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Antibacterial Potential of Radish Extract (*Raphanus sativus* L.) against Fish Spoilage Bacteria

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Radish (*Raphanus sativus* L.) root is commonly used as flavor enhancing additive or side dish. Previous research revealed the presence of active compound in which could inhibit bacterial growth. Thus, a research concerning natural antibacterial for fish products that are categorized as high-risk food being contaminated by spoilage bacteria (*Pseudomonas aeruginosa, Bacillus cereus,* and *Staphylococcus aureus*) was done. Radish root extraction was made by using ethyl acetate (semi polar) for 3 days. Well diffusion was performed using 4 extract concentration (10, 20, 30, and 40% (w/v)) against three fish spoilage bacteria. Based on our results, 30% concentration was the best concentration which inhibit more than 10 mm in inhibition zone with minimum inhibitary concentration (MIC) and minimum bactericidal concentration (MBC). The scores were of 0.06% and 0.24% (*P. aeruginosa*), 0.13% and 0.50% (*S. aureus*), and 0.12% and 0.48% (*B. cereus*). Moreover, based on stability test against heating temperature showed that this extract concentration was more stable in 80 °C with duration times for 5 minutes and pH 3 which resulting the lowest inhibition zone reduction compares to control extract. Selected radish extract was categorized as low toxic compound (LC_{50} = 839.52 ppm) and by GCMS test its functioning in antibacterial compound containing major antibacterial compound (bis(2-ethylhexyl) phthalate, 1,2-benzenedicarboxylic acid, 9,12,15-octadecatrienoic acid), fatty acid (n-hexadecanoic acid, butanedioic acid), carboxylic acid (isobutyric acid, malic acid, oleic acid), and minor antibacterial compound (n-Hydroxymethylacetamide, 2,4-bis(1,1-dimethylethyl), 2,4-pentanedione,2-Cyclohexen-1-one, hydrazine, cyclohexene oxide, gamma-sitosterol).

Key words: antibacterial, pH, *Raphanus sativus*, stability, temperature, time

Umbi lobak (*Raphanus sativus*) umumnya dimanfaatkan sebagai bahan tambahan peningkat rasa dan aroma atau pendamping makanan utama. Penelitian terdahulu mengungkap adanya komponen aktif yang dapat menghambat pertumbuhan bakteri. Hal ini mendorong dilakukannya penelitian mengenai senyawa antibakteri alami untuk produk ikan yang merupakan jenis pangan berisiko tinggi terkontaminasi bakteri pembusuk (*Pseudomonas aeruginosa, Bacillus cereus,* dan *Staphylococcus aureus*). Ekstraksi dilakukan terhadap umbi lobak dengan etil asetat (semi polar) selama 3 hari. Pengujian difusi sumur dilakukan dengan empat konsentrasi ekstrak (10, 20, 30, dan 40% (b/v) pada ketiga spesies bakteri pembusuk ikan. Konsentrasi 30% ditentukan sebagai konsentrasi ekstrak terbaik penghasil zona hambat lebih dari 10 mm dengan nila *minimum inhibitary concentration* (MIC) and *minimum bactericidal concentration* (MBC) berurutan sebesar 0,06% dan 0,24% (*P. aeruginosa*), 0,13% dan 0,50% (*S. aureus*), dan 0,12% dan 0,48% (*B. cereus*). Konsentrasi terpilih digunakan pada tahap pengujian stabilitas ekstrak terhadap suhu pemanasan (80 °C dan 100 °C), waktu pemanasan (5, 10, dan 15 menit), dan nilai pH (3, 4 [kontrol], 5, 6, dan 7). Perlakuan panas dan perubahan nilai pH menyebabkan ketidakstabilan ekstrak. Ekstrak lobak lebih stabil pada suhu 80 °C selama 5 menit dan pH 3 menghasilkan ekstrak dengan penurunan zona hambat terkecil terhadap nilai penghambatan ekstrak kontrol. Ekstrak lobak termasuk dalam kategori senyawa toksik rendah (LC_{50} = 839,52 ppm) dalam fungsinya sebagai senyawa antibakteri yang mengandung senyawa antibakteri mayor (*bis(2-ethylhexyl) phthalate*, *1,2-benzenedicarboxylic acid*, *9,12,15-octadecatrienoic acid*), *fatty acid* (*n-hexadecanoic acid*, *butanedioic acid*), *carboxylic acid* (*isobutyric acid*, *malic acid*, *oleic acid*), dan senyawa antibakteri minor (*n-Hydroxymethylacetamide*, *2,4-bis(1,1-dimethylethyl)*, *2,4-pentanedione*, *2-Cyclohexen-1-one*, *hydrazine*, *cyclohexene oxide*, *gamma-sitosterol*).

Kata kunci: antibakteri, pH, *Raphanus sativus*, stabilitas, suhu, waktu

Radish (*Raphanus sativus* L.) is medicinal plant that commonly used as food, additive, or side dish. Beside its nutritional contain radish also has bioactive compound such as tannin, flavonoids, saponin, raphanin, and essential oil which made this plant more potential for natural antimicrobial. Sukhla (2011) in his research found that raphanin in radish was effective in the inhibition of pathogenic microbes such as *Escherichia coli*, *Pseudomonas pyocyaneus*, *Salmonella typhi*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococci*, *P. Listeria*, *Micrococcus*,

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Enterococcus, *Lactobacillus*, and *Pedicoccus*. In addition, Janjua (2013) found that 100 mg mL^{-1} of radish extract was effective in the inhibition of Gram positive and negative bacteria. However, this antibacterial potency needs to be improved in order to expand in the food sector.

In our study, we focused in antibacterial potential of radish root against three species bacteria which often causes damage to fish (*Pseudomonas aeruginosa*, *Bacillus cereus*, and *Staphylococcus aureus*). Related analysis such as Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), toxicity analysis using Brine Shrimp Lethality Test (BSLT), and Gas Chromatography-Mass Spectrometry (GC-MS) were also assessed. Based our study, we expect that this research gives more information source concerning optimal condition of radish extract which can be used in food industry.

MATERIALS AND METHODS

Radish extract was obtained from maceration process using ethyl acetate (semi polar) for 3 days. Extract with 4 different of concentrations (10, 20, 30, and 40%) were tested using well diffusion method towards three species of bacteria test. On further, the stability test based on heating temperature was assessed by heating this extract in 80 °C and 100 °C with duration times: 5, 10, and 15 minutes and pH value (3, 4 [control], 5, 6, and 7). Temperature level and heating time were determined based on Syamsir (2002) research, which found that phenolic compound was stable on 121 °C for 15 minutes; and pH level was determined based on the acidity characteristic of food in daily lives, started from taste senses' acceptable acidity (3.0) to neutral condition (7.0).

Material. White radish (*Raphanus sativus* L.), distilled water, K_2SO_4, selenium, H_2SO_4 96%, H_2O_2 35%, boric acid 4%, NaOH, mix indicator, HCl, hexane, buffer solution pH 4 and pH 7, phenolphthalein, $AlCl_3$ 2%, NA, NB, dilution solution, spoilage microorganism culture: *Staphylococcus aureus* (Gram positive bacteria), *Pseudomonas aeruaeruginosaginosa* (Gram negative bacteria), and *Bacillus cereus* (spore bacteria).

Sample Preparation Phase Methods. Sample preparation phase was the phase used to make powder from radish root (*Raphanus sativus* L.), that are washed, peeled, cut (3 mm), dried (oven; 60 °C; 21 hours), size reduction (dry blender), and sieved (35 mesh). Radish root powder was then proximately analyzed. A growth curve was carried out on each test bacterium to determine the age of the bacteria to be used in the well diffusion test, where the number of bacteria used in the well diffusion test was 10^8 cfu mL^{-1}.

Phase I Methods. Phase I research (Fig 1) started by maceration process of radish root powder (1:10; 20-25 °C) using ethyl acetate for 3 days. Extraction was executed by constant shaking at 150 rpm. Filtrate evaporation and filtration (45 °C; 35 rpm; 1 hour) was done in order to produce antibacterial compound extract. Extract was then diluted to 4 level of concentration (10, 20, 30, and 40% (w/v)). Antimicrobial activity test was done using well diffusion method in order to produce selected extract which was the best extract concentration.

Phase II Methods. In phase II, the stability test was performed by put the extract in different temperature (80°C and 100°C) with different heating time (5, 10, and 15 minutes), and pH (3, 4 [control], 5, 6, and 7); MIC and MBC test; component analysis using GC-MS; and toxicity test (Atmoko 2009) were also performed done toward selected extract from phase I research.

Analysis. Proximate analysis in phase I research was used to determine the content of water, ashes, protein, fat, carbohydrate (AOAC 2005). MIC and MBC in Phase II research were used to analyze the minimum value needed to inhibit and kill 90% growth of test bacteria (Bloomfield 1991). In addition, to asses the cytotoxicity of extract, we used BSLT method (Atmoko 2009), whilst GC-MS test is used to analyze major antibacterial compound in extract.

Experimental Design. Experimental design used in phase I research was Completely Randomized Design with one factor and four levels (10% [A_1], 20% [A_2], 30% [A_3], and 40% [A_4]) and three repetition. Experimental design used in phase II research for stability against heating temperature and time was Completely Randomized Design with two factor and three repetition. Temperature factor used two levels which were: 80 °C (A_1) dan 100 °C (A_2); while heating time used three levels which were: 5 minutes (B_1), 10 minutes (B_2), and 15 minutes (B_3). Stability test against pH value was done using Completely Randomized Design with one factor and three repetition. pH levels were: 3 (A_1), 4 [control] (A_2), 5 (A_3), 6 (A_4), and 7 (A_5).

RESULTS

Phase I. Based on proximate analysis on radish root extract, we found that this extracts contain

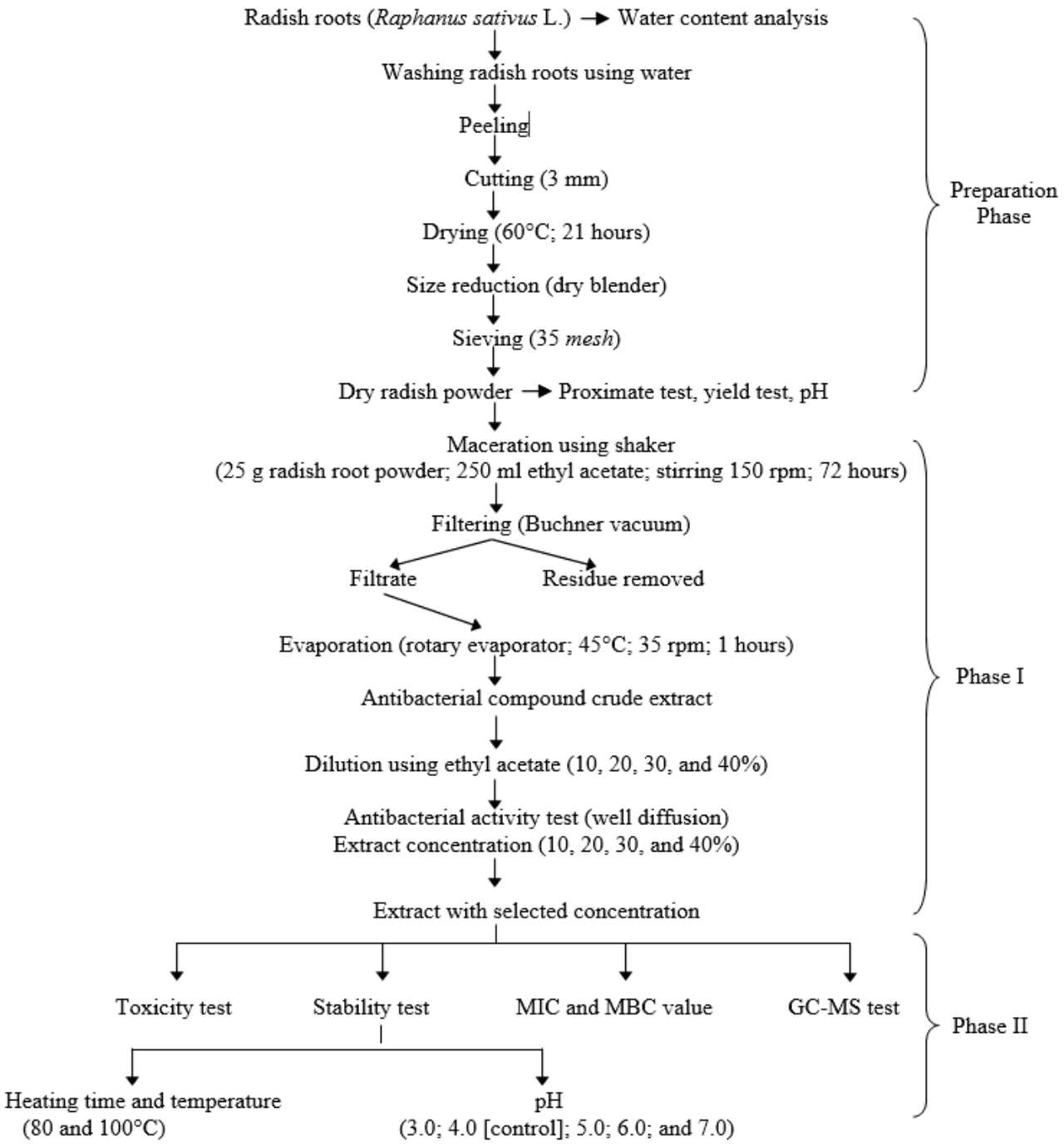

Fig 1 Flowchart of radish root antibacterial activity research.
Source: Modification from Astuti (2007); Janjua (2013); Nuriani (2007)

18.00±0.16% water, 11.27±0.09% ashes, protein 16.72±1.25% protein, 2.50±0.30% fat, 51.51±1.23% carbohydrate. Radish root powder had 79.45±1.29% yield and pH of 4.90±0.10.

The Bacteria Used. Bacteria used in test were in log phase after 6 hours of incubation period. Total colony used for well diffusion in this research were 10^8 cfu mL^{-1}, total colony of *Staphylococcus aureus, Pseudomonas aeruginosaginosa,* and *Bacillus cereus* used in test were $5.10x10^8$ cfu mL^{-1}, $2.61x10^8$ cfu mL^{-1}, and$4.30x10^8$ cfu mL^{-1}.

Inhibition Diameter Based on Extract Concentration. According to Chandra (2011), antibacterial activity could be divided into four categories based on its inhibition diameter, that were no activity (<6 mm), weak (6-7 mm), medium (7-10 mm), and strong (>10 mm). Antibacterial activity test result of radish root extract using ethyl acetate was proven effectively inhibit the growth of fish spoilage bacteria (Table 1). Table 1 showed that extract concentration

significantly affecting inhibition diameter ($p<0.05$). The bigger the extract concentration would result in bigger inhibition diameter. According to Mpila *et al.* (2012), bioactive compound contained in higher concentrated extract was higher than those in lower concentrated, affecting bacteria growth inhibition. Table 1 also showed that 30% extract concentration was able to inhibit the bacteria with more than 10 mm inhibition diameter, it was strong inhibition according to Chandra (2011); thus, the extract concentration for the next phase was 30%.

Phase II. Phase II Research was done in order to determine the stability against heating temperature (80 °C and 100 °C) and heating time (5, 10, and 15 minutes), and pH (3, 4 [control], 5, 6, and 7). In this step, analysis of selected extract from phase I was also done such as MIC and MBC test, toxicity test, and component analysis using GC-MS.

Extract Stability Based on Heating Temperature and Time. Statistical test results showed that heating temperature and time interacted affected inhibition diameter of the three bacteria test ($p<0.05$). Both factors affected the inhibition diameter so that the extract stability could be indicated. Table 2 showed that heating temperature and time caused the reduction of the formed inhibition diameter, moreover at 100 °C there was no inhibition diameter formed. It could be indicated that heat treatment made the extract unstable and the presence of heat treatment caused radish extract antibacterial activity only reach medium category (7-10 mm).

Extract Stability Based on pH. The change in pH affect the inhibition diameter of the three test bacteria ($p<0.05$). The addition of HCl 0.1M as the acid conditioner and the addition of NaOH 0.1M as base conditioner on extract gave significant difference compared to control extract with pH ~4,0 (the extract without acid or base conditioning). Table 3 showed that the increase in pH from control reduce the size of inhibition diameter, while the decrement of pH value will increase the size of inhibition diameter.

MIC and MBC. MIC and MBC value was determined based on the selected extract inhibition zone value (30% concentration). MIC value was the minimum value needed to inhibit 90% growth of test bacteria, while MBC value was the minimum concentration needed to kill 90% of test bacteria (Shahid *et al.* 2013). MIC and MBC test results for *S. aureus* were 0.13% and 0.50%, for *P. aureginosa* were 0.06% and 0.24%, and for *B. cereus* were 0.12% and 0.48% (Table 1). Usually, Gram negative bacteria had higher MIC and MBC value compared to Gram positive bacteria, but in this research the MIC and MBC of Gram positive bacteria (*S. aureus* and *B. cereus*) were higher.

Toxicity. Toxicity value (LC_{50}) indicates the safety level of extract to be applied in food products. This test was done as the first step in other complex toxicity test. Based on the principle of Brine Shrimp Lethality Test (BSLT), the more compound needed to kill 50% of shrimp larvae, then the compound is categorized as non-toxic. Juniarti (2009) divides the toxicity level of LC_{50} into three categories that are: $LC_{50}>1000$: non-toxic, $30<LC_{50}<1000$ ppm: low toxic, dan $LC_{50}<1000$ ppm: toxic. Test results showed that radish root extract had LC_{50} of 839.52 ppm (low toxic).

GC-MS. The extract contain major antibacterial compound (bis(2-ethylhexyl) phthalate, 1,2-benzenedicarboxylic acid, 9,12,15-octadecatrienoic acid), fatty acid (n-hexadecanoic acid, butanedioic acid), carboxylic acid (isobutyric acid, malic acid, oleic acid), and minor antibacterial compound (n-Hydroxymethylacetamide, 2,4-bis(1,1-dimethylethyl), 2,4-pentanedione,2-Cyclohexen-1-one, hydrazine, cyclohexene oxide, gamma-sitosterol).

DISCUSSION

Radish root extract made using ethyl acetate with 3 days extraction time and 30% concentration effectively gave the best inhibition toward three fish spoilage bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus cereus*). MIC and MBC score against the three test bacteria respectively were: 0.13% and 0.50% (*Staphylococcus aureus*), 0.06% and 0.26% (*Pseudomonas aeruginosa*), and 0.12% and 0.48% (*Bacillus cereus*). According to Davidson (2009) and Yunilla (2016), even though Gram negative bacteria has more complex cell membrane, Gram positive bacteria has membrane composed of peptidoglycan which is polar and bigger (90%) compared to peptidoglycan layer of Gram negative bacteria (10-50%). This would result in the phytochemical compound that are mostly non-polar getting hard to invade and destroy Gram positive bacteria cell, thus more compound is needed to inhibit and kill bacteria.

Radish root extract was not stable against heat treatment and change in pH value; but 80 °C heat treatment for 5 min gave the closest results to control's inhibition strength. According to Syamsir (2002), the reduction of inhibition rate was caused by the decrement of phenolic compound effectivity which act

Table 1 Phase I analysis results (inhibition diameter based on extract concentration)

Concentration (%)	Inhibition diameter (mm)		
	S. aureus	*P. aeruginosaginosa*	*B. cereus*
10	8.36 ± 0.19^{a}	8.88 ± 0.29^{k}	8.73 ± 0.34^{w}
20	10.11 ± 0.34^{b}	11.14 ± 0.39^{l}	9.92 ± 0.31^{x}
30	11.39 ± 0.21^{c}	12.73 ± 0.25^{m}	12.73 ± 0.52^{y}
40	13.77 ± 0.30^{d}	13.64 ± 0.61^{n}	13.83 ± 0.40^{z}

Different notation showed that there was significant difference ($p<0.05$); not compared between bacteria

Table 2 Phase II analysis results (extract stability based on heating temperature and time)

Heating temperature (°C)	Heating time (minutes)	Inhibition diameter (mm)		
		S. aureus	*P. aeruginosaginosa*	*B. cereus*
Control (no heat treatment)		11.39 ± 0.20	12.73 ± 0.25	12.73 ± 0.52
80	5	7.81 ± 0.28^{d}	9.97 ± 0.31^{n}	10.80 ± 0.16^{z}
	10	7.05 ± 0.05^{c}	8.72 ± 0.37^{m}	9.78 ± 0.36^{y}
	15	6.23 ± 0.15^{b}	7.11 ± 0.27^{l}	8.60 ± 0.44^{x}
100	5	0.00 ± 00^{a}	0.00 ± 00^{k}	0.00 ± 00^{w}
	10	0.00 ± 00^{a}	0.00 ± 00^{k}	0.00 ± 00^{w}
	15	0.00 ± 00^{a}	0.00 ± 00^{k}	0.00 ± 00^{w}

Different notation showed that there was significant difference ($p<0.05$); not compared between bacteria

Table 3 Phase II analysis results (extract stability based on pH)

pH	Inhibition diameter (mm)		
	S. aureus	*P. aeruginosaginosa*	*B. cereus*
3.0	11.70 ± 0.00^{d}	13.90 ± 0.60^{n}	14.40 ± 0.38^{z}
~4.0 (control)	11.38 ± 0.20^{c}	12.73 ± 0.25^{m}	12.72 ± 0.32^{y}
5.0	5.13 ± 0.60^{b}	6.20 ± 0.20^{l}	6.50 ± 0.30^{x}
6.0	0.00 ± 00^{a}	0.00 ± 00^{k}	0.00 ± 00^{w}
7.0	0.00 ± 00^{a}	0.00 ± 00^{k}	0.00 ± 00^{w}

Different notation showed that there was significant difference ($p<0.05$); not compared between bacteria

as antibacterial in the extract. Phenolic compound is stable on 120 °C for 15 min, but in several phytochemical compound with low molecular weight, evaporation could easily happen when heated. This could result in the instability of extract because of the evaporation of active component. Naufalin *et al.* (2007) added that the loss of inhibition potential of a flavonoid compound could happen because of degradation of flavonoid starting from 60 °C such as radish drying. At pH of 3, the inhibition radish root extract was increase from the inhibition strength of control extract. According to Naufalin *et al.* (2007), there are several factors that increase the inhibition of bacteria in acid condition. The first is substitution between antibacterial compound with HCl as halogen affecting the breakdown of membrane. Cl^- ion made bacteria cell spend more energy making it more susceptible against radish extract antibacterial compound. The second is that phenolic compound work actively in low pH condition because alkylation and hydroxylation happen easily in acid condition, improving the phenol group of bacteria whether it is in water or lipid phase. The third is that bacteria cell maintain constant pH in cell when making contact with

acid. In low pH, proton of H^+ ion is going to enter the cytoplasm through trans membrane proton gradient making the cytoplasm pH decrease and causing the enzyme work to return internal cell pH to normal. This would happen because the proton causing acid condition could also cause denaturation of cell component. The process to return pH to normal need a lot of energy from bacteria and could affect cell metabolism causing the cell to die.

Soetan and Oyewole (2009) and Yunilla (2016), stated that natural antinutritional compound could be formed by plant metabolism such as glycoside, tannin, lignin, lignin, triterpenoid, oxalate, dan amino acid. Yunilla also said that low toxicity is also categorized as safe and is not dangerous if consumed as herbal intake, thus radish root extract was categorized as safe (not dangerous) and could be consumed at certain dose.

Selected radish root extract was categorized as low toxic compound (LC_{50} = 839.52 ppm) in its function as antibacterial compound that contains as follows (in the order of the best antibacterial potential): major antibacterial compound (bis(2-ethylhexyl) phthalate, 1,2-benzenedicarboxylic acid, 9,12,15-octadecatrienoic acid), fatty acid (n-hexadecanoic acid, butanedioic acid), carboxylic acid (isobutyric acid, malic acid, oleic acid), and minor antibacterial compound (n-Hydroxymethylacetamide, 2,4-bis(1,1-dimethylethyl), 2,4-pentanedione,2-Cyclohexen-1-one, hydrazine, cyclohexene oxide, gamma-sitosterol). All component were categorized as essential oil that could act as antibacterial agent (Hussain *et al.* 2011; Agoramoorthy *et al*. 2007; Pringgenies 2010; Rajeswari *et al*. 2012; Rahminiwati *et al.* 2010; Soni *et al.* 2014).

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