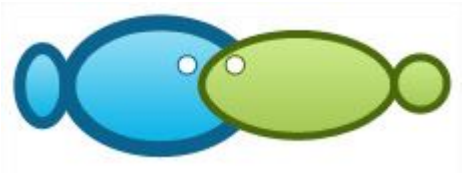

Bioactive components, antibacterial activity, and toxicity of mangrove *Bruguiera gymnorrhiza* fruit extract

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Abstract. Mangrove forest in Indonesia is the largest in the world, either in number of areas or number of species. Plant extract and natural material-derived compounds recently became popular because of their low side effects and strong influence on some resistant microorganisms. *Bruguiera gymnorrhiza* is known as a wide leaf-mangrove that is a potential source of bioactive compounds. This study aims to know the bioactive compounds, antibacterial activity, and toxicity of mangrove *B. gymnorrhiza* fruit extract. Extraction was carried out through maceration in 96% ethyl acetate solution. The bioactive components were tested through phytochemical screening. The extract concentrations for antibacterial test were set at 10%, 20%, 40%, 60%, and 80%, then analyzed with ANOVA. The antibacterial activity was measured using agar diffusion method (Kirby and Bauermethod) using disc paper, while toxicity test of LC50 used probit analysis. Results showed that the extract of *B. gymnorrhiza* fruit contained flavonoid, tannin, saponin, steroid, triterpenoid, and phenol. ANOVA indicated that all treatment concentrations inhibited the bacterial growth in the range of strong to very strong category. Ethyl acetated-extracted mangrove *B. gymnorrhiza* fruit had toxicity on brine shrimp larvae with LC50 of 271.6439 ppm.
Key Words: brine shrimp larvae, diffusion method, LC50, maceration, phytochemistry.

Introduction. Mangrove forest in Indonesia is the largest in the world, either in number of areas (±42,550 sq. km) or number of species (±45 species) (Spalding et al 2001). Mangrove forests of Indonesia reach 50% of total mangrove forests in Asia and nearly 25% of total world mangrove forests, about 3.7 million ha (Onrizal 2010). Most of mangrove plants are useful as medicinal material. The extract and raw materials of the mangrove have been benefited by coastal communities as natural medicine (Purnobasuki 2004). Previous studies have been conducted on some mangrove species such as *Sonneratia alba,* to examine its antibacterial activity to inhibit the growth of bacterium *Vibrio alginolyticus* (Karim et al 2018) and leaf extract of *Aegiceras corniculatum* as antibacterial against V*ibrio harveyi* and V*ibrio parahaemolyticus* (Triyanto et al 2004).

Scientists' interest in plant extract and the compounds isolated from natural materials have recently become a trend because they have low side effects and strong influence on some resistant macroorganisms (Saad et al 2013). The number of antimicrobial substances contained in mangrove plants is very high, but these substances have not been maximally utilized yet, and thus further exploration is needed. Mohamad et al (2017) stated that nature has provided source of medicinal agents since the last thousands years. During the last several decades, search for natural compounds has increased, especially from marine environment that is well-known as rich in biological and chemical diversity. The compounds isolated from marine organisms have proved their potential as medicine due to their extensive spectrum against a variety of diseases, without dangerous side effects (Bordbar et al 2011).

Bruguiera gymnorrhiza, known as wide-leafed mangrove, is one of the most remarkable mangrove species and widely distributed in the Pacific region, living in the

intertidal areas from the coastline of Southeast Asia to Ryukyu Island, Japan (Allen & Duke 2006). *B. gymnorrhiza* is a potential source of bioactive compounds (Kunwar et al 2013; Analuddin et al 2019). Several previous studies were done on bioactive components extracted from stem, leaf, and root (Saad et al 2013; Dia et al 2015; Nurjanah et al 2015), but only few active components explorations in *B. gymnorrhiza* fruit were conducted. This study aims to find out the secondary metabolites potency, the antibacterial ability, and the toxicity of the mangrove *B. gymnorrhiza* fruit extract.

Material and Method

Materials. The main research material was brownish-colored mangrove fruit of *B. gymnorrhiza* collected in Tiwoho village, North Minahasa Regency, the Province of North Sulawesi, in May and September 2017 (Figure 1). Further chemical analyses were done in the laboratory.

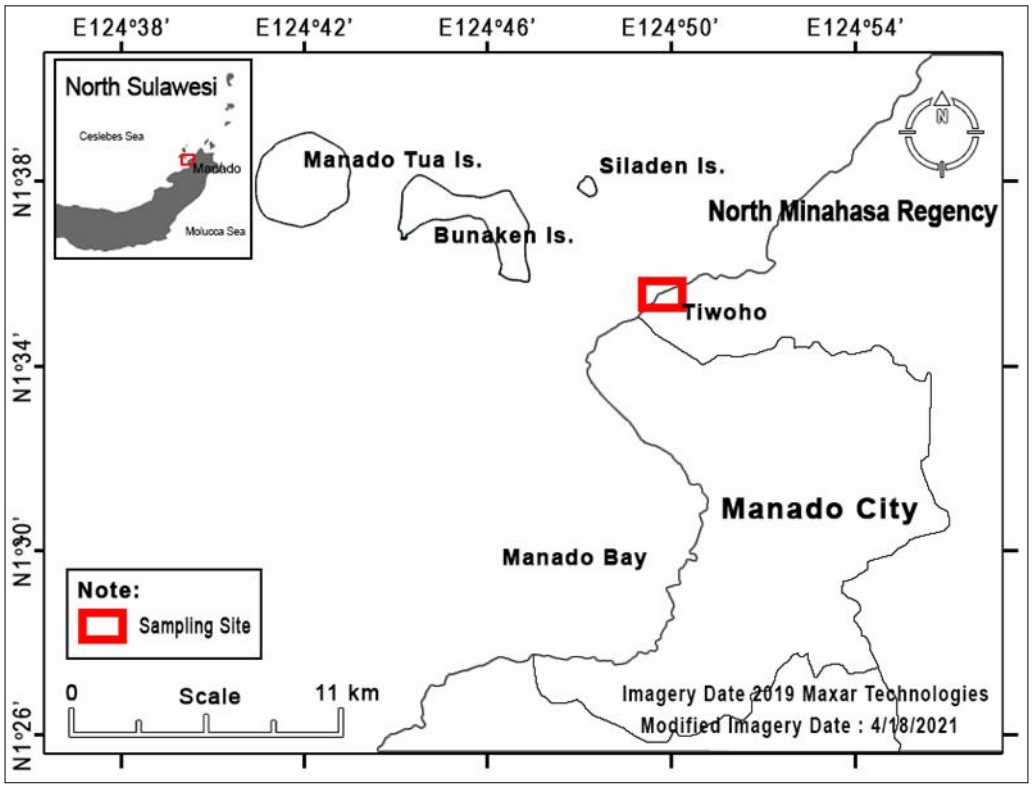

Figure 1. Map of sampling location.

Active compounds extraction. Active compound extraction used maceration method in ethyl acetate solvent. Extraction, phytochemical screening, antibacterial activity, and toxicity test were accomplished in the Laboratory of Faculty of Fisheries and Marine Science, UNSRAT, Manado. Ripe fruit of mangrove *B. gymnorrhiza* was weighed as much as ±3 kg, placed in polyethylene plastic bag, and wind-dried at room temperature up to having ±15% water content (Prihanto et al 2018). The dry sample was then ground, macerated in ethyl acetate at the ratio of 1:3 for 72 hours, and filtered through Whatman 42 filter paper. The filtrate was then dried using a rotary vacuum evaporator at 50^{o}C.

Phytochemical screening. The phytochemical content of *B. gymnorrhiza* fruit was analyzed following Richardson & Harborne (1985) and presented in table. Sample was added with chloroform and some drops of ammonia. Chloroform fraction was then separated and acidified with 10 drops of 2M H_2SO_4. The acid fraction was then taken and added with Dragendorf, Meyer, and Wagner reagents. The presence of alkaloid is indicated with white deposit for Meyer reagent, red deposit for Dragendorf reagent, and brown deposit for Wagner reagent. Flavonoid compound was detected using 1 g of

sample added with 30% methanol then heated until a concentrated filtrate was obtained. Afterwards, the filtrate was put on a spot plate and added with H_2SO_4. Red color formation indicates the presence of flavonoid. Saponin compounds were detected with foam test in hot water. For this, one gram of sample was added with water and heated in boiling water for 5 min, cooled, and shaken. If the foam appears and stays longer than 10 min, it indicates the presence of saponin.

Steroid and triterpenoid compounds were detected as follows: as much as 2 g of sample were added with 25 mL of 30% ethanol, then heated (50^{o}C) and filtered. The filtrate was then evaporated and added with ether. The formed ether layer was pipetted and put on the spot plate, then added with Lieberman Burchard reagent. The presence of triterpenoid is shown by purple or red color and steroid by green or blue color.

Antibacterial activity test. The antibacterial activity was examined by measuring the inhibition zone diameter using agar diffusion method (Kirby-Bauer method) (Trisia et al 2018). As much as 20 µL of each liquid isolate of bacteria *Escherichia coli, Staphylococcus aureus*, and *Pseudomonas aeruginosa* at a density of 10^7 CFU mL^{-1} was poured on Mueller Hinton Agar (MHA) media, then distributed with a spreader. A separate 6 mm disc paper was immersed for 15 min in *B. gymnorrhiza* fruit extract at each concentration of 10%, 20%, 40%, 60%, and 80%, and then pasted on the MHA media. Positive control was set with a disc paper immersed in an antibiotic, namely chloramphenicol, while the negative control was prepared as neutral disc paper (dipped in distilled water). After incubated for 24 hours, the inhibition zone diameter formed around the disc paper was measured using a caliper. This measurement was done twice and presented in mean value. It is intended to know the potential of antibacterial activity in the mangrove *B. gymnorrhiza* fruit extract against the bacteria *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* using agar diffusion method.

The effect of ethyl acetate-extracted mangrove *B. gymnorrhiza* fruit on the inhibition zone diameter against the growth of *E. coli*, *S. aureus* and *P. aeruginosa* was examined using One-way ANOVA facilitated with Statistical Product Services Solution (SPSS 23) program at 95% significance level, then continued with Duncan test.

Toxicity test. *Artemia salina* larvae preparation started from egg hatching carried out by immersing 100 mg of eggs in sea water-containing tank under 25 watt-light. The eggs hatched to larvae after approximately 24 hours. When the larvae reached 48 hours old, they were ready to use for toxicity test (Widjaya et al 2019). The toxicity test of *B. gymnorrhiza* fruit extract followed Brine Shrimp Lethality Test (BSLT) method of Meyer et al (1982). Ten experimental tanks were prepared for 4 treatments and control, each with 2 replications. Each treatment concentration contained 10 shrimp larvae. Mortality was recorded during 24 hours with 6 hour intervals, i.e. at hour-6, hour-12, hour-18, and hour-24. Larval mortality was calculated as follows:

$$\% \text{ Mortality} = \frac{\text{Number of dead larvae}}{\text{Number of test larvae}} \times 100$$

If larva mortality occurs in the control treatment, it can be corrected following Abbot's formula:

$$\% \text{ Mortality} = \frac{\text{No. dead larvae (test larvae – control)}}{\text{No. test larvae – No. dead in control treatment}} \times 100$$

Standard criterion to determine the mortality of *A. salina* larvae is no movement shown by the larvae after several seconds. The toxicity data were analyzed using probit analysis (Finney 1952) with SPSS 23.0 for Windows and presented with table and figure to determine LC_{50}. The extract is categorized to be toxic if the LC_{50} value is below or same as 500 ppm.

Results and Discussion

Phytochemical screening. Maceration method is used because it is easy and inexpensive. Longer maceration could yield more extract, since cell wall and membrane lysis occur as a result of pressure difference between inside and outside the cell, so that the secondary metabolites in the cytoplasm will dissolve in the ethyl acetate.

Phytochemical screening is aimed to know the presence of active compounds potential as antibacterial, such as phenol, flavonoid, tannin, saponin, alkaloid, and terpenoid (Ciptaningrum & Putri 2019). These active compounds can act as defense to the environmental pressures, disease, and predation. Several secondary metabolites can be produced in different phases and paths of the metabolisms. In the present study, the phytochemical compounds of mangrove *B. gymnorrhiza* fruit extract are given in Table 1.

Table 1

Chemical compounds in mangrove fruit *Bruguiera gymnorrhiza* extract

Group	*Outcome*	*Color change*
Alkaloid (Dragendorf, Wagner, Meyer)	-	No color change
Flavonoid	+	Red
Tannin	+	Green
Saponin	+	Foam
Steroid	+	Blue
Triterpenoid	+	Orange
Phenolic	+	Brown orange

Notes: - absent; + present.

According to Hanin & Pratiwi (2017) and Prihantini et al (2018), different composition and amount of chemical compounds can give different effect on the bioactivity. Dalimunthe & Rachmawan (2017) stated that basic composing component of the secondary metabolite was primary metabolite that played its role in photosynthesis and respiration. The plant part that has important role in photosynthesis is leaf, because leaf contains chloroplast functioning to capture the sunlight (Hanin & Pratiwi 2017). Green color in the filtrate indicates the presence of chlorophyll or tetrapyrrole group, brownish yellow color indicates the presence of flavonoid or O-heterocyclic group, reddish brown indicates the presence of phenolic compound, yellowish brown indicates the presence of flavonoid, and blackish brown indicates the presence of alkaloid. These findings are in agreement with Bandaranayake (2002) that these compounds are found in mangrove plant and have important toxic, pharmacological, and ecological effects. Several studies on mangrove plants found that they contained bioactive compounds, such as saponin, tannin, flavonoid, diterpenoid, and phenol as active antimicrobials (Prihanto et al 2018). The presence of bioactive components in plants is affected by many factors, such as habitat type, geography, climate, topology, and parts of plant, and plants of the same genus or family tend to contain similar chemical compounds, but in different quantities.

Mangrove plants are resistant to highly extreme environmental conditions. Phenolic compound is one produced by plant as response to environmental stress. The presence of phenolic compound in the sample is indicated by the formation of green, red, purple, blue or black color (Richardson & Harborne 1985; Tahir et al 2017). Phenol functions as protection from UV-B light and cell mortality to protect DNA from dimerization and damage (Dhurhania & Novianto 2018). The presence of phenolic compounds functions to prevent damages or to maintain the survival ability (Purwaningsih et al 2013). The hydroxyl group of phenol is capable of scavenging the free radicals and able to dampen the radical feature of the reactive oxygen compounds, such as superoxide, peroxide radical, hydroxyl radicals, and peroxide nitrite (Zuraida et al 2017). Phenolic compounds protect the plant from grazing, competition, and damages from excessive light exposure, and support reproduction as well (Purwaningsih et al 2013). The component in this compound is known to have important role as preventive

and treatment agents against several disease disorders, such as arteriosclerosis, brain dysfunction, diabetes, and cancer.

Flavonoid is an acidic phenolic compound that changes to typical color for each group with the presence of ammonia (Markham 1988). Therefore, if it is not mixed with other pigment, flavonoid can be detected with ammonia vapor. Flavanone and flavonol give reddish yellow color. Anthocyanin yields red blue color, while flavanone gives orange or brown color. Red and crimson colors that appear at the sudden in alkaline condition could result from the presence of chalcone or aurone (Robinson 1995).

Tannin functions as astringent, anti diarrhea, antibacterial, and antioxidant (Widi & Indriati 2007). This compound generally occurs in the plant stem, skin, and fruit. Tannin acts as fruit protection, and it will disappear when the fruit is mature (Pari 1990). Desmiaty et al (2008) stated that tannin is a very complex organic component consisting of phenolic substances that are difficult to separate and crytalize, precipitate protein from the solution and bind it. According to Artati & Fadilah (2007), tannin has complex biological roles from protein precipitating to metal chelating and can also function as an antioxidant.

Saponin is a glycoside, a mixture of simple carbohydrate and aglycone occurring in various plants. Saponin is separated based on hydrolysis to carbohydrate and sapogenin, in which sapogenin is divided into two groups, saponin steroid and saponin triterpenoid. Saponin has foam-like characteristic, so that when it is reacted with water and shaken, the foam will be formed and stays for long. It is soluble in the water, but insoluble in ether, has bitter taste, and causes sneezing and irritation of mucous membranes. Saponin is a toxin that can rupture the blood cell or cause hemolysis, and toxic to cold-blood animals (Suharto et al 2012).

Steroid is one of the important substances in pharmacy and widely used in treatment, such as antibacterial, anti-inflamamation, and painkiller (Firdiyani et al 2015). Triterpenoid is a crystal colorless compound that often has high melting point (Harborne 1984) and is beneficial for treating several types of human diseases, including various types of cancer (Patlolla & Rao 2012).

Antibacterial activity test. Table 2 shows the effect of treatment concentration on the inhibition zone diameter against the growth of *E. coli*, *S. aureus*, and *P. aeruginosa*. The inhibition zone increases with treatment concentration, except that it declines against the growth of *S. aureus* at 80% extract concentration.

Table 2

Inhibition zone diameter of *B. gymnorrhiza* fruit extract against bacteria *E. coli*, *S. aureus*, and *P. aeruginosa*

Extract concentrations (%)	*Mean diameter of inhibition zone against bacterial growth (mm)*					
	E. coli	*C (+)*	*S. aureus*	*C (+)*	*P. aeruginosa*	*C (+)*
10	12.8	24.1	16.5	24.1	18.5	27.7
20	20.4	22.0	21.4	20.3	21.8	26.1
40	20.8	22.3	21.7	23.9	23.6	25.7
60	22.7	23.1	22.1	22.7	20.6	26.0
80	24.8	33.6	19.0	20.7	22.1	23.2

Note: C – control treatment (chloramphenicol).

All treatments yield smaller inhibition zone to that of positive control, chloramphenycol. According to Rusmini (2015), chloramphenicol is a wide spectral antibiotic that is bacteriostatic and active against aerobic and anaerobic Gram positive and Gram negative bacteria. It is also a strong inhibitor in the synthesis of microbial protein since it can reversibly bind with 50s ribosomal subunit of the bacteria and inhibit peptide formation. The study results showed that the extract of *B. gymnorrhiza* fruit could be used as antibacterial substance. Higher concentration gave bigger inhibition zone diameter against bacteria *E. coli.* For *S. aureus,* higher extract concentrations up to 60% yield larger inhibition zone, but then the inhibition zone declines at higher extract

concentration. The effect of mangrove *B. gymnorrhiza* fruit extract on *P. aeruginosa* growth tends to rise at the concentrations of 10%, 20%, 40%, then the inhibition zone declines at the concentration of 60%, and rises again at the concentration of 80%.

Furthermore, ANOVA shows significantly different effect of *B. gymnorrhiza* fruit extract on the test bacteria, in which all treatment concentrations gave inhibition activity against the bacterial growth. Duncan test indicates that only treatment concentrations of 20% and 40% yield non-significantly different inhibition zone against *E. coli*, but different from the other treatments. The extract concentration of 10% gave significantly different inhibition zone from other treatments, and concentration of 60% has significantly different effect from that of 80% as well (Table 3).

Table 3

Inhibition zone (mm) of mangrove *B. gymnorrhiza* fruit extract against the growth of *Escherichia coli, S. aureus, and P. aeruginosa.*

Bacteria	*Extract concentrations (%)*				
	10	*20*	*40*	*60*	*80*
E. coli	12.8 ± 0.000^{a}	20.4 ± 0.000^{b}	20.8 ± 0.000^{b}	22.7 ± 0.282^{c}	24.8 ± 1.697^{d}
S. aureus	16.5 ± 0.000^{a}	22.0 ± 0.000^{b}	21.7 ± 0.000^{b}	22.2 ± 0.282^{b}	19.0 ± 1.697^{c}
P. aeruginosa	18.5 ± 0.000^{a}	21.8 ± 0.070^{b}	23.6 ± 0.000^{c}	20.6 ± 0.000^{d}	22.1 ± 1.414^{e}

Note: the same letter on the same row indicates no significant difference at α = 0.05.

The treatment concentration of 10% *B. gymnorrhiza* fruit extract has significantly different effect on the inhibition zone against the growth of *S. aureus* from all other treatments. The extract concentrations of 20%, 40%, and 60% do not have significantly different inhibition zone against the growth of *S. aureus*, but different inhibition zone from that of 10% and 80%. The inhibition zone against the growth of *P. aeruginosa* is significantly different among the treatment concentrations (Table 3).

The ability to inhibit the bacterial growth is influenced by several factors, such as concentration, type of antimicrobial, and active compounds of the extract, such as flavonoid, tannin, saponin, steroid, terpenoid, and phenol. The criteria of the antibacterial inhibition zone are as follows: the diameter of ≤ 5 mm is categorized as weak, 5-10 mm as moderate, 10-20 mm as strong, and > 20 mm as very strong (Sari & Ferdinan 2017; Morales et al 2003). Therefore, the present study confirms that the antibacterial ability of mangrove *B. gymnorrhiza* fruit extract against *E. coli* and *P. aeruginosa* is strong at the concentration of 10%, and very strong at the concentration of 20%, 40%, 60% and 80%, while against *S. aureus,* the strong ability was recorded at the concentration of 10% and 80% and very strong at the concentration of 20%, 40%, and 60%.

Toxicity test is one of the toxicological evaluations of herbal medicinal extracts done before clinical test (Sharwan et al 2015). Lethal concentration of 50% (LC_{50}) is used to examine the acute toxicity (Cyrus et al 2008; Surya 2018), the concentration that causes 50% mortality. Mortality observations at 24 hours showed that mangrove *B. gymnorrhiza* fruit extract could be toxic to brine shrimp *A. salina* larvae at the lowest treatment concentration of 250 ppm to the highest one (1,000 ppm), in which higher extract concentration application caused higher mortality (Table 4).

Table 4

Toxicity test of *B. gymnorrhiza* fruit extract on *Artemia salina* at 24 hours

Dose (ppm)	*log ppm*	*Probit (Y)*	*Mortality*	*% Mortality*
0	0	0	0	0
250	2.398	4.01	5	16
500	2.699	4.56	10	33
750	2.875	4.90	14	46
1000	3.000	8.09	30	100

The toxicity effect of *B. gymnorrhiza* fruit extract on *A. salina* was obtained through probit analysis (Table 4). Lethal concentration (LC_{50}) was estimated using regression line of Y = a + bx. The relationship between the extract concentration and the larval mortality at 24 hours is indicated as Y = 2.0839x – 0.2609 and R^2 = 0.8101 (Figure 3). The correlation coefficient (R^2) reveals that 81% of the extract concentration could explain the percent mortality of the brine shrimp *A. salina* larvae.

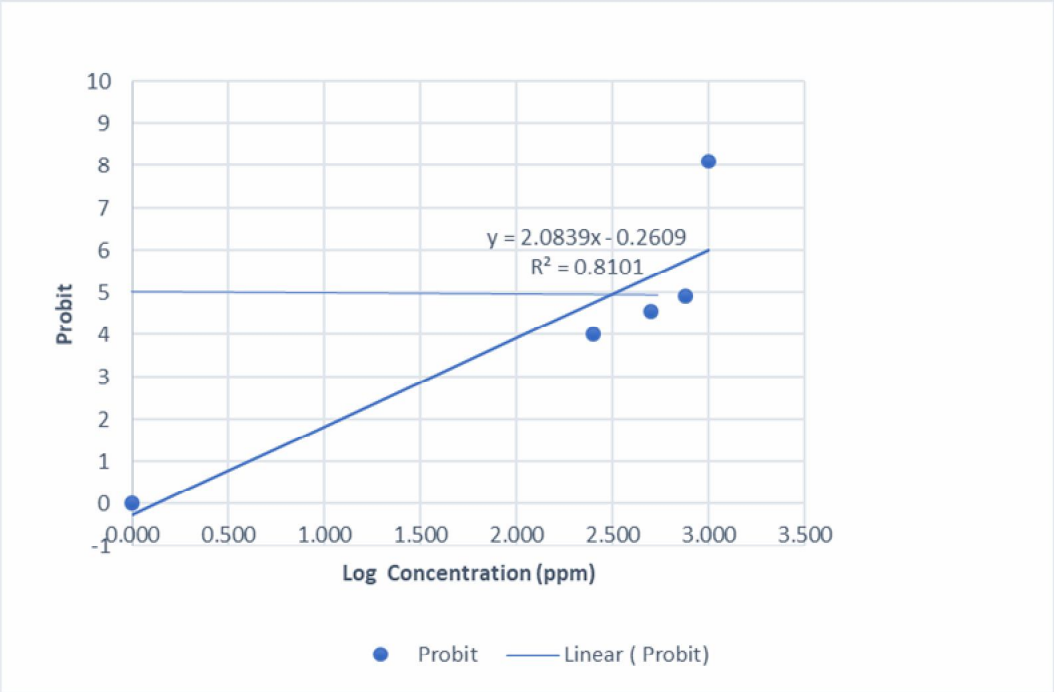

Figure 3. Relationship between mangrove *B. gymnorrhiza* fruit extract and mortality of *Artemia salina* larvae.

LC_{50} calculation was done using the probit value (Y) of 5 to represent 50% mortality, so that the equation becomes 5 = 2.0839x - 0.2609, then x value was obtained, 2.526537. The antilog of x is 334.559 meaning that LC_{50} of *B. gymnorrhiza* fruit extract effect on *A. salina* is 334.559 ppm (Figure 3). This result is consistent with Meyer et al (1982) that the fruit extract of mangrove *B. gymnorrhiza* possesses toxicity activity on the brine shrimp.

Conclusions. Ethyl acetate-extracted mangrove *B. gymnorrhiza* fruit had secondary metabolite of flavonoid, tanin, saponin, steroid, triterpenoid, and phenol. The antibacterial activity of *B. gymnorrhiza* fruit extract against bacteria *E. coli, S. aureus*, and *P. aeruginosa* is categorized from strong to very strong at the concentration applications of 10%, 20%, 40%, 60%, and 80%. This indication reconfirms the potentiality of mangrove extract as active compound. However, it could also be toxic to brime shrimp larvae at lower concentration with LC_{50} of 334.559 ppm.

Conflict of interest. The authors declare that there is no conflict of interest.

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