AgNO_3$, Carbopol, Propylenglycol, Triethanolamin, and Glycerin are obtained from "Bratachem".

Instrumentation

Instruments used in this study are, particle size analyzer (HORIBA Scientific, Japan), Spectrophotometer UV-VIS (Shimadzu, JAPAN), pH meter, and Viscosimeter Rheosys (Model: Merlin VR).

Preparation of Tempeh Extract

The tempeh extract was prepared by tempeh with three days fermentation under the brand name "Muchlar". Tempeh was cut 5 cm long and 6.5 cm wide and 1 cm thick. Tempeh extract was made with the ratio of tempeh and aquadest which is 1: 2. Three hundred grams of tempeh was added into 600 mL of distilled water, then heated to a temperature of 90˚C. Maintained the temperature remained 90˚C for 30 minutes then the extract cooled to a temperature 30˚C then filter with filter paper.

Preparation of Lipid Nanoparticles of Tempeh Extract as the Active Substances

Lipid nanoparticles were made by weighing soybean lecithin by 12 grams and then minimized by mortar and stamper. The refined soy lecithin was then homogeneously dispersed in 200 mL of aquabidest at 60˚C. The soy lecithin dispersion was then blended at high speed for sixty seconds. The soy lecithin suspension was maintained at 60˚C and then homogenized with ultraturax for one minute on 4 scale. Furthermore, soy lecithin suspension was put in the bath sonicator together with tempeh extract as much as 80 mL. The sonicator bath is set to a temperature of 60˚C for 30 minutes (Dwiastuti, Noegrohati, Istyastono, *et al.* 2016).

Preparation of Silver Nanoparticles Using Tempeh Extract as Bioreduction

Silver nanoparticles were made by weighing 0.034 grams of silver nitrate ($AgNO_3$) in 200 mL aquabidest (1mM) silver nitrate solution. The silver nitrate solution was heated to a temperature of 90˚C. Then it was added with tempeh extract 80 mL and kept at 90˚C while stirring 600 rpm for 30 minutes (Ramadon and Mun'im 2016; Ariyanta 2014).

Preparation of Gel and Physical Properties Testing

The preparation of this formula began with the swelling of carbopol. It was prepared in 100 mL lipid nanoparticles or 100 mL silver nanoparticles with 3 grams of carbopol for 24 hours. Then, 3 grams of carbopol 3% w/v as much as 50 grams and added TEA to the

mortar and stirred until homogeneous for about 5 minutes. Next, put the mixture of carbopol and TEA into the blender and add propylene glycol and glycerin and mixing or three minutes at low speed.

Table I. Gel Formula of Lipid and Siver Nanoparticles Gel

Ingredients	Formula
R/ Carbopol 3% b/v (gram)	50
Propyleneglycol (gram)	30
Glycerin (gram)	60
Triethanolamin (TEA) (gram)	2,4

Scattering Test. The scatter power test was carried out 24 hours after manufacture by putted one gram of gel and placed in the middle of a large round glass. On top of the gel was placed another round glass and ballast with a total weight of 125 grams then allowed to stand for one minute and note the spread diameter. *Viscosity Test.* The viscosity test was carried out 24 hours after preparing the gel using the Rheosys cone and plate Merlin VR model. *pH test.* The pH test carried out 24 hours after the gel prepared using a pH-meter. The pH test began with putted one gram of gel and then dissolved it in 10 mL aquadest. Furthermore, the pH meter inserted into the aquadest and then putted into a gel then the pH meter will show the pH value.

Wavelength of Silver Nanoparticles.

Measurement of the maximum wavelength is one of the initial steps to determine silver nanoparticles. The indicator of silver nanoparticles is the wavelength with maximum absorbance in the range of 400-450 nm (Ariyanta, 2014; Ayu 2015).

Particles Size of Lipid Nanoparticles and Silver Nanoparticles.

This measurement is done by conducting a DLS particle size analyzer (Horiba SZ 100, Japan).

Data analysis

Particle size data and physical properties test results obtained in this study were then performed statistical tests with the R computational statistical program. The T test used to find out whether there are significant differences in physical properties results between lipid nanoparticles with silver nanoparticles preparations.

RESULTS AND DISCUSSION

Tempeh extract contains a lot of isoflavones with a function as a wound healing (Danciu *et al.* 2012). Tempeh extract was prepared with water solvent so that the tempeh extract can be used as a bioreduction in the formation of silver nanoparticles. One of the bioreduction requirements in the formation of silver nanoparticles is a water-soluble extract. That is expected to dissolve and react with $AgNO_3$ solution. While in the addition of lipid nanoparticles, tempeh extract functions as an active substance.

Physical Appearance of Tempeh Extract Lipid Nanoparticles and Tempeh Extract Silver Nanoparticles

The description of lipid nanoparticle was a turbid white color and unique smelled of soy lecithin. The silver nanoparticle preparations had a clear-reddish-brown and unique smelled of tempeh extract. Clear-reddish-brown in the aqueous solution formed from excitation. The reduction of silver ion causes it; there are indicated the formation of silver nanoparticles (Jain, Daima, *et al.* 2009). This physical appearance of difference nanoparticle preparation and gel nanoparticle is presented in Figure 1 and 2.

The Particle Size of Tempeh Extract Lipid Nanoparticle and Tempeh Extract Silver Nanoparticles

The lipid nanoparticles formation can be known after the Particle Size Analyzer (PSA) test have been done. The formation of silver nanoparticles can be recognized immediately by measuring the maximum wavelength using UV Vis spectrophotometer. If the wavelength is between 400 - 450 nm, it means that silver nanoparticles are known (Maharini *et al.* 2017). This is one of the advantages of silver nanoparticles compared to lipid nanoparticles, namely the success of the preparation formulation can be known after manufacture.

In this study, wavelength measurements were made after 24 hours of storage. The average wavelength measurements of silver nanoparticles with three replications after 24 hours of storage at room temperature were obtained 406 nm. The results of these wavelength measurements indicate that silver nanoparticles can be formed the extract of tempeh as bioreduction at a temperature of 90^0C and 30 minutes (Sari Purwo Ismaya *et al.* 2017).

PSA test was conducted to determine the size of lipid nanoparticles (Dwiastuti, Noegrohati, Istyastono, *et al.* 2016) and silver nanoparticles. PSA test results are shown in Table II.

The measurement results in Table II showed that the silver nanoparticle formula could produce particle sizes less than 100 nm, while the lipid nanoparticle formulas produce sizes more than 100 nm. Tempeh Extract in lipid nanoparticle as an active substance make colloidal dispersion could not be form completely so that affect particles size. Tempeh extract in silver nanoparticle will act as bioreductor in silver nitrate and could produce nanoparticle. Analysis with T-test at 95% confidence level obtained p-value of 0.21. Thus the average particle size of tempeh extract lipid nanoparticles was the same as the average particle size of tempeh extract silver nanoparticles and not significantly different. This phenomena could be happen because in silver nanoparticle extract tempeh will initiate reduction reaction of silver nitrate and reduce particle size, but in lipid nanoparticle, particle will be reduce by the reaction of colloidal dispersion from soy lecithin (Dwiastuti, Noegrohati, Istyastono, *et al.* 2016).

Figure 1. Tempeh extract nanoparticle preparation of lipid nanoparticles (a) and silver nanoparticles (b)

(a) (b)

Figure 2. Gel nanoparticle of lipid nanoparticle (a) and silver nanoparticle (b)

Table II. *Particle Size Analyzer* (PSA) result of Lipid and Silver Nanoparticles with Tempeh Extract

Replication	Tempeh Extract Nanoparticle Lipid (nm)	Tempeh Extract Silver Nanoparticles (nm)
Replication 1	129,00	128,10
Replication 2	124,00	87,00
Replication 3	124,20	69,20
Average	130,03 ± 6,41	94,76 ± 30,20

Table III. Viscosity, Spread ability, and pH Value Result of Lipid and Silver Nanoparticles

Parameter	Lipid Nanoparticle	Silver Nanoparticle	*p value*	Statistical Result
Viscosity (d.Pa.s)	4,02 ± 0,20	4,22 ± 0,33	0,59	Not Significantly Different
Spread ability (cm)	4,37 ± 0,11	4,05 ± 0,02	0,99	Not Significantly Different
pH	7,70 ± 0,10	7,33 ± 0,05	0,98	Not Significantly Different

Physical Properties of Tempeh Extract Lipid Nanoparticles Gel and Tempeh Extract Silver Nanoparticle Gel Preparations

Preparation of tempeh extract lipid nanoparticles and tempeh extract silver nanoparticles were tested for physical properties with parameters including: viscosity, spread ability, and pH value. Physical test results of lipid nanoparticle gel and silver nanoparticle gel preparations showed physical properties test results with pH parameters. The results of the physical properties test were followed by an analysis of the T-test with a 95% confidence level to see differences in physical properties of the two preparations.

The results of the viscosity testing (Table III) after 24 hours of preparation of lipid nanoparticles and tempeh extracts of silver nanoparticles indicated no different results. This result is strengthened by the results of statistical tests using the T-test. The analytical results showed p-value is 0,59 so that it can be said that the viscosity of the two preparations that are not significantly different. Viscosity is influenced by the carbopol composition, because carbopol acts as gelling agent that will form gel-forming matrix (Maheswara 2008). The carbopol composition of Lipid Nanoparticle Gel and Silver Nanoparticle Gel have same composition, thus the result of the viscosity testing are not significantly different

The similar analysis results were also found in the spread ability (Table III) and pH response of lipid nanoparticle gel and tempeh extract silver nanoparticles. Statistical tests with the T-test obtained that the p value of the spread ability test is 0.99 and the p-value of the pH test is 0.98. It can be explained that the spread ability and pH of the preparations resulting from the formulation of lipid nanoparticles and silver nanoparticles of tempeh extract have no significantly different results. This result can be obtained because the amount of gelling agent and humectant used for the preparation of gel nanoparticle lipid and silver nanoparticle gel preparations uses the same amount. The physical properties of gel preparation are influenced by the gelling agent and humectants used in the formulation. Carbopol act as gelling agent and Propylene glycol act as humectant. Gelling agent will form gel-forming matrix. Humectant will maintain the stability of dosage form by absorbing moisture from the environment and reducing the evaporation of water from the preparation. Because of that, spread ability and viscosity will influence dominantly by carbopol and propylene glycol will influence the stability of dosage form (Maheswara 2008).

CONCLUSIONS

Lipid and silver nanoparticles of tempeh extract can be formulated and the average

particle size of lipid nanoparticles was 130.03 nm and silver nanoparticle was 94.76 nm. The average viscosity of lipid nanoparticles gel was 4.02 d.Pa.s and silver nanoparticles was 4.22 d.Pa.s.. The average spreadability of lipid nanoparticles gel was 4.37 cm and silver nanoparticles is 4.05 cm. The average pH value of tempeh extract lipid nanoparticles was 7.70 and silver nanoparticles was 7.33.

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ANTIOXIDANT AND ANTICANCER ACTIVITIES OF MURBEI (*Morus alba L.*) STEM EXTRACT ON IN VITRO WiDr CANCER CELLS

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ABSTRACT

Mulberry is considered as an important plant in traditional Chinese medicine, due to its various compounds, including phenols and flavonoids. These flavonoids have antioxidant activities so that can be a potential anticancer candidate. The aims of this study were to determining antioxidant activity, phenolic content, and potential anticancer activity in Mulberry stem extract. The extraction was carried out by maceration using ethanol as the solvent, antioxidant activity test using ABTS (*2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid*) method, phenolic content determination using Folin-Ciocalteu reagents, and anticancer activity test using the MTT *(3-(4,5- dimetiltiazol-2-il)-2,5-difenil tetrazolium bromida)* method on WiDr cancer cells and Vero cells. The result of total phenolic Mulberry stem extract was 35.9%, the antioxidant activity value was 83.18 µg / mL, the IC_{50} value for anticancer activity for WiDr cells was 71.24 µg / mL and Vero cells IC_{50} value was 154.241 µg / mL. It could be concluded that the Mulberry stem ethanol extract had strong antioxidant activity and had the potential anticancer selectively against cancer cells WiDr.

Keywords: anticancer; antioxidant; IC_{50}; mulberry stem extract

INTRODUCTION

Cancer is one of the leading causes of death in the worldwide. The number of cancer patients is predicted to increase each year. Cancer patients reach 23.6 million cases per year in 2030 (Ministry of Health RI, 2016). One of the plants used by the community as medicine is Mulberry (*Morus Alba* L.) which has many potentials, including reduce blood cholesterol, antidiabetes, and antihypertension (Mallaleng *et al.,* 2011).

The plants generally contain phenolic compounds such as flavanoids, synamic acid derivatives, coumarins, and tocopherols (Gupita and Rahayuni, 2012). Antioxidant compounds produced as vitamin C, vitamin E, carotene, groups of phenolic compounds-especially polyphenols and flavonoids-are known to have the potential to reduce the risk of degenerative diseases (Kuntorini and Astuti, 2010).

Antioxidant activity is thought to inhibit the growth of cancer cells, due to the similarity of the mechanism of resistance at the cellular level (Anam *et al.*, 2014). Compounds that act as antioxidants include flavonoids, alkaloids, tannins, and also phenolic. The content is thought to have anticancer activities such as flavonoids which work by inhibiting carcinogenesis inactivation, cell cycle inhibition, inhibition of angiogenesis, cell proliferation and apoptotic mechanisms (Ahmad *et al*., 2014; Meiyanto, *et al.*, 2008).

Burhan A. and Aisyah (2018), studied about the toxicity test of mulberry stem extract with various solvents, found that the IC_{50} values for ethanol extract was 2.8045 µg / mL.

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This result showed the potential of mulberry stems as anti-cancer.

Based on all of those studies above, The aim of this study was to determine antioxidant activity by the method of ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid*)* and to determine anti-cancer activity using WIDR cells and vero cells.

METHODS

Instrument

UV-Vis spectrophotometer (Shimadzu®), ELISA reader (Thermo fischer Scientific®), rotary evaporator (Buchi®), microplate (Iwaki®).

Materials

MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolium bromida) (Sigma®), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (Merck®), n-hexane (Brataco Chemica®), ethanol (Onemed®), Follin-Cioucalteu (Merck®), Phosphate Buffer-Saline (PBS) (Gibco®), gallic acid (Sigma®), Sodium dodesil sulfat (SDS) (Merck®).

Extraction of mulberry steam

The preparation of the material begins with taking mulberry stems which is 60-90 days old in The Mountainous Area of Sidenreng Rappang, South Sulawesi. Extraction was carried out by maceration method using ethanol solvent, then extract was applied with rotary evaporator until it got thick extract.

Phytochemical screening

Phytochemical screening includes examination of alkaloids, flavonoids, tannins, phenols and saponins.

Quantitative testing of antioxidant activity using the ABTS method. Samples were made in series with concentrations of 40 ppm, 80 ppm, 120 ppm, 160 ppm, each concentration mixed with ABTS solution and then let stand for 12 hours, then measured by UV-Vis spectrophotometry with a wavelength of 750 nm, the comparison used in this experiment was vitamins C. Total phenol was tested using the UV-Vis spectrophotometer method with Follin-Cioucalteu reagent with a wavelength of 714 nm and gallic acid as a control.

In Vitro Test of Anticancer Activity

In vitro test of anticancer was carried out using WiDr cells and vero cells, cells were inserted into the wellplate, then the cells were incubated for 24 hours, after the cell was ready, the well plate was taken from the incubator then discarded the cell media, then inserted 100 µL PBS into the well cells have been filled, then discard PBS. The concentration of the sample was included in the well (triplo) then incubated in a 5% CO_2 incubator for 24 hours at 37ºC. The MTT 100 µL reagent was added to the well. Cell suspension was incubated again for 2-4 hours in a CO_2 incubator. SDS 100 µL was added when formazan was clearly formed, then wrapped in paper and stored overnight at room temperature in a dark place, the color intensity that occurred was read by ELISA reader at a wavelength of 595 nm.

RESULTS AND DISCUSSION

The extraction process was carried out by maceration using ethanol solvents with a yield value of 4.91%. The study which was carried out by Burhan A. and Aisyah (2018), showing the yield obtained in the extraction of mulberry stems in 3 solvents namely non-melt, ethyl 70% acetate and ethanol, ie 0.34%, 0.859% and 1.51% of the yield obtained showed that the larger polar solvents produced yields, but with the amount of yield obtained not necessarily proportional to the quality of the extract obtained.

Table I. Results of phytochemical screening of mulberry stem extract

No.	Compounds	Result
1	Alkaloid	+
2	Fenol	+
3	Flavanoid	+
4	Tanin	-
5	Saponin	+

Table II. Results of the antioxidant activity of stem extract using the ABTS method

Sample concentrations (ppm)	Absorbance	% inhibition	Probit	IC_{50} Value (ppm)	Equation linear regression
40	0.550	18.599	4.12	83,18	y = 2.8979x – 0.559
80	0.351	48.002	4.95		
120	0.270	60.089	5.25		
160	0.111	83.572	5.99		

Screening of phytochemical compounds aimed to determine the class of compounds contained in mulberry stem extracts. The results of phytochemical screening of mulberry stem extract can be seen in Table I. The results of phytochemical screening Mulberry stem extract showed that the plant contained flavonoid compounds, alkaloids, phenols and saponins. The presence of flavonoid compounds causes mulberry stem extract to have antioxidant potential. Flavonoid compounds have many hydroxyl (OH) groups, hydroxyl atoms can be donated to unstable radical compounds so that radical compounds can be stable, alkaloid compounds also have OH groups so that they can also donate to radical compounds as well as phenol compounds.

Total phenolic test with quantitative test using spectrophotometric method with Folin–Ciocalteu reagent and comparison of gallic acid. Gallic acid is used because gallic acid is a derivative of benzoate hydroxyl acid which is classified as simple phenolic acid and also as a stable and pure standard (Rahmawati, 2009). From the total phenolic test, mulberry stem extract was obtained by 35.9%. According to several studies it is known that the phenolic component has high antioxidant activity, therefore to determine the potential of compounds that act as antioxidants from mulberry stem extract, activity tests were carried out using the ABTS method.

Test for antioxidant activity using the ABTS method. Antioxidant activity testing using the ABTS method, antioxidant activity is known by looking at how much IC_{50} produced by mulberry stem extract in reducing ABTS free radical compounds, IC_{50} shows 50% reduction of free radicals, smaller the IC_{50} is obtained the greater the potential of the extract as an antioxidant. The comparison used is vitamin C as it is known that vitamin C is a compound that is used as one of the natural antioxidants. The result of antioxidant activity can be seen in Table II.

Obtaining IC_{50} from the extract can be calculated using a linear regression equation. The results of IC_{50} obtained at 83.18 µg / mL. From the results of mulberry stem extract measurements IC_{50} results were 83.18 µg / mL, according to Blois (1985), a compound has a very strong antioxidant if the IC_{50} value is <50 ppm, strong if the IC_{50} is worth 50-100 ppm, if the IC_{50} is worth 101-150 ppm, and weak if the IC_{50} is worth 151-200 ppm, according to the classification above the mulberry stem mulberry extract is in the strong category.

Table III. Results of cytotoxic activity of mulberry stem extract on WiDr cancer cells and normal vero cells

Sample concentration (ppm)	% death		IC_{50} value (µg/ml)		Equation linear regression	
	WiDr cells	vero cells	WiDr cells	vero cells	WiDr cells	vero cells
7,8	22,90	1,73	71,24	154,24	y = 1,6724x + 1,9015	y = 1,4603x + 1,8045
15,6	30,49	8,36				
31,2	36,08	18,30				
62,5	44,04	35,30				
125	58,41	43,84				
250	75,03	66,21				
500	93,45	70,21				
1000	97,66	88,35				

One way to prevent the formation of free radicals is to use nutrients that can act as antioxidants such as vitamin E, carotene, vitamin C and other drugs that can capture these free radicals. Free radicals are considered dangerous because they become very reactive in an effort to get an electron pair, the damage that can be caused by free radicals includes damage to cell membranes, proteins, DNA and lipids. The damage can cause various kinds of degenerative diseases, one of which is cancer (Auroma, 1994). To find out the anticancer activity in extracts there are various methods used, one of which is the MTT method.

Anticancer activity test. Anticancer activity test using MTT method using WiDr cells and Vero cells. Anticancer activity is known by looking at IC_{50} values, IC_{50} obtained using probit analysis, probit analysis is usually used to determine the response of the subject under study. The calculation results obtained in testing the anticancer activity of mulberry extract with WIDR cells and Vero cells can be seen in Table III. The toxicity test results of mulberry stem extract on WiDr cells and vero cells can be seen in Table II, showing IC_{50} values for WiDr cells 71,24 µg / ml and for vero cells having IC_{50} values 154,241 µg/mL, toxic boundary determination of this study using National criteria Cancer Institute (NCI) 2009, stated that an extract was declared to have active activity having an IC_{50} value <30 µg/mL, moderate active if it had an IC_{50} value ≥ 30 µg /mL and was said to be inactive if $IC_{50} > 100$ µg /mL, from the calculation results IC_{50} value obtained from mulberry stem extract against WiDr cancer cells has a value of 71.24 µg/mL, the value obtained is in the moderate active range. In this study it was found that mulberry stem extract was moderate active, but did not work selectively because of the results of the selectivity comparison between WiDr cancer cells and vero cell 2.16. A chemotherapy agent is said to have a high selectivity if the SI value is ≥ 3, and is said to be less selective if the SI value is <3 (Rahmawaty, I, 2016).

Compounds that are thought to play an anticancer role in mulberry plants are quercetin and anthocyanin which are potential substances as chemo preventive agents. The anthocyanin type which has the effect of being a chemo preventive agent is cyanidin-3-O-glucoside. In vitro, cyanidin-3-O-glucoside is known to be able to reduce invasion of A549 lung cancer cells and reduce cell motility (Chen, 2006) and quercetin is known to significantly inhibit HL-60 cell growth and can induce differentiation HL-60 cells to express CD 66B and CD 14 antigens (Kim *et al.*, 2000). Quercetin is also known to be able to inhibit development, adhesion and

migration of hela cells and can trigger apoptosis in hela cell cultures.

CONCLUSION

It can be concluded that mulberry stem extract has the value of total phenolic was 35.9 %, while the value of antioxidant activity was 83.18 µg / mL. The value of anticancer activity for WiDr cells IC_{50} value 71.24 µg / mL and for vero cells has IC_{50} value 154.241 µg / mL.

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PROFILE OF ANTICANCER ACTIVITIES OF BROTOWALI (*Tinospora crispa L.*) PLANTS OFVARIOUS REGIONS IN EAST JAWA

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ABSTRACT

Brotowali (*Tinospora crispa L*) is a plant which has potential to be a chemopreventive agent. This study aims to determine the profile of anticancer activity of brotowali stem extracts (*Tinospora crispa L.*), from several regions in East Java. The extraction was carried out by maceration method using 80% ethanol solvent. Then, anticancer activity test was carried out on MCF-7 breast cancer cell model using the Microtetrazolium (MTT) Assay method. The results of the anticancer activity test showed that 15 brotowali stem extracts taken from 5 locations in East Java had significant differences in anticancer activity ($P < 0.05$). Brotowali extracts from Kanigoro, Blitar city, had the highest anticancer activity with an IC_{50} value of 30.64 µg / mL.

Keywords: Anticancer profile; Brotowali (*Tinospora crispa L.*); East Java; Microtetrazolium (MTT) Assay method

INTRODUCTION

Cancer is abnormal and uncontrolled cell growth. These cells can grow further and spread to other parts and can cause death. The type of cancer that mostly attacks women is breast cancer (Siegel *et al*., 2017). In 2013, breast cancer in East Java region ranked second in Indonesia after Central Java, with the incidence number of 61,230 patients (Ministry of Health of the Republic of Indonesia, 2015).

Cancer treatment in general is carried out by surgery, radiotherapy, and chemotherapy (Mutiah, 2017). However, some of the treatments still have limitations, including the occurrence of resistance and the side effects of drugs (Haryanti and Yuli, 2017). Therefore, it is necessary to develop a new drug that has relatively few side effects and is selective for cancer cells; one of which is by using natural materials (Isparning *et al*., 2015).

Brotowali (*Tinospora crispa L.*) is one of the plants that is potential as an anticancer. Previous research proved that the ethanol extract from brotowali plant (*Tinospora crispa L.*) has a cytotoxic effect on MCF-7 cells, HeLa cells, and Caov-3 cells (Adnan, 2016). Brotowali has been known to contain quaternary flavonoids and alkaloids, including apigenin, berberine, palmatine, borapetol a, borapetol b, borapetosid a, borapetosid b and pikroretin (Adnan, 2016). The largest active compound in brotowali (*Tinospora crispa L.*) plant is berberine compounds group. Berberine compounds have the most prominent pharmacological activity in various cancer cells (Rahmatullah, 2014). This compounds can inhibit the growth of MCF-7 and MDA-MB-23 breast cancer cell (Utami *et al*., 2015). This study can be used as a reference that brotowali plants (*Tinospora crispa L.*) are potential for anticancer

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treatment so that they can be developed as phytopharmaceutical drugs.

One obstacle in developing phytopharmaceutical is the variability of compounds content in the medicinal raw material (Verma and Shukla, 2015), that has different activities probably due to external factors and internal factors in each plant (Kim *et al*., 2011). The internal factors of plants are genetic factors and physiological variations, while the external factors (environment) are temperature, humidity, light intensity, water intake, mineral content, sun exposure, and rainfall (Verma and Shukla, 2015). The growing location can also be a possible reason of potential differences in the composition of the chemical content of plants that can influence pharmacological activity (Meisarani & Ramadhania, 2016).

To optimize the quality of raw materials, it is necessary to examine the potential of anticancer in brotowali plants for treatments. This study aims to profile the anticancer activity of brotowali (*Tinospora crispa L.*) plant extracts from several locations in East Java (Tulungagung, Blitar, Malang, Pasuruan and Jombang) against MCF-7 breast cancer cells. By doing so, it can be seen in which areas brotowali (*Tinospora crispa L.*) plants have the most potential anticancer activity against breast cancer cells. Moreover, this study can also be made as a reference for taking raw materials for breast cancer.

METHODS

Materials

The subjects of this research were brotowali(*Tinospora crispa L.*) stems, aged around 8-12 months old which were taken from 5 different regions in East Java, namely Tulungagung (Ngunut, called TcTN; Besuki, called TcTB; Sendang, called TcTS), Blitar (Garum, called TcBG; Kanigoro, called TcBK; Selopuro, called TcBS), Malang (Kedungkandang, called TcMK; Lowokwaru, called TcML; Singosari, called TcMS), Pasuruan (Purwodadi, called TcPPW; Pandaan, called TcPPD; Sukorejo, called TcPS), Jombang (Kabuh, called TcJK, Megaluh, called TcJM; Ploso, called TcJP) . The plant determination was carried out at Materia Medika Batu, East Java, Indonesia with the number of 074 / 354A / 102.7 / 2018.

The solvents used in this study were 80% ethanol (Merck, Germany), chloroform (Merck, Germany), methanol (Merck, Germany), 10% sulfuric acid (Merck, Germany), aquadest, silica gel GF254 (Sigma Aldrich Chemie GmbH, Germany), pure doxorubicin (Sanbe Farma 2 mg/ml, Indonesia). MCF-7 breast cancer cells were obtained from the Parasitology Laboratory of the Faculty of Medicine of Gajah Mada University, Yogyakarta. The cell culture media used were Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA), Dimethyl sulfoxide (DMSO) (Sigma Aldrich Chemie GmbH, Germany), Phosphate buffer saline (PBS) (Gibco, USA), Mikrotetrazolium (Sigma Chemical, USA), Sodium Dodecyl Sulfate (Sigma Co, USA), 10% in 0,1 N HCl and Incubator (Heraeus) at 5% CO_2, 95% O_2 at 37 ° C.

Sample Preparation

A hundred grams of brotowali stem from each region was washed, sorted, and dried. The drying process wascarried out by aerating the stem sunder the sun indirectly, by covering the stems with black cloth for 4-5 days. After the steams were dried, they were grinded.

Moisture Analysis

The moisture analysis was carried out by weighing 0.5 g of simplicia. Then,

the moisture content was measured using the moisture content analyzer (Ohaus, Indonesia). The working principle of this tool is by evaporating the water contained in the sample. The result of water evaporation was then measured as a percentage of moisture content.

Extraction

The extraction of brotowali stem simplicia was carried out using maceration method with Ultrasonic Assisted Extraction(UAE) (Sonica, USA), using 80% ethanol solvent. The ratio between simplicia and solvent was 1:10. ± 10 grams of simplicia was dissolved in 100 ml of 80% ethanol solvent which was divided into 3 macerations. The first maceration was 10 grams of simplicia, dissolved in 35 ml of 80% ethanol in the UAE for 3x2 minutes, and filtered. The filtrate was collected and the residue was macerated again using 35 ml of 80% ethanol in the UAE for 3x2 minutes, then filtered and accommodated. The residue was re-macerated with 30 ml of 80% ethanol. After the residue was re-macerated, it was extracted again with the UAE for 3x2 minutes. Then the filtrate was collected and evaporated using a rotary evaporator (IKA RV10 DIGITAL V, Germany) to produce a thick extract.

Identification of compounds using thin-layer chromatography (TLC)

The identification of compounds was carried out to detect the content of compounds in 80% ethanol extracts of brotowali (Tinospora crispa L.) stems. The identification of compounds was determined using the thin layer chromatography (TLC) and the spots were visualized using TLC Visualizer (CAMAG, Switzerland). The stationary phase used was silica gel F_{254} which was polar. However, the mobile phase used was chloroform: methanol with a ratio of 9.5: 0.5.

The ethanol extract of 10 mg brotowali (*Tinospora crispa L.*) stems was weighed and dissolved in 1 ml 80% ethanol. Then, the solution was bottled in the stationary phase (silica gel F_{254}) as much as 2μm. Then, it was eluted in the chamber which contained the saturated mobile phase (eluant). The plate was sprayed using a 10% H_2SO_4 stain in the fume hood for visualization. Then, it was heated on TLC plate heater (CAMAG, Switzerland) at 105 ° C for 5 minutes then observed under UV lamps with wavelengths of 254 and 366 nm.

Anticancer activity test using MTT method

Anticancer activity tests were conducted in the laboratory of Parasitology, Faculty of Medicine, Gadjah Mada University, Yogyakarta. The MCF-7 cells were cultured with DMEM media and then harvested. Before the cells were planted on 96-well plat, they were counted first with the hermocytometer and the result was $160x10^4$/ml. Based on that, the calculation of how many cells will be planted per well obtained the result of $5x10^4$ cells/well. Then, the MCF-7 cells were distributed to the well plate 96.After that, 100 μl of DMSO solution was added to the extract and doxorubicin (positive control) was made with a certain series of concentrations. Moreover, cell control group was given culture media, the incubation was carried out for 24 hours in an incubator with a flow of 5% CO_2 and 95% O_2. At the end of the incubation, the culture media containing the sample was discarded and then washed with 100 μl of PBS.Then, each well was added by 100 μl of DMEM culture media containing MTT 0.5 mg / ml (dilution 10 x MTT stock 5mg / ml). It was incubated for 4 hours at 37 °C and CO_2 5% flow. Then, the cell condition was observed with a microscope. The observation showed that the living cells reacted with MTT by

forming purple formazan crystals. After 4 hours, the MTT reaction was stopped by adding 100 µl of SDS stopper. After that, the microplate was wrapped and incubated overnight at the darkroom with normal temperature. The test results were read by ELISA reader (Benchmark, Lithonia;Georgia) at a wavelength of 595 nm (Mutiah, 2014).

Data Analysis

The data obtained in the form of absorbance of each well was converted into cell viability percentage by using this formula:

Living cell percentage (%):

$$\frac{(\text{Abs treatment} - \text{Abs media control})}{(\text{Abs cell control} - \text{Abs control media control})} \times 100\%$$

Notes: Abs: absorbance

The percentage of living cells was calculated to obtain the IC_{50} value of concentration. This value of concentration could cause a growth inhibition of 50% of the cell population this is cytotoxic potential could be seen. IC_{50} values are determined by probit analysis using Statistical Product and Service Solution (SPSS) 24.0 for Windows statistics (Mutiah, 2017).

IC_{50} values are divided into three categories: if the IC_{50} values <50 µg / ml, they are categorized as having a strong cytotoxic effect; if IC_{50} values 50 µg / ml - <200 µg / ml, they are categorized as having a moderate cytotoxic effect; if IC_{50} 200 µg / ml - <1000 µg / ml, they are categorized as having a weak cytotoxic effect, and;if IC_{50} values>1000 µg / ml, they are categorized as having no cytotoxic effect (Kuete, 2017).

RESULTS AND DISCUSSION

Moisture Analysis

Moisture content analysis aims to determine the quality and stability of the material. The high water content in simplicia can be a medium for the growth of molds and fungi. In addition, the high water content can also cause an enzymatic reaction that is able to decipher the active substances in simplicia (Salamah and Widyasari, 2015). The results of the analysis of water content in this study showed that the water content of brotowali (*Tinospora crispa L.*) stem powders which were obtained from the average brotowali stem powder was below 10%. Therefore, the results of the analysis of the water content of 15 brotowali (*Tinospora crispa L.*) stem powder can be said to have fulfilled the requirements set by BPOM. It is stated that water content requirements for solid preparations for natural medicine must have a moisture content of ≤10%, except for efferent moisture content, that is ≤5% (Ministry of Health Republic of Indonesia, 2013). The results of the water content analysis can be seen in Table I.

Extraction

The results obtained from maceration extraction were in the form of concentrated dark brown extracts. The yield value of each extract is a parameter to find out the size of the product resulted from the extraction process. However, the calculation was done by dividing the number of products by the number of materials used (Warsono *et al.*, 2013). The results of the rendement calculation can be seen in Table I.

Based on the results of the calculations as shownin Table I, the extract of TcPPW (Purwodadi) has the highest yield value compared to other extracts.It can be seen that the extract of TcPPW (Purwodadi) has many compounds. It is because the higher the yield, the more compound producted (Warsono *et al.*, 2013).

Table I. Results of Maceration Extract of Brotowali Stem (*Tinospora crispa L.*)

Samples	Water content (%) b/b)	Simplicia (grams)	The color of the concentrated extract	The weight of the concentrated extract (gram)	rendement (%) (b / b)
TcTN	9.24	9.059	Deep brown	0.711	7.848
TcTB	9.45	7.000	Deep brown	1.025	14.642
TcTS	7.19	3.316	Deep brown	0.411	12.394
Tc BG	8.40	9.002	Deep brown	1.267	14.075
TcBK	7.69	5.027	Deep brown	0.729	14.502
TcBS	8.09	4.000	Deep brown	0.445	11.125
TcMK	7.85	7.097	Deep brown	0.493	6.946
TcML	8.73	6.402	Deep brown	0.551	8.606
TcMS	7.95	10.226	Deep brown	0.803	7.852
TcPPW	8.76	10.254	Deep brown	1.632	15.916
TcPPD	7.22	4.976	Deep brown	0.383	7.696
TcPS	8.17	3.040	Deep brown	0.043	1.414
TcJK	8.64	10.705	Deep brown	0.320	2.989
TcJM	9.17	10.364	Deep brown	0.381	3.675
TcJP	7.39	6.331	Deep brown	0.415	6.555

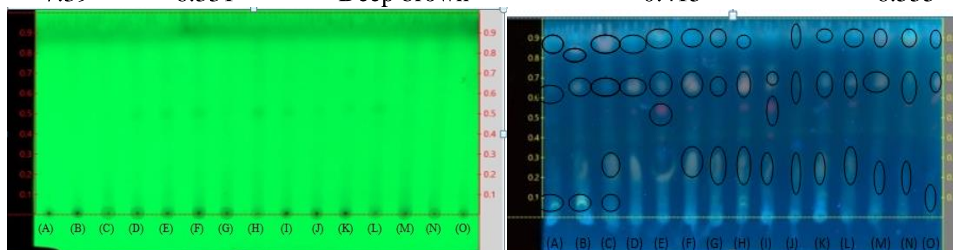

Figure 1. The TLC test results of 80% ethanol extract of brotowali stems using the mobile phase of chloroform: methanol (9.5: 0.5) in the observation of TLC visualizer with wavelengths of 254 nm and 366 nm. (A)TcTN (Ngunut), (B) TcTB(Besuki), (C)TcTS(Sendang), (D)TcBG(Garum), (E)TcBK(Kanigoro), (F)TcBS(Selopuro), (G)TcMK(Kedungkandang), (H)TcML(Lowokwaru), (I)TcMS(Singosari), (J)TcPPW(Purwodadi), (K)TcPPD(Pandaan), (L)TcPS(Sukorejo), (M)TcJK (Kabuh), (N)TcJM(Megaluh), (O)TcJP(Ploso).

The Identification of compounds using thin layer chromatography (TLC)

The results of the observations on compound identification tests showed that there were yellow stains on all brotowali plant extracts from several locations in East Java. It shows the presence of alkaloid compounds in brotowali plants taken from several regions in East Java (Wagner & Bladt, 2001). The results of the observations are shown in Figure 1.

Anticancer activity of Brotowali (*Tinospora crispa. L*) from several regions in East Java

Cytotoxic tests in this study were carried out by using the MTT method. The purpose of the cytotoxic test was to determine the potential of the toxicity of brotowali ethanol extracts on the MCF-7 breast cancer cells. The results of the observations carried out under an inverted microscope showed that there were differences in the morphology of MCF-7 breast cancer cells after the administration of brotowali stem ethanol extracts. The shape of living cells appeared like leaves and sticked to the bottom of the well, while the shape of the dead cells appeared to be round and floated on the surface of the well (Machana *et al*. 2011).

Cytotoxic test of brotowali stem ethanol extracts on MCF-7 breast cancer cells resulted in a reduction of cell

viability (number of living cells) along with increased concentration levels. The higher the concentration, the lower the cellviability. The results of viability of MCF-7 breast cancer cells are shown in Figure 2.

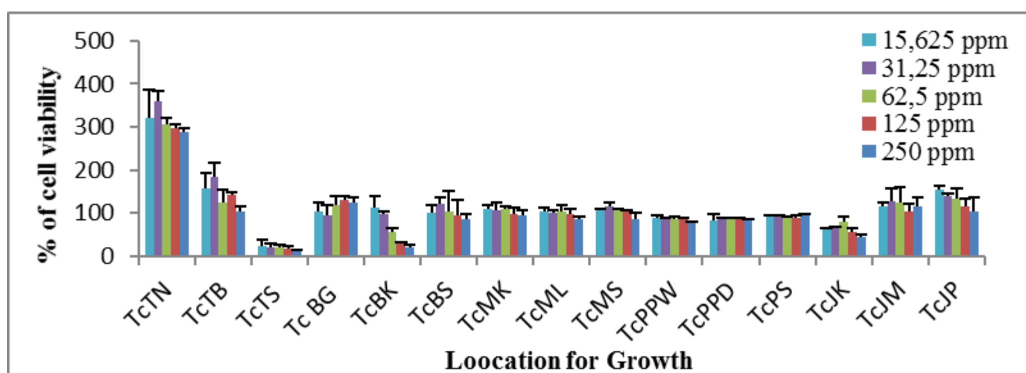

Figure 2. Cell viability of MCF-7 breast cancer cells after administration of brotowali extracts taken from several regions in East Java

Table II. The results of IC_{50} Value of Ethanol Extracts of Brotowali stems from 5 Regions in East Java

Plants	**IC_{50} (µg/ml)±SD**
TcTN	131,48±4,64
TcTB	126,80±9,66
TcTS	140,32±12,28
Tc BG	197,95±12,65
TcBK	30,64±2,18
TcBS	177,12±35,13
TcMK	243,08±63,60
TcML	198,33±9,40
TcMS	254,15±30,77
TcPPW	172,37±16,17
TcPPD	234,89±16,03
TcPS	187,96±19,76
TcJK	177,58±12,25
TcJM	178,95±12,51
TcJP	245,43±25,12
Doxo	21,11±3,047

The results of the data analysis using SPSS 24.0 which show the IC_{50} values of 15 samples of brotowali stem ethanol extracts are presented in Table II.

Based on the data from Table II, the results of IC_{50} values can be categorized as follows: the extracts that are from Kanigoro, Blitar have the IC_{50} value of 30.64 µg/ml and are categorized as having strong cytotoxic potential. Moreover, extracts which are categorized as having moderate cytotoxic potential come from the following regions. Tulungagung: Besuki (IC_{50}126.80µg/ml), Ngunut (IC_{50}131,48µg/ml), Sendang (IC_{50}140.32 µg/ml); Blitar city: Garum (IC_{50}197.95 µg/ml), Selopuro (IC_{50} 177.12 µg/ml); Malang city: Lowokwaru (IC_{50}198.33 µg/ml); Pasuruan city: Purwodadi (IC_{50}172.37 µg/ml) and Sukorejo (IC_{50}187.96 µg / ml); Jombang city: Kabuh (IC_{50}177.58µg/ml), Megaluh (IC_{50}178.95 µg/ml) and Ploso (IC_{50}245.43 µg/ml). The 4 other extracts, namely extracts from Malang city: Kedung Kandang (IC_{50}243.08 µg/ml) and Singosari (IC_{50}254.15 µg/ml); Pasuruan city: Pandaan (IC_{50}234.89 µg/ml); Jombang: Ploso (IC_{50}245.43 µg/ml) are categorized as having weak cytotoxic potential.

The results of the data analysis showed that 15 extracts from 15 sub-districts had significant differences in

their anticancer activity ($p < 0.05$). The variance in anticancer activity was caused by growth location differences, which differ in their altitude, temperature, rainfall, climate, and soil type. The growth location difference is a factor that can influence the content of secondary metabolites of brotowali plants. Furthermore, it also influences their pharmacological activity (Kim *et al.*, 2011). The location characteristics of where the samples were taken can be seen in Table III.

Table III. Location Characteristics Where the Samples were Taken

No.	Samples	Altitude (MDPL)	Average Temperature (ºC)	Rainfall (mm)	Climate	Soil Type
1.	TcTN	106	25,3	1735	Aw	Brownish alluvial gray and alluvial grayish brown; Brownish Regosol
2.	TcTB	146	25,2	1897	Am	Alluvial; Association of Brownish alluvial gray and alluvial; Litosol
3.	TcTS	102	25,4	1742	Aw	Association of Brownish alluvial gray and alluvial; Mediterranean litosol and resina
4.	TcBG	236	24,2	2085	Am	Regosol; Litosol
5.	TcBK	174	24,5	1954	Am	Regosol; Litosol
6.	TcBS	184	24,3	2040	Am	Regosol; Litosol
7.	TcMK	454	23,7	2090	Am	Alluvial; Mediteran; Association of reddish-brown or grayish latosol; Gray chocolate and humus association
8.	TcML	459	23,8	2101	Am	Andosol
9.	TcMS	486	23,8	2203	Am	Alluvial; Mediteran; Association of reddish-brown or grayish latosol; Gray chocolate and humus association
10.	TcPPW	255	25,1	1844	Aw	Alluvial; Mediteran; Labosal; Grumasol; Andosol
11.	TcPPD	345	24,6	2003	Am	Alluvial; Mediteran; Labosal; Grumasol; Andosol
12.	TcPS	254	26,2	1740	Af	Regosol, Gumusol
13.	TcJK	36	26,5	1727	Aw	Grumasol taupe
14.	TcJM	35	26,4	1686	Aw	Mediterranean brown and latosol complex
15.	TcJP	35	26,4	1723	Aw	Association of Gray alluvial and grayish alluvial

Based on the data of the location characteristics where the samples were taken in table 3, there are differences from each location. The results showed that the extract from in Kanigoro sub-district, Blitar city, had the highest anticancer activity (IC_{50}30.64µg / ml) compared to the extracts from 14 other cities. This is due to differences in the content of the compounds that are influenced by internal factors and external factors of each plant (Heuberger *et al.*, 2014). From the results obtained, when it is associated with the characteristics of the location of sampling, altitude can affect the content of compounds contained in plants (Nurnasari, 2010). However, in this study, it has not been proven that altitude can affect the content of plant compounds. This is possible because microorganisms help the process of the plant growth. Therefore, extract from kanigoro had the highest anticancer activity compared to other location (Meisarani & Ramadhania, 2016).

Meanwhile, if it is seen from the type of soil, there is also a difference. Nevertheless, the difference is not quite significant. The differences of soil type result in the different characteristics of one species. Besides, the temperature, rainfall, and also heat correspond to the location. Therefore, these factors can be various (Shukla, 2015). The anticancer activity that plays a role in brotowali plants is the composition of alkaloids that can inhibit DNA topoisomerase II activity (Zuhair and Subchan, 2010). DNA topoisomerase II is an enzyme that removes positive DNA supercoiling which occurs during DNA replication. The mechanism of alkaloid compounds as anticancer also plays a role in activating caspase (Macabeo *et al.*, 2008). The activation of caspase is an alternative to kill cancer cells (Prescott, 2006). Berberine compounds in brotowali also play a role specifically in binding nucleic acids (DNA or RNA) and inducing DNA damage in cancer cells through regulation of DNA topoisomerase activity.Therefore, it can cause cell of cancer to die(Wang *et al.*, 2016).

CONCLUSION

Based on the results of the study, it was found that the ethanol extracts of brotowali (Tinospora crispa L.) stems obtained from several locations in East Java had different anticancer activities. The extract which hadthe highest anticancer activity wasthe extract obtained from Kanigoro, Blitar with an IC_{50} value of 30.64 µg / ml.

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ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC, AND FLAVONOID CONTENTS OF THE EXTRACT OF ENDOPHYTIC FUNGI DERIVED FROM TURMERIC (*Curcuma longa*) LEAVES

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ABSTRACT

Air pollution can increase free radicals that may worsen some diseases. Antioxidants such as phenol and flavonoid compounds are known to counteract these free radicals. Long term use of synthetic antioxidants is known to cause bad effects on the body. Therefore, the necessity to search for natural antioxidants from plants and its endophytic microbes continues with the hope to obtain potential natural antioxidants with minimum side effects. The purpose of this study was to determine the antioxidant activity, total phenolic, and flavonoid contents of the extract of endophytic fungi which were derived from the Bogor-originated turmeric leaves via in vitro isolation. DPPH free radical scavenging method was used to determine in vitro antioxidant activity. The total phenolic and flavonoid contents assays were based on the Follin-Ciocalteu and aluminium chloride reagents, respectively. The extract of endophytic fungi of Bo.Ci.Cl.D1 and Bo.Ci.Cl.D2 showed antioxidant activity with IC_{50} value at 24.04 and 96.08 mg/L, respectively. Total phenolic content of Bo.Ci.Cl.D1 and Bo.Ci.Cl.D2 extracts were 113.47 and 81.83 mg gallic acid equivalent/g extract respectively. Total flavonoid content of Bo.Cl.D1 and Bo.Ci.Cl.D2 extracts were 41.79 and 38.50 mg quercetin equivalent/g extract, respectively. Based on these assays, it could be concluded that the extract of Bo.Ci.Cl.D1 has better antioxidant activities than Bo.Ci.Cl.D2.

Keywords: antioxidant; *Curcuma longa* leaves; endophytic fungi; total flavonoid; total phenolic

INTRODUCTION

Heavy air pollution from motor vehicle fumes and cigarettes can increase the formation of free radicals in the body. Free radicals give impact on the pathogenesis of several diseases in humans due to the occurrence of oxidative cell stress (Sitorus *et al*., 2017). Under normal circumstances, radicals in the body will be eliminated by the body's natural defence mechanism. Materials that can counteract free radicals are called antioxidants. Antioxidants become so important nowadays because of their ability to reduce free radicals and inhibit lipid peroxidation so that they can protect the human body from attacks of several diseases caused by free radical reactions (Khani *et al*., 2017).

The use of synthetic antioxidants to prevent any damage caused by free radicals has been reported to give toxic side effects. Therefore, it is necessary to search for new sources of antioxidants, preferably from nature (Rico *et al*., 2013). One of the natural ingredients reported to have a high antioxidant activity is turmeric (Tanvir *et*

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al., 2017). Other than turmeric's rhizome, the leaves of the turmeric plant also reported showing antioxidant activity (Priya *et al*., 2012). The antioxidant ability of turmeric leaves extract is influenced by the content of phenolic compounds (Faujan *et al*., 2015), especially flavonoid compounds (Gruyal & Rosario, 2013).

Within the tissue of turmeric leaves, the endophytic fungi can also be found. These endophytes are predicted to have the same antioxidant activity. Endophytic fungi were reported to have a high content of polyphenolic compounds (Yadav *et al*., 2014). Endophytic fungi are fungi that live in the host plant tissues without causing disease and form mutualism association (Nicoletti & Fiorentino, 2015). Some endophytic fungi isolated from turmeric roots, stems, rhizomes, and tubers were reported to produce antioxidant compounds (Bustanussalam *et al*., 2015). This study aimed to examine the antioxidant activity, total phenol, and total flavonoid contents obtained from the in vitro extract of turmeric leaves endophytic fungi from Bogor using the DPPH free radical scavenging method.

METHODS

Materials

Endophytic fungi isolated from the leaves of some turmeric plants were obtained from the private garden of residents in sub-district of Tanah Sareal, Bogor. Two isolates were distinguished based on macroscopic, morphological characteristics. The identity of plant was confirmed at the Herbarium Bogoriense, Research Center for Biology, LIPI with number of certificate confirmation of 1145/IPH.1.02/If.8/VII/2012. Reagents and solvents used in this research: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma), ascorbic acid (Sigma), Follin-Ciocalteu (Merck) reagents, gallic acid (Sigma, >97%), sodium carbonate (Merck, 99%), quercetin (Sigma, >95%), aluminum chloride (Merck, >98%), sodium acetate (Merck, 99%), methanol (Merck, for analysis 99%), ethanol (Merck, for analysis 99%), 96% technical ethyl acetate, Potato Dextrose Agar (PDA) (Difco), Potato Dextrose Broth (PDB) (Difco), and aquades. The tools used in this study were rotary vacuum evaporator (Stuart), UV-vis spectrophotometer (Hitachi U-3900H), cuvette (Hellma), laminar air flow, analytical balance (Precisa 240A), water bath incubator (Grant), oven (Jouan), magnetic hotplate stirrer (Thermolyne), sonicator (Branson), shaker (Thermolyne), Buchner funnel, Erlenmeyer, microtube (Axygen), scale test tube, volumetric flask, micropipette (Eppendorf), micropropylene tip, autoclave (Tomy) and other glassware.

Endophytic Fungi Fermentation and Extraction

Isolation of endophytic fungi was performed using the surface sterilization method and grown on PDA, both later called by their isolate codes of Bo.Ci.Cl.D1 and Bo.Ci.Cl.D2, which were distinguished based on the morphological character macroscopically. Fungal colonies were rejuvenated on PDA and incubated for seven days at room temperature. The endophytic fungi isolates, which were seven days old, were then transferred into 100 mL of PDB in 250 mL Erlenmeyer flask. For making endophytic fungi growth curves, fermentation was carried out on a shaker 120 rpm for 22 days at room temperature and then harvested every two days. The biomass obtained was then dried and weighed. Fermentation to obtain the extract was carried out on a shaker 120 rpm for 14 days (stationary phase) at room temperature. After 14 days, the endophytic fungi filtrate and biomass were separated by filtration using sterile filter paper in a vacuum Buchner funnel. The filtrate was then extracted three times using ethyl acetate in the separated funnels and concentrated using a rotary evaporator until dried (Salini *et al*., 2015).

Antioxidant Activity

The antioxidant activity assay was performed by free radical scavenging method (Tiwari *et al*., 2006) using DPPH with a modification on its wavelength of 517 nm. The concentrations of the test solution were 5, 10, 25, 50, and 100 mg/L. Ascorbic acid (vitamin C) as the standard was prepared in level concentrations of 1, 3, 5, 7, and 9 mg/L, whereas the concentration of DPPH as control was 0.4 mM. All samples, control, and standard solutions were incubated at 37 °C for 30 minutes for then measured at 517 nm. Antioxidant activity was obtained using the equation below and IC_{50} value, a number that shows the concentration of test samples capability to inhibit the oxidation process by 50%, which was obtained by making a linear curve between the concentration of the test solution (x-axis) and antioxidant activity (y-axis).

$$\% \text{ Inhibition} = (A - B) / A \times 100\% \quad \ldots(1)$$

Note: A = blank absorption
B = absorption of test material

Total Phenolic and Flavonoid Contents

The concentration of ethyl acetate extract from isolated endophytic fungi in ethanol was 1000 µg/mL. The total phenolic assay was started by making the standard solution of gallic acid in distilled water with concentration series of 20; 40; 60; 80; 100; and 120 mg/L to make the standard curve of gallic acid. A total of 1 mL of extract (test sample) and gallic acid (standard) were put into separate test tubes and later 0.1 mL of Follin-Ciocalteu reagent and 0.9 mL of distilled water were added into each tube. Each mixture was then incubated at room temperature for 5 minutes. 1 mL of sodium carbonate 7% and 0.4 mL of distilled water were added into the test tube and incubated for 30 minutes at room temperature. The uptake of the mixture was measured at 765 nm. Total phenolic content is expressed as mg gallic acid equivalent to per gram of dried extract (mg GAE/g dry weight) (Pekal & Pyrzynska, 2014).

The standard quercetin solution in ethanol with concentration series of 10; 15; 20; 25; 30; 35; and 40 mg/L was measured to make the standard curve of quercetin in the assay of total flavonoid content. 500 µL of extract (test sample), quercetin (standard), and ethanol (blank) were put into separate test tubes and later 1.5 mL of ethanol was added into each tube. Then, 0.1 mL of aluminum chloride solution (0.1 g / mL), 0.1 mL of sodium acetate (1 M) and 2.8 mL of distilled water were also added. Each mixture was then incubated for 30 minutes at room temperature and then measured at 415 nm. Total flavonoid content is expressed as mg quercetin equivalent to per gram of dried extract (mg QE/g dry weight) (Kaur & Singh, 2015).

RESULTS AND DISCUSSION

Endophytic Fungi Fermentation and Extraction

Fermentation was done by shaken culture fermentation method to maintain aeration and agitation. Aeration is needed to supply endophytic fungi oxygen while agitation or stirring aims to increase oxygen supply in the medium as well as to maintain temperature homogeneity (Kumala & Pratiwi, 2014). The isolates were spherical when grown in liquid fermentation media and the color of mycelium was identical to the color when grown on agar media. Bo.Ci.Cl.D1 isolate mycelium was black, while Bo.Ci.Cl.D2 isolate was yellowish pink. The harvesting of endophytic fungi was carried out during the stationary growth phase (Figure 1) because, in general, fungi will produce secondary metabolites during this phase (Basha *et al*., 2012). Secondary metabolite compounds will be released into fermentation media. Therefore, the filtrate extract of endophytic fungi contains more secondary metabolites in terms of weight than the mycelia or biomass (Bustanussalam *et al*., 2015).

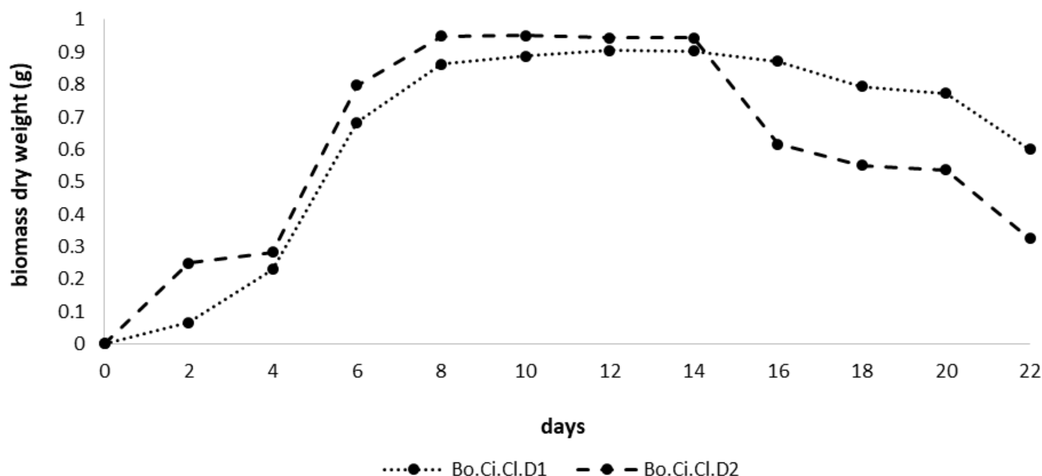

Figure 1. Growth curve of endophytic fungi derived from turmeric leaves of isolate 1 (Bo.Ci.Cl.D1) and isolate 2 (Bo.Ci.Cl.D2)

Antioxidant Activity

The assay of antioxidant activity showed that both ethyl acetate extract of the filtrate isolates have antioxidant activity (Table I). The test results also showed that both extracts have an inhibition above 50% at the concentration of the test material of 100 mg/L. The extract of Bo.Ci.Cl.D1 isolate had a lower IC_{50} value compared to the extract of Bo.Ci.Cl.D2 isolate; hence, the extract of Bo.Ci.Cl.D1 isolate has a better antioxidant activity (Table I).

The strength of antioxidant activity are categorised into very active categories (IC_{50} <10 mg/L), active (IC_{50} <100 mg/L), and inactive (IC_{50}> 100 mg/L) (Minami *et al*., 1994). The antioxidant activity of both extracts are categorised as active since the IC_{50} value of both isolates were <100 mg/L. However, both showed lower activity compared to ascorbic acid as the positive control with an IC_{50} value of 3.88 mg/L which is in the very active category (Minami *et al*., 1994). The extract of endophytic fungi of Bo.Ci.Cl.D1 showed a better antioxidant activity compared to the ethanol extract of turmeric leaves, obtained from the previous study conducted by Faujan *et al*. (2015), which IC_{50} value was 85 mg/L.

DPPH scavenging method is commonly used in antioxidant studies. The antioxidant effect in the DPPH free radical scavenging method occurs because of the ability of a compound to donate hydrogen (Babu *et al*., 2013). The extract of endophytic fungi isolated from *Rhodiola crenulata*, *R. angusta*, and *R. sachalinensis* plants were reported to have good antioxidant abilities (Cui *et al*., 2015). Antioxidant activity of Bo.Ci.Cl.D1 extract showed a better activity with IC_{50} value of 24.04 mg/L, compared with the activity of endophytic fungi extract from turmeric plant rhizomes with the IC_{50} value of 32.28 mg/L, reported in the previous study (Septiana *et al*., 2017).

Table I. Antioxidant activity from endophytic fungi extracts derived from turmeric leaves

No	Sample code	Concentration (mg/L)	Inhibition (%)±SD	IC_{50} (mg/L) ±SD
1	Bo.Ci.Cl.D1	5	19.36±0.18	
		10	31.28±0.36	
		25	50.83±0.27	24.04±0.11
		50	73.08±0.54	
		100	76.54±0.18	
2	Bo.Ci.Cl.D2	5	1.54±0.36	
		10	1.60±0.63	
		25	12.11±0.27	96.08±0.06
		50	28.78±0.09	
		100	50.83±0.09	
3	Vit. C	1	22.64±2.67	
		3	41.73±5.11	
		5	69.60±1.07	3.88±0.14
		7	90.35±0.53	
		9	93.75±0.91	

Table II. Total phenolic and flavonoid contents of endophytic fungi extracts derived from turmeric leaves

No.	Sample code	Total Phenolic Content (mg gallic acid equivalent/g dry weight) ±SD	Total Flavonoid Content (mg quercetin equivalent/g dry weight) ±SD
1	Bo.Ci.Cl.D1	113.47±0.25	41.79±0.07
2	Bo.Ci.Cl.D2	81.83±0.14	38.50±0.13

Total Phenolic and Flavonoid Contents

The ability of endophytic fungi extract to give positive results in the antioxidant activity assay is related to the composition of its chemical compounds. Some of the chemical compounds in endophytic fungi that are active as antioxidants are phenols and flavonoids. Phenol and flavonoid compounds, contained in some endophytic fungi from plants *Calotropis procera,* are acting as antioxidants (Nagda *et al*., 2017).

The phenolic and flavonoid contents of Bo.Ci.Cl.D1 extract were higher than Bo.Ci.Cl.D2 (Table II). The result supported the antioxidant activity result, in which Bo.Ci.Cl.D1 also showed better activity than Bo.Ci.Cl.D2 (Table I). The results of this study are in line with previous studies which stated that antioxidant activity has a very close relationship with the levels of phenols and total flavonoids. The higher content of total phenols and flavonoids, the higher the antioxidant activity (Esmaelli *et al*., 2015).

The phenols (in mg GAE/g DW) and flavonoids (in mg QE/g DW) in an endophytic fungi extract can be divided into several levels. Endophytic fungi extract with total phenol content of <20 mg/g are categorised in the low category, 20-40 mg/g in the moderate category, and >40 mg/g in the high category. Endophytic fungi extract with a total flavonoid content of <15 mg/g are in the low category, 15-30 mg/g in the moderate category, and >30 mg/g in the high category (Zohri *et al*., 2017). Therefore, the total phenolic and flavonoid contents of the two extracts in this study could be categorised as high due to their total phenolic contents which was >40 mg/g and flavonoid contents which was >30 mg/g (Table II).

The phenolic contents of both endophytic fungi Bo.Ci.Cl.D1 and Bo.Ci.Cl.D2 extracts are higher than the phenolic contents of turmeric leaves in ethanol extract reported in the previous study which was only 8.86 mg GAE/g DW (Faujan *et al*., 2015). However, the flavonoid contents of both extracts were

still lower than those of turmeric leaves in ethanol extract from the same study, which reported to contain 141.09 mg QE/g DW (Gruyal & Rosario, 2013). The mechanism of flavonoids as antioxidants can occur through several mechanisms. One possible mechanism is the ability of flavonoids to bind free radical immediately by donating a hydrogen atom or transfer a single electron (Prochazkova *et al*., 2011). In this study, each extract showed different ability to reduce free radicals, despite being originated from the same part of the plant. The different antioxidant abilities are caused by the fact that each endophyte may produce different compounds with the same function or different amounts depending on their interaction with the host plant (Selim *et al*., 2012).

CONCLUSION

The ethyl acetate extract of endophytic fungus of Bo.Ci.Cl.D1 from turmeric leaves cultivated in Bogor showed a better antioxidant activity and higher total phenolic and flavonoid contents compared to Bo.Ci.Cl.D2.

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THE SUB-CHRONIC TOXICITY TEST OF MENIRAN *(Phyllanthus niruri L.)* AND PEGAGAN *(Centella asiatica)* EXTRACT IN WISTAR STRAIN RATS ON LIVER AND KIDNEY FUNCTION

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ABSTRACT

Meniran (Phyllantus niruri L.) and Pegagan (Centella asiatica L.) was proven had activity that considered as an antioxidant. However, the safety was not confirmed. This research is intended to evaluate sub-chronic toxicity of standardized combination extract of meniran and pegagan on liver and kidney function. This research used post-test-control design. Twenty female and twenty male Wistar strain rats divided into 4 groups. The first group was controlled in dosage of CMC Na 0.5%. The others were given a combination of meniran and pegagan extract in ratio with different dosage (50:50; 250:250; and 1250:1250 mg/KgBW). Sub-chronic toxicity test of meniran and pegagan combination was given orally once a day for 28 consecutive days. On the 29th day, all the rats were sacrificed and blood samples were analyzed using automatic analyzer. SGOT, SGPT, BUN, and creatinine value were statistically analyzed using one way ANOVA with post hoc LSD ($p<0.05$). The results show that treatment of meniran and gotu kola combination had no significantly different of SGOT and creatinine value of male and female rats, and SGPT value of male rats ($p>0.05$). The treatment of meniran and gotu kola combination had significantly different of BUN value of male and female rats, and SGPT value of female rats ($p<0.05$) but still within normal range. Based on this study, it can be concluded that orally administered of meniran and gotu kola extract combination at doses 50:50 mg/KgBW; 250:250 mg/KgBW; and 1250:1250 mg/KgBW had no affected on SGOT, SGPT, BUN and creatinine value of Wistar strain rats on sub-chronic administration for 28 consecutive days.

Keywords: *Phyllantus niruri* L.; *Centella asiatica* L.; subchronic toxicity; liver and kidney function

INTRODUCTION

Meniran (*Phyllantus niruri* L.) and *Pegagan* (*Centella asiatica* L.) are the most found plants in Indonesia as herbal or traditional cure. *Pegagan* has substances, comprising of triterpenoid saponin, flavonoid, essential oil, phytosterol and other active materials (Kristanti, 2010). Additionally, *Meniran* has many advantages derived from substances of various chemical substances, such as alkaloid (securinine), flavonoid (quercetin, quercitrin, isoquercitrin, astragalin, niruri, rutine, leucodelphinidin, and galocatecine), and lignan (filantine and hipofilantine) (Mangunwardoyo *et al.*, 2009).

Utilization of both plants as traditional cure require further research to prove feasibility and security. A study of Fitrianingsih (2017) postulated that combination of *meniran* and celery at dosage of 100:50 mg/KgBW can decrease significantly the extent of blood urea nitrogen (BUN) ($p>0,05$), while, at

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dosage of 100:100 and 50:100 mg/KgBW, it has not decreased the level of BUN and creatinine of rats induced by gentamycin significantly (p>0,05). Other researches stated that there are components of *meniran* and *pegagan* having adverse effects on body's organ. Research performed by Praptiwi (2010) argued that saponin substance is main component of *pegagan* extract that can cause hemolysis and decrease surface pressure, so that it can result degeneration and sinusoid congestion, color changing and there is white spot in liver caused by cell destruction and leucocyte accumulation. Further, a study done by Mulyadi (2010) set forth that tannin substance in *meniran* can provide worse depiction of histopathology against mice's gastric and duodenum at dosage administer up to 2.000 mg/kgBW. Anfiandi (2013) then reported that infuse of *pegagan* leaves at dosage of 1.500 mg/kgBW can provide teratogenic effect against mice, such as physical defect, stunting, and hemorrhagic on mice's fetus. Therefore, it is necessary to design a test to acknowledge security from utilization of *meniran* and *pegagan* extract combination.

Sub-chronic toxicity testing of *meniran* extract at dosage of 2.000 mg/KgBW and 5.000 mg/KgBW showed that there is no significant difference against the content of *serum glutamic oxaloacetic transaminase* (SGOT) and *serum glutamic pyruvic transaminase* (SGPT) of rats after 14 days of administering (Asare *et al.,* 2011). While, Sujono *et al.* (2015) stated that administering of ethanol-*meniran* extract at dosage of 50 mg/KgBW and 250 mg/KgBW does not cause significant difference against decreasing SGPT level measured in the 0-day and 90-day at Sprague Dawley strain rats. Another research depicted that repeated dosage of *pegagan* extract at 9.07 mg/20 grams to mice or equivalent to 63.49 mg/200 grams of rats can cause changes of liver and kidney (Praptiwi, 2010).

Based on above description, this research is significantly to be performed aimed to analyze sub-chronic toxicity against liver and kidney function from both *meniran* and *pegagan* extract combination since both extracts have activity as anti-oxidant.

METHOD

Materials and Tools

Materials employed in this research were standardized dried *meniran* extract (number of bets 049PP02.2) and non-specifically standardized dried *pegagan* (number of bets 056PP01.2) obtained from PT. Industri Jamu Borobudur, *sodium carboxymethil cellulose* (CMC Na) and aquadest derived from Laboratory of Pharmaceutical Technic of Ahmad Dahlan University, reagent tube obtained from Laboratory of Parahita, and male and female Wistar strain rats aged 2-3 moths and weighted 100-300 grams obtained from Integrated Testing and Research Laboratory, Universitas Gadjah Mada, Yogyakarta.

In addition, tools used was measuring glass (*Pyrex*, Indonesia), volumetric flask (Iwaki, Indonesia), measuring pipette (Iwaki, Indonesia), beaker glass (*Pyrex*, Indonesia), spatula, orally injection needle, 5-cc syringe, analytic weight (Sartorius, Indonesia), electric stove, a series of surgery tools (nippers, scalpel, blade, scissor), flacon, vacutainer tube, and automatic analyzer (Sysmex XT-2000i).

Method

Designing 0.5 % of CMC Na

CMC Na with concentration 0.5 % was used to suspense dried *meniran* and *pegagan* extract. The manufacturing, 0.5% of CMC Na, was by weighting 0.5% of CMC Na as 0.5 grams and solved it little by little into 100,0 mL of heated aquadest. Such mixing was then

homogenously stirred until colorless liquid was obtained. Further, 0.5% solution of CMC Na utilized to suspense dried extract was always made freshly every time it would be used.

Designing of *Meniran* and *Pegagan* Suspension Extract

The design of suspension extract was always made freshly as it would be administered to test rats for each group. It was made from stock solution by concentration of 25% b/v. At dosage of 1250 mg/kgBW, it was made by weighing each extract of 12.5 grams. Next, it was suspended with 0.5% of CMC Na and 50.0 mL of ad, so that 25% b/v of concentration was obtained. Further, this design of suspension at dosage of 250 mg/kgBW and 50 mg/kgBW used following formulation:

$$V_1M_1 = V_2M_2 \quad (1)$$

Solution at dosage of 50 mg/kgBW was made by taking 1,0 mL of suspension stock and 25,0 mL of ad using 0,5% of CMC Na, so that 1% b/v of concentration was obtained. The design of suspension at dosage of 250 mg/kgBW was made by taking 5,0 mL of suspension stock and 25,0 mL of ad, using 0,5% of CMC Na, so that 5% b/v of concentration was obtained. As an example of calculation for dosage administered to every 200 grams of rat at dosage of 50 mg/KgBW, it can be seen in following equation:

$$\frac{Dosage\ of\ extract\ (mg)}{1000\ grams} = \frac{Dosage\ given}{200\ grams} \quad (2)$$

$$\frac{50\ mg}{1000\ grams} = \frac{Dosage\ given}{200\ grams}$$

Dosage given = 5 mg

As an example of calculation for administering volume for every 200 grams of rat at dosage of 50 mg/KgBW, it can be seen in following equation:

$$Administering\ volume = \frac{Dosage\left(\frac{mg}{KgBB}\right) x\ 0{,}2\ Kg}{Extract\ concentration} \quad (3)$$

$$Administering\ volume = \frac{50\ \frac{mg}{KgBB}\ x\ 0{,}2\ Kg}{1\%\ b/v}$$

$$Administering\ volume = \frac{50\ \frac{mg}{KgBB}\ x\ 0{,}2\ Kg}{10\ mg/mL}$$

Administering volume = 1 mL

Treatment of Test Rats

The research was performed in the Laboratory of Faculty of Pharmacy, Ahmad Dahlan University, Yogyakarta. Administratively, this procedure had obtained letter of statement of Committee of Research Ethics, Ahmad Dahlan University under No. 011803043. This study, then, was according to Regulation of Head of Food and Drug Supervisory of the Republic of Indonesia Number 7 of 2014 on Manual of Non-Clinical Toxicity Test by In Vivo (Anonim, 2014). Wistar strain rats thus were selected randomly, and 5 rats were classified into male and female group, respectively. Test rats were previously adapted for 1 week prior to treatment process. Treatment of each test group can be seen in following Table I.

Table I. Treatment of Test Rats

No.	Group	Description
1.	Group I	Administered feed + drink and suspension of 0,5% of CMC Na
2.	Group II	Administered feed + drink and extract combination of *meniran* and *pegagan* at dosage of 50:50 mg/kgBW
3.	Group III	feed + drink and extract combination of *meniran* and *pegagan* at dosage of 250:250 mg/kgBW
4.	Group IV	feed + drink and extract combination of *meniran* and *pegagan* at dosage of 1250:1250 mg/kgBW

Clinically Bio-chemical Test

At the 29th day, sampling through *sinus orbitalis* was performed, which the blood was contained into vacutainer tube. It then was tested in the Laboratory of Parahita to know the value of SGOT, SGPT, BUN, and creatinine.

Data Analysis

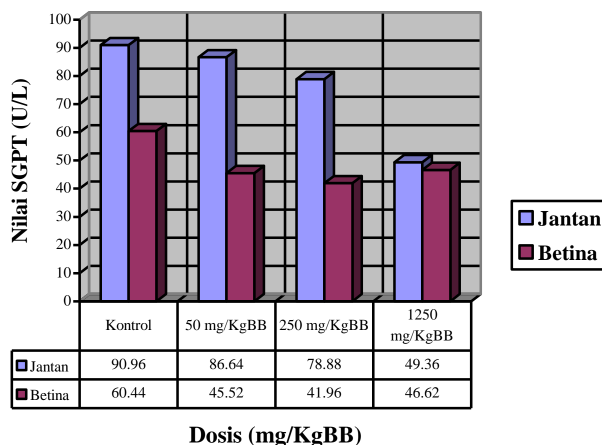
The result of this research gained data comprising of the level of SGOT, SGPT, BUN, and creatinine for each group, respectively. Such data was statistically analyzed. Data analysis of clinically bio-chemical parameter employed SPSS (statistic product and service solutions). The first analysis was distribution test using the Shapiro Wilk test to examine data distribution, and the Levene test was aimed to see data homogeneity. If data was normally and homogenously distributed, parametrically statistical test of one-way ANOVA was done using SPSS by reliable degree of 95% to acknowledge which group showed difference.

RESULT AND DISCUSSION

This study is aimed to know sub-chronic toxicity of *meniran* and *pegagan* extract combination against liver and kidney function during 28 days of orally administering with various dosage levels. It utilized test rats of male and female Wistar strain as many as 40 rats.

SGPT was mostly found in liver, so that SGPT was more specific to detect disease than SGOT. The measurement result of SGPT for male and female can be seen in following Figure 1, and for female, it can be viewed from Figure 2.

According to Figure 1, the analysis result of one-way ANOVA depicts that there was no significant difference toward SGPT value of male rats, showed by its significant value of 0,105 (p>0,05). Average value of SGPT from male rats in controlled group was 90.96 ± 24.22 U/L. The SGPT had consecutive decreasing value than controlling group did. The higher dosage of *meniran* and *pegagan* extract combination, the more decreasing the SGPT level of test rats. Based on such result, the extract combination of *meniran* and *pegagan* could prevent the increasing value of SGPT on male rats. It is probably because *meniran* and *pegagan* contain polyphenol and triterpenoid, having activity as antioxidant, so that it can decrease enzyme level produced by liver, as cell necrosis takes place (Hashim *et al.,* 2011; Hu, 2011).



	Kontrol	50 mg/KgBB	250 mg/KgBB	1250 mg/KgBB
Jantan	90.96	86.64	78.88	49.36
Betina	60.44	45.52	41.96	46.62

Figure 1. The average SGPT value of male and female rats after the administration of a combination of Meniran and Gotu kola extract for 28 days

Meanwhile, the analysis result of one-way ANOVA in the Figure 1 shows that there was no significant difference in the SGPT value of female rats, depicted by its significant value of 0,059 (p>0,05). Average SGPT value of female rats in the controlled group was 60.44 ± 15.45 U/L. The SGPT value of each group administered extract combination was lower than controlling group. However, such decreasing was in normal level of rats' SGPT, which was 35.9–81.6 U/L (Wicaksono, 2010). Based on such result, extract combination of *meniran* and *pegagan* could prevent increasing value of SGPT on female rats. Further, the decreasing value of SGPT in treatment group under administering of *meniran* and *pegagan* extract combination could happen since *meniran* contains filantine, having activity as antioxidant, so that it can protect liver from cell degeneration or impairment.

In detail, based on Figure 1, the SGPT value of male group was higher than female group. This was caused by physiology factor of those test rats. While, statistical result demonstrates that there was no significant difference on decreasing value of SGPT on male and female rats (p>0.05). Further, it can be said that administering of *meniran* and *pegagan* extract combination to male and female Wistar strain rats would not interfere the SGPT value.

Subsequently, SGOT is enzyme existed in body to promptly detect periphery circulation, if there is trauma or necrosis in certain tissue. Also, SGOT is greatly found in any tissue other than liver, such as heart. In general, changing level of SGOT is related to liver disease, but it is also possible that the SGOT changes also take place due to heart disease (Yuwono *et al.*, 2016). The calculation result of SGOT for group of male rats is shown in Figure 2, while female rats is in Figure 2.

According to Figure 2, the analysis result of one-way ANOVA in the Figure 2 describes that there was no significant difference of the SGOT value of male rats, depicted by its significant value of 0.354 (p>0,05). Averagely, the rate value of SGOT from female rats in controlled group was 173.76 ± 34.87 U/L. Moreover, the SGOT value derived from administering of extract combination at dosage of 250:250 mg/kgBW was higher than controlling group, which was 180.48 ± 16.85 U/L. Nonetheless, such increasing was insignificant. Liver impairment can be seen from three folds increasing of SGOT and SGPT parameter from normally upper range (Maria, 2014). Then, the result obtained from the measurement of male's SGOT level (Figure 2) is that increasing of SGOT level had not reached more than three folds of normally upper range, so, as consequence, it can be said that there was no impairment of liver.

In addition, the analysis result of one-way ANOVA in the Figure 2 depicts that there was significant difference at the SGOT value of female rats, shown by its significant value of 0,026 (p<0,05). In average, the SGOT value of female rats under controlled group was 174.5 ± 23.37 U/L. The SGOT value in each group administered with extract combination was lower than controlling group, yet it was still under normal range (35.7-168 U/L) (Wicaksono, 2010). Additionally, significant difference existing between controlling group against group administered with *meniran* and *pegagan* extract at dosage of 1250:1250 mg/KgBW was shown by its significant value of 0.003 (p<0.05).

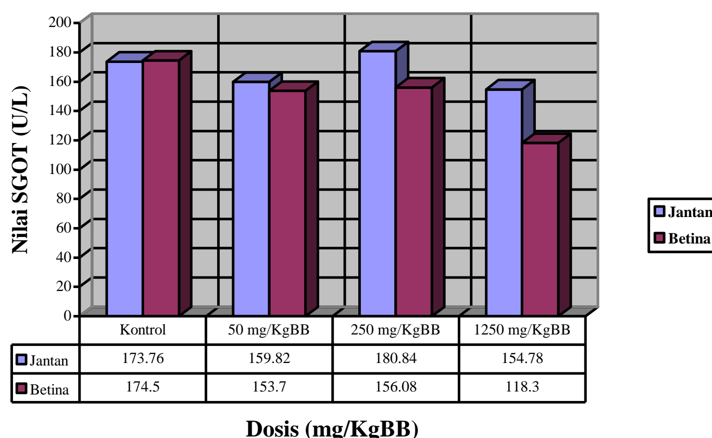


Figure 2. The average SGOT value of male and female rats after the administration of a combination of Meniran and Gotu kola extract for 28 days

Hence, according to such results, extract combination of *meniran* and *pegagan* could prevent increasing value of the SGOT at female rats. While, decreasing value of the SGOT was probably caused by polyphenol, contained in *pegagan* (Sutardi, 2016). Polyphenol has substance included in class of primary antioxidants, such as phenol substances enabling to cut off chain reaction of fatty-acid-free radical formation (Hu, 2011).

According to Figure 2, further, the SGOT value at male group was higher than female group was. It was probably caused that male rats had heavier muscle mass. Similarly, Tripathi and Hall (2016) argued that SGOT is mostly found in muscle, skeletal, heart, and brain. Therefore, the SGOT value of male rats is higher than female rats. Meanwhile, the statistical result demonstrates that there was no significant difference of the SGOT value found in male rats (p=0.354). Contrastingly, the statistical result of the SGOT value found in female rats had significant difference against controlling group (p=0.026). However, such decreasing was still in normal range, so that it can be concluded that administering of *meniran* and *pegagan* extract combination at male and female Wistar strain could not interfere the SGOT value.

Moreover, the level of BUN is final product from protein metabolism synthesized inside liver and secreted almost 75% through kidney without experiencing molecule changes. Increasing of BUN level in blood could show impairment of kidney function. The result of the BUN level derived from this research is then shown in following figures as attached.

Based on Figure 3, significant value from statistical test of one-way ANOVA was 0.025 (p<0.05), meaning that there was significant difference. This result depicts that average value of BUN at 14.20 ± 2.61 was lower than controlling group (0,5% of CMC Na), while the group III and IV demonstrated more significant increasing of BUN's average value than controlling group, which was 20.16 ± 4.23 and 19.00 ± 1.97, respectively, with increasing value of BUN where was still under normal range. In addition, normal value of BUN on mice was 13.9-28.3 mg/dL (Anshar, 2015). Based on above result, increasing value of BUN was necessarily cautioned since it could show impairment of kidney function (Haryoto *et al.*, 2015). Main function of kidney is to regulate liquid and electrolyte balance and body's basal and acid to create figure environment for tissue and cell metabolism (Miriam and Wallace, 1998).

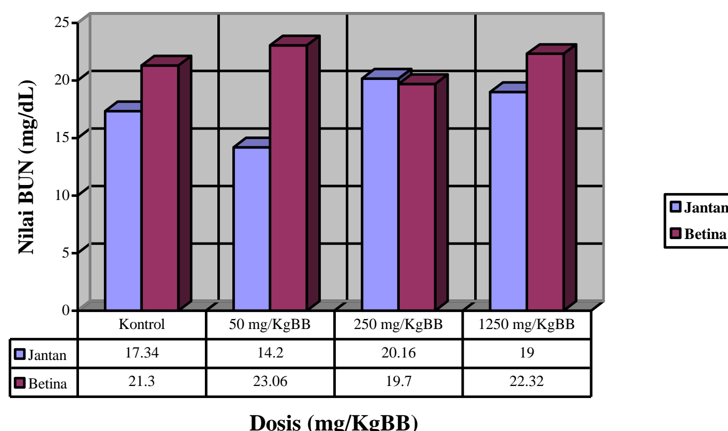


Figure 3. The average BUN value of male and female rats after the administration of a combination of Meniran and Gotu kola extract for 28 days

Figure 3 postulates that value of statistical for the BUN value on female rats was 0.031 (p>0.05), meaning that there was significant difference. Thus, from Figure 3, it is known that group administered with *meniran* and *pegagan* at dosage of 250:250 mg/KgBW showed decreasing value of the BUN as 19.70 ± 0.52 rather than controlling group (0.5% of CMC Na), whereas the group II had BUN value of 23.06 ± 0.82; and the group IV had 22.32 ± 0.67. Improvement of the BUN value was occurred on both groups than controlling group. Such increasing happened in the group II and IV of female rats, yet it was still in normal range.

Next, based on Figure 4, the analysis result of one-way ANOVA gained was significant value of 0,590 (p>0.05). It means that there was no significant difference. Such result shows that there was increasing of creatinine value of three groups than controlling group (0.5% of CMC Na). The group II demonstrated creatinine's average value of 0.56 ± 0.07; the group III was 0.52 ±0.09; and the group IV was 0.56 ± 0.08. Creatinine's reference value of Wistar rats was 0.2-0.8 mg/dL and it was secured due to below normal range (Amir *et al.*, 2015).

Improvement of creatinine serum became impairment index and kidney disfunction. Kidney ability to filter non-protein nitrogen (NPN) (metabolism product of phosphate creatinine) is lessen during kidney disfunction. It was due to declining glomerulus filtration (Reddy *et al.*, 2012).

Lastly, based on Figure 4, it postulates that significance value of statistical test with one-way ANOVA as 0,181 (p>0.05). It means that there was no significant difference. Moreover, the result depicts that the group II was more improving than controlling group (0.5% of CMC Na). Thus, average value of creatinine at the group II was 0.61 ± 0.08, while the controlling group was 0.56 ± 0.09. The average value of creatinine at the group III was 0.51 ± 0.03, and the group IV was 0.56 ± 0.06. Hence, there was decreasing occurred in the group III and IV, yet it was insignificant.

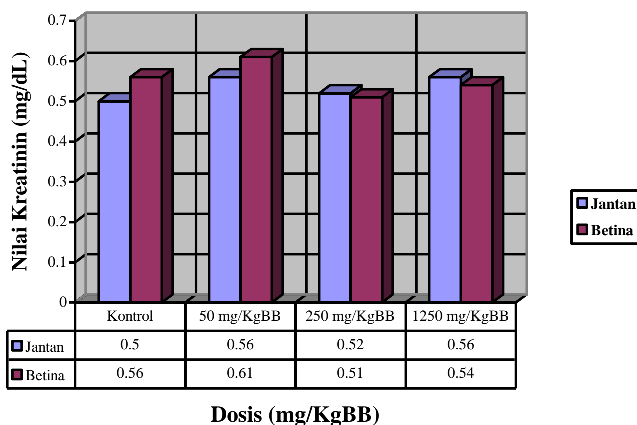


Figure 4. The average creatinine values of male and female rats after administration of a combination of Meniran and Gotu kola extract for 28 days

Any impairment of kidney function in determining minor, moderate, or major severity could be known from total value of glomerulus filtration (GFR) (Hutagaol, 2017). Also, other supporting impairment of kidney, such as kidney histopathology, usually depicted inflammation or focally lymphocyte cells infiltration and bleeding (Winarno and Sundari, 2011).

From above research's findings, administering of *meniran* and *pegagan* extract combination had not result adverse effect to liver and kidney function as seen in the level parameter of SGOT, SGPT, BUN, and creatinine, so it was secured and did not cause toxicity.

CONCLUSION

In sum up, according to research's findings, it can be concluded that orally administering of *meniran* and *pegagan* extract combination at dosage of 50:50; 250:250; and 1250:1250 mg/KgBW had not affected SGOT, SGPT, BUN, and creatinine value in Wistar strain rats after sub-chronic toxicity test, administered for 28 days.

It is further necessary to perform histopathology to acknowledge liver and kidney in detail and research using more variative dosages in *meniran* and *pegagan* extract combination.

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THE CORRELATION OF TOTAL FLAVONOID AND TOTAL PHENOLIC WITH ANTIOXIDANT ACTIVITY OF SINGLE BULB GARLIC (*Allium sativum*) FROM TAWANGMANGU AND MAGETAN

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ABSTRACT

Flavonoids and phenolics are compounds with hydroxyl groups (-OH) bound to aromatic rings which enable them to react with reactive oxygen species and eliminate free radical activity. Single bulb garlic (*Allium sativum* var. solo garlic) is known to have antioxidant activity which comes from the phenolic groups. This study aims to determine the correlation of total flavonoid and phenolic levels with the antioxidant activity of ethanolic extracts from single bulb garlic grown in Magetan and Tawangmangu regions. This study included an observational analytic study with a cross-sectional design. Total flavonoid levels were measured by colorimetric method and total phenolic levels were measured by Folin-Ciocalteu method using Spectrophotometry UV-Vis. Antioxidant activity was measured by DPPH method at a wavelength of 517 nm. The data analysis used was multiple linear regression. The results showed that the extract of single bulb garlic from Magetan had total flavonoids of 12.1833 ± 0.1943 mg QE/gram, total phenolics of 70.244 mg GAE/gram, and antioxidant activity with an IC_{50} value of 20,216 ppm. The extract of single bulb garlic from Tawangmangu contained total flavonoids of 14.4833 ± 0.5911 mg QE/gram, total phenolics of 92.222 mg GAE/gram, and antioxidant activity with an IC_{50} value of 13.777 ppm. The conclusion of this study is that there is a significant correlation of total flavonoid and total phenolic content with antioxidant activity.

Keywords: antioxidant; flavonoid; garlic; phenolic

INTRODUCTION

Natural antioxidants can protect the body from free radical attacks and can slow the occurrence of chronic diseases caused by an increase in reactive oxygen species (ROS) especially hydroxyl radicals and superoxide radicals (Wahdaningsih *et al.*, 2011). According to Paravicini and Touyz (2008), the increase in ROS production, known as oxidative stress, affects various diseases such as hypertension, diabetes, heart failure, stroke, atherosclerosis, and other chronic diseases. It is predicted that in 2030 more than two thirds (70%) of the population or 52 million deaths per year will be due to degenerative diseases (Kemenkes RI, 2012).

One of the plants known to produce potential antioxidant activity is single bulb garlic (*Allium sativum* var.solo garlic) because it has the main phytochemicals content of sulfur compounds, peptides, steroids, terpenoids, flavonoids, and phenols (Agarwal, 1996). According to a study carried out by Prasonto *et al.*, (2017), single bulb garlic has IC_{50} values of 10.61 mg/ml which means that it has stronger antioxidant effect and is significantly different from IC_{50} values of garlic which is 13.61 mg/ml. IC_{50} values are influenced by the content of secondary metabolites such as phenolics. Phenolics have the ability as antioxidants because they can transfer an electron to a radical compound (Zuraida *et al.*, 2017). The production of secondary

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metabolites and antioxidant activity is influenced by different growth sites where different growth places affect the environmental temperature and biochemical processes found in plants causing the content of secondary metabolites such as phenolic will be different (Sholekah, 2017). If the total phenolic content in ethanolic extract of single bulb garlic is different, it can affect its antioxidant activity.

Based on the description above, a study investigating the correlation of total flavonoid and total phenolic content with the antioxidant activity of single bulb garlic ethanolic extract (Allium sativum var.solo garlic) is essential to carry out. Single bulb garlic used in this study is originated from Magetan and Tawangmangu area with considerations of the growing habitat temperature, soil pH, air humidity, and altitude of different places.

METHODS

Instrumentations and Materials

Instrumentations used in this research were glassware, rotary evaporator (Heidolph), water bath (Memmert), moisture analyzer (Shimadzu), analytical balance (Mettler Toledo), and spectrophotometer UV-Vis (Genesys 10 UV *scanning Thermo electron* co). The materials used in this research were single garlic bulb, 96% ethanol (E. Merck), DPPH (2.2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich.), aquadest (technical), 95% quercetin (Sigma. Co.), 97.5-102.5% gallic acid (Sigma. Co.), 99.9% Folin-Ciocalteau (E. Merck), 98.5% Mg powder (E. Merck), 99.9% Methanol (E. Merck), 37% HCl solution (E. Merck), 10% $FeCl_3$ (E. Merck) and 10% $AlCl_3$ (E. Merck).

Identification of the Plant

The specifications of the selected *Allium sativum* var solo garlic are having white color, single bulb, and fresh bulb. The origins of the plants are from Magetan (Wonomulyo village) with an altitude of 1,300 meters and Tawangmangu (Gondosuli village) with an altitude of 1,200 meters. Plants identification took place at Biology Laboratory, Universitas Negeri Semarang.

Extract Production

Allium sativum var solo garlic obtained from Tawangmangu and Magetan were cleaned using running water and dried with aerated. The fresh bulb was chopped up with ethanol 96% then it was extracted by maceration method using ethanol 96% for 3x24 hours while stirring periodically. The liquid extract was filtered then concentrated using rotary evaporator. The process continued with water bath until viscous extract was obtained.

Characterization of the Extract

Organoleptic and phytochemical screening method was chosen for characterization of the extract. Organoleptic test was administered using human senses starting from the shape, smell, and color.

Phytochemical Screening of Flavonoid and Phenolic

One gram of single garlic bulb ethanolic extract was added with 5 drops of ethanol then shaken until it got homogeneous. This mixture was added with Mg powder and HCl. Flavonoid content was identified by color changing into green, yellow, or red (Atmoko & Ma’ruf, 2009).

As much as 0.5 gram of single garlic bulb ethanolic extract was dissolved with methanol then shaken until it became homogeneous. This mixture was added with $FeCl_3$ reagent. Phenolic content could be identified by color changing into green, blue, or black (Atmoko and Ma’ruf, 2009).

Determination of Total Flavonoid

Total flavonoid content was determined using colorimetric assay with modification based on the procedure of (Chang *et al*., 2002). Firstly, preparation for stock solution quercetin standard was completed. 1 mg of quercetin was dissolved in 10 ml of ethanol p.a (100 ppm) and was subsequently diluted to 10, 20, 30, 40 and 50 ppm. Quercetin as standard solution (1 ml) was added with 0.1 ml $AlCl_3$ 10% and 0.1 mL potassium acetate 1M. It was then incubated for 30 minutes at room temperature. In addition, absorbance was measured at maximum wavelength of 434 nm. Secondly, preparation for extract solution was accomplished. 100 mg extract was dissolved in 10 mL of ethanol p.a. (10.000 ppm). 1 ml stock solution was taken and the volume was made into 10 ml by adding ethanol p.a. with 0.1 mL (1000 ppm). Each quercetin solution was added with 0.1 ml $AlCl_3$ 10% and 0.1 mL potassium acetate 1M. It was then incubated for 30 minutes. Absorbance was measured at maximum wavelength 434 nm. The extract solution was made in three replications. The TFC was expressed in mg Quercetin Equivalent (QE)/gram. Data obtained from the absorbance value of each sample were plotted into standard quercetin and calculated using the following formula.

$$TFC = \frac{c \times V}{m}$$

In the above formula, c is concentration from calibration curve, V is extract volume, and m is extract mass.

Determination of Total Phenolic Content

The total phenolic content (TPC) was determined using Folin-Ciocalteou method. The first step was the preparation of gallic-acid solution as standard solution. The stock solution was made into 100 ppm gallic-acid standard and was subsequently diluted to 10, 20, 30, 40 and 50 ppm. Each standard of solution (2 ml) was added with 5 mL aquadest, 0.5 mL Folin Ciocalteau 50% reagent mixture with vortex. Next, it was incubated at temperature between 20-25°C for 5 minutes. The mixture was added with 1 mL Na_2CO_3 20% and incubated at room temperature for 60 minutes. Absorbance was also measured at a maximum wavelength of 784 nm.

The second step was preparation of *Allium sativum* var solo garlic solution extract weighed 15 mg. It was then dissolved in 10 mL of ethanol p.a. (150 ppm). The 2 ml of solution was added with 5 mL aquadest and 0.5 mL Folin Ciocalteau 50% reagent mixture with vortex. It was incubated at 20-25°C for 5 minutes. That mixture was added with 1 mL Na_2CO_3 20% and was incubated at room temperature for 60 minutes. The extract solution was made in three replications. Furthermore, absorbance was measured at a maximum wavelength of 784 nm. The TPC (total phenolic content) was expressed in mg Gallic-acid Equivalent (GAE)/gram. TPC data obtained from the absorbance value of each sample were plotted into standard Gallic-acid and then calculated using the following formula.

$$TPC = \frac{c \times V}{m}$$

In the above formula, c is concentration from calibration curve, V is extract volume and m is extract mass (Zuraida *et al*., 2017).

Antioxidant Activity Assay with DPPH method

One hundred mg extract was diluted with 100 mL ethanol p.a (stock solution). Sample from the extract stock solution and vitamin C were prepared in five different concentrations. 1 ml sample was added with 3 ml DPPH and ethanol until it reached 5.0 ml. The mixture was kept in the dark for 30 minutes. The absorbance at λ maximum was recorded to determine the concentration of the remaining DPPH. The DPPH inhibition percentage of the test sample and the known solution were calculated using the following formula.

$$Scavenging\ effect\ (1\%) = (1 - \frac{Ai}{Ac}) x\ 100 \quad \text{......... (1)}$$

In the above formula, Ai is absorbance of the sample and Ac is absorbance of the blank at λ maximum (Amin, 2015) .

RESULTS AND DISCUSSION

The identification results of *Allium sativum* are species of *Allium sativum* L., varieties of *Allium sativum* var. Sativum, and cultivar of *Allium sativum* L. Ctv. Solo (Biology Lab Universitas Negeri Semarang) with letter determination number of 657/UN/37.1.4.5/LT/2018.

Phytochemical Screening of Flavonoid and Phenolic

Single garlic bulb ethanolic extract had a specific smell, viscous extract, and brown-yellow color. The extract was identified as flavonoid because when it was added with Mg powder and HCl, it made bubble (H_2 gas) and flavilium sodium until it changed into yellow. The extract was also identified as phenolic because it resulted in a blue coloration within the addition of ferric chloride (Atmoko & Ma'ruf, 2009).

Total Flavonoid Content (TFC)

Determination of TFC was completed using a UV-Vis spectrophotometer with maximum absorption of 434 nm. Quercetin was chosen as a standard because it is reported from Ouzounidou *et al*. (2011) that *Allium* vegetables, particularly common onion and garlic, are extremely abundant in flavonoids especially quercetin. TFC was determined based on colorimetric method using $AlCl_3$. Colorimetric is the common method for calculating total flavonoid content such as in purified extract of *Piper crocatum* Ruiz and Pav (Januarti & Wijayanti, 2018) and teak leaf ethanolic extract (Januarti *et al*., 2017). The principle is that $AlCl_3$ forms an acid complex which is stable with C-4 ketone groups, C-3 or C-5 hydroxyl groups from flavones and flavonols (Chang *et al*., 2002). TFC of single garlic bulb extract from Magetan and Tawangmangu is presented in Table I.

Table I. TFC and TPC from Magetan and Tawangmangu

Origin	TFC (mg QE/g) ± SD	TPC (mg GAE/g) ± SD
Magetan	12.1833 ± 0.1943	70.2444 ± 1.2006
Tawangmangu	14.4833 ± 0.5991	92.2222 ± 1.8201

TFC of single garlic bulb extract from Tawangmangu was higher than those from Magetan. TFC could be different because single garlic bulb extract from Magetan and Tawangmangu grew in different altitude, environment temperature, soil pH, and air humidity. Single garlic bulb in Tawangmangu grew in altitude of 1,200 meters, environment temperature of 20°C, soil pH of 6.8, and air humidity of 80%. Single garlic bulb in Magetan grew in altitude of 1,300 meters, environment temperature of 18°C, soil pH of 6.5 and air humidity of 77%.

The correlation was that TFC was decreasing from lower to higher altitudinal zones. Malinikova, *et al*. (2013) reported similar results from *Fragaria vesca* species with strong correlation between TFC and altitude. TFC and distribution of flavonoids are influenced by not only the altitude of the growth place but also genetic and various environmental factors such as light, humidity, and soil fertility (Malinikova *et al*., 2013).

Total Phenolic content (TPC)

Method used to determine total phenolic content is based on the strength of reducing the hydroxy groups of phenolic compounds. All phenolic compounds, including simple phenols, can react with Folin-Ciocalteu reagents (Alfian & Susanti, 2012). Gallic acid is used as a comparative compound because gallic acid is a heteropoly acid which has 3-hydroxy phenolic groups. The hydroxy phenolate group will be oxidized by Folin-Ciocalteu reagent in an alkaline atmosphere. The hydroxyl group has a function as a

contributor to hydrogen atoms when reacting with radical compounds through an electron transfer mechanism so that the oxidation process can be inhibited. Therefore, TPC is intended to find out the number of phenolic compounds in single garlic bulb extract which has antioxidant activity. TPC of single garlic bulb extract from Tawangmangu is higher than Magetan based on Table I.

Different TPC between single garlic bulb from Tawangmangu and Magetan could be due to different growth habitat. Single garlic bulb in Tawangmangu grew in altitude of 1,200 meters, environment temperature of 20°C, soil pH of 6.8 and air humidity of 80%. Single garlic bulb in Magetan grew in altitude of 1,300 meters, environment temperature of 18°C, soil pH of 6.5 and air humidity of 77%.

Chen *et al*. (2013) reported similar results that variability of TPC and TFAC in bulbs of different cultivars could be attributed to various cultivar characteristics. In agreement with our results, previous reports have shown that different garlic cultivars had different yields, allicin content (Khar *et al*., 2011), and polyphenolic content (Lu *et al*., 2011). These results are in accordance with total phenolic acid content of a local garlic cultivar grown near Namhae-gun, Korea. Its total phenolic acid content was 17.86 mg/kg of dry matter (dm) and the total flavonoid content was 29.95 mg/kg dm (Kim JS & Kang OJ, 2013). The total phenolic content varied from 3.4 mg gallic acid equivalents (GAE)/g dm to 10.8 mg GAE/g dm in different garlic cultivars grown at four locations in Andalusia, Spain (Beato VM *et al*., 2011). Total phenolics of red onion var. Rouge Amposta were reported by Benkeblia (2005) from 18 to 20 mg/100 g fresh weight. The content of phenolic compounds in garlic, thus, varies greatly within genetic, agronomic, and environmental factors (Beato VM *et al*., 2011).

Antioxidant Activity

Antioxidant activity of single garlic bulb extract is presented in Table II and is classified as very strong because it has IC_{50} value of less than 50 ppm (Mardawati *et al*., 2008). Antioxidant activity of single garlic bulb extract from Tawangmangu was stronger than Magetan in correlation with TFC and TPC. The correlation was that antioxidant activity was increasing along with TFC and TPC from higher to lower concentration.

Table II. IC_{50} values of Single Garlic Bulb Extract

Replicates	IC_{50} (ppm)	
	Magetan	Tawangmangu
1	20.3333	13.8759
2	20.1626	13.8281
3	20.1600	13.6153
Average	20.2186	13.7731
SD	0.0993	0.1387

Our results are in agreement with the report from Benkeblia (2005) stating that garlic extract showed higher radical scavenging activity and reducing capacity than green onion extract. These properties were significantly correlated to total phenolics content which was high in garlic. Sulfur compounds could be involved in the assessment of the antioxidant properties.

Correlation between total flavonoids content and total phenolic contents, and antioxidant Activity

Garlic possesses potential health-promoting effects due to its high phenolic phytochemical content and is a source of natural antioxidants (Nuutila AM *et al*., 2003). Garlic is a source of various biologically active phytomolecules including organosulfur compounds, phenolic acids, allyl thiosulfinates, flavonoids, and vitamins. Antioxidant activity of flavonoids and phenolic is influenced by functional group that binds to its main structure. Flavonoids has mechanism of capturing free radicals (hydroxyl radicals, superoxide, and peroxyl) and inhibiting various oxidation reactions because they can produce phenolic radicals which are stabilized by

resonance effect of aromatic rings (Ahmad *et al*., 2015). In other words, flavonoids stabilize reactive oxygen species by reacting with reactive compound of the radical (Hamidu *et al.*, 2018).

Data analysis to show the correlation between TFC and TPC, and antioxidant activity was accomplished using multiple regression analysis. The results were presented in Table III. The analysis showed that there was a correlation between TFC and TPC, and antioxidant activity of single bulb garlic from Magetan and Tawangmangu. This is indicated by the significant value ($p<0.05$).

Shahwar *et al*. (2010) also reported that antioxidant activity was positively correlated with TFC and TPC. TFC and TPC were increasingly relevant to its antioxidant activity. The pharmacological activity of garlic depends on its bioactive compounds and more especially on its phenolic compounds (Lanzotti, 2006) (Corzo-Martı´nez & Corzo, 2007) in which interesting pharmacological properties are present in relatively high amounts (Beato VM *et al*., 2011).

Antioxidant activity in garlic was researched in protection against oxidative DNA damage, decreasing fibrinogen, reducing the risk of chronic diseases, preventing disease progression, and preventing cancer (Park & Park, 2009; Shukla, 2007 ; Jastrzebski *et al*., 2007). Mohsen and Ammar's (2009) research shows that the radical capture activity tested on flavonoids and phenolics is related to the number and position of the hydroxyl group (OH) bonds in the molecule. Increasing number of hydroxyl groups substituted in the molecule causes higher antioxidant ability because many hydrogen atoms can be donated (Yu Lin *et al*., 2009).

According to Zuraida *et al*., (2017), phenolic compounds contribute to antioxidant activity. Benkeblia (2000) reported similar results for garlic extract with high correlation between reducing power and total phenolics content with a determination coefficient (r^2) of 1.7. Statistical analysis also showed that radical scavenging activity, reducing capacity, scavenging of hydrogen peroxide and chain-breaking activity (initial and after heating) were highly correlated with total phenolics content of garlic extracts with coefficients of determination (r^2) ranging from 0.90 to 0.95.

CONCLUSION

Based on the research that has been conducted, antioxidant activity of single bulb garlic (*Allium sativum* var.solo garlic) from Tawangmangu and Magetan was correlated with the presence of total flavonoid and total phenolic content. It was shown that single garlic bulb from Tawangmangu had higher TFC and TPC than those from Magetan, which was consistent with their antioxidant activity.

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Table III. Multiple Regression Analysis of EEUBL Magetan and Tawangmangu

EEUBL	Total Flavonoid toward Antioxidant Activity	Total Phenolic toward Antioxidant Activity	Total Flavonoid and Total Phenolic toward Antioxidant Activity
Magetan	0.037*	0.022*	0.000*
Tawangmangu	0.014*	0.027*	0.001*

*significant ($p<0.05$)

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