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Antibacterial Potential of Star Anise (*Illicium verum* Hook. f.) Against Food Pathogen Bacteria

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Star anise (*Illicium verum* Hook. f.) is commonly used as spice and flavor enhancer in food. Previous research revealed the presence of active compound which could inhibit bacterial growth. Thus, in order to apply star anise as natural antibacterial agent in food product, a further research concerning antibacterial activity and stability of star anise was conducted. Crude extract of star anise was obtained using ethanol and acetone with maceration method for 3 days, then diluted to 10, 20, 30, 40, and 50% (w/v). Well diffusion was conducted against three food spoilage bacteria (*Staphylococcus aureus*, *Escherichia coli*, and *Bacillus cereus*). Extract from ethanol with 30% concentration was selected as the best extract in which inhibit more than 6 mm inhibition zone with MIC and MBC value: 1.59% and 6.36% (*S. aureus*), 1.04% and 4.18% (*E. coli*), and 0.59% and 2.39% (*B. cereus*). This selected extract was used to test the extract stability against 4 levels of heating temperature (60, 70, 80, and 90°C) for 2 levels of heating time (15 and 30 minutes), and 4 levels of pH (4, 5, 6, and 7). Based on our results, different heating treatment and pH caused extract instability. Star anise extract was more stable at 60°C for 15 minutes heating treatment and pH 4, which resulting the lowest inhibition zone reduction compared to control extract. Star anise extract was categorized as low toxic compound (LC_{50} = 212.09 ppm). Terpenoids (anethole, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-2-norpinene, β-caryophyllene, β-bisabolene) was founded as major antibacterial compound in star anise extract; fatty acid (6-octadecenoic acid, hexadecanoic acid, stearic acid) and benzaldehyde (4-anisaldehyde, p-allylanisole) were also founded as minor compound.

Key words: antibacterial, *Illicium verum* Hook. f., pH, stability, temperature, time

Bunga lawang (*Illicium verum* Hook. f.) umumnya dimanfaatkan sebagai rempah dan perisa pada makanan. Penelitian terdahulu mengungkap adanya senyawa aktif yang berpotensi menghambat pertumbuhan bakteri. Hal ini mendorong dilakukan penelitian lebih lanjut agar diketahui aktivitas dan stabilitas bunga lawang sebagai senyawa antibakteri alami yang dapat diterapkan dalam produk pangan. Ekstrak kasar bunga lawang diperoleh dari ekstraksi dengan etanol dan aseton selama 3 hari pada konsentasi 10, 20, 30, 40, dan 50% (b/v). Pengujian difusi sumur dilakukan terhadap tiga bakteri perusak pangan (*Staphylococcus aureus*, *Escherichia coli*, dan *Bacillus cereus*). Ekstrak 30% dengan pelarut etanol merupakan ekstrak terpilih penghasil zona hambat lebih dari 6 mm dengan MIC dan MBC berurutan sebesar 1,59% dan 6,36% (*S. aureus*), 1,04% dan 4,18% (*E. coli*), 0,59% dan 2,39% (*B. cereus*). Ekstrak terpilih digunakan dalam tahap pengujian stabilitas ekstrak terhadap suhu pemananasan (60, 70, 80, dan 90°C), waku pemanasan (15 dan 30 menit), dan pH (4, 5, 6, dan 7). Perlakuan panas dan perubahan pH menyebabkan ketidakstabilan ekstrak. Ekstrak bunga lawang lebih stabil pada suhu 60°C selama 15 menit dan pH 4, kondisi ini menghasilkan ekstrak dengan penurunan zona hambat terkecil terhadap nilai penghambatan ekstrak kontrol. Ekstrak bunga lawang termasuk dalam kategori senyawa toksik rendah (LC_{50} = 212,09 ppm) dalam fungsinya sebagai senyawa antibakteri yang mengandung senyawa antibakteri mayor berupa golongan terpenoid (anethole, 2,6-*dimethyl*-6-(4-*methyl*-3-*pentenyl*)-2-*norpinene*, *β-caryophyllene*, *β-bisabolene*); dan senyawa antibakteri minor berupa golongan asam lemak (*6-octadecenoic acid*, *hexadecanoic acid, stearic acid*) dan golongan benzaldehide (*4-anisaldehyde*, *p-allylanisole*).

Kata kunci: antibakteri, *Illicium verum* Hook. f., pH, stabilitas, suhu, waktu

Star anise (*Illicium verum* Hook. f.) is spice which commonly used as traditional medicine. This plant is commonly found at tropical and subtropical area in Asia (Ahmad and Youssef, 2015; Sivakumar *et al.* 2016). Star anise's essential oil consists of 89.5% trans-anethole, which acts as antimicrobial, antimicotoxins, antioxidants, and insecticide compound (Aly *et al.* 2016; Wei *et al.* 2014). The potency of star anise as antimicrobial agent then encouraged further research to increase the possibility to use star anise as natural antibacterial agent in food industry.

Crude extract of star anise was obtained using maceration method with ethanol (polarity index 5.2) and acetone (polarity index 5.1) as solvent for 3 days. The crude extract then diluted into 5 levels of concentration (10, 20, 30, 40, and 50% (w/v)) and tested with well diffusion method against 3 species of

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bacteria (*S. aureus*, *E. coli*, and *B. cereus*). Previous research regarding solvent used in antibacterial compound extraction to apply in food product by Ahmad and Youssef (2015), Dasgupta and Klein (2014), and Das and Kumar (2013) showed that ethanol and acetone work effectively to extract phytochemical compound (flavonoids, alkaloids, triterpenoids, tannins, steroids, and glycosides) which can be used as antibacterial agent against *E. coli*, *S. aureus*, and *B. subtilis*. Aguda and Chen (2016) added that both ethanol and acetone were categorized as Generally Recognized as Safe (GRAS) by Food and Drug Administration (FDA).

The selected concentration was then used to test extract stability against 4 levels of heating temperature (60, 70, 80, and 90 °C) for 2 levels of heating time (15 and 30 minutes), and 4 levels of pH (4, 5, 6, and 7). The heating temperature and time were determined based on previous research by Peter (2001) regarding the stability of trans-anethole in fennel extract (*Foeniculum vulgare*) at 70°C for 15 minutes heating treatment. Research by Handayani and Sriherfyna (2016) and Surono *et al.* (2016) also showed that bioactive compounds were commonly degraded at temperature more than 50°C, also the common temperature used in food processing starts around 63°C for 30 minutes. Level of pH used in this research was determined based on the pH of food product (3.5-7.0) and the ability of pathogenic microbes to grow at pH 4.0-9.5 (Surono *et al.* 2016). Analysis about 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 conducted in this research. The results of this research hopefully can be used as source of information, especially regarding optimum conditions of star anise extract for application in food industry.

MATERIALS AND METHODS

Material. Dry star anise ("SAJP" brand), ethanol, acetone, alcohol, dimethyl sulfoxide (DMSO), hexane, NaCl 0.85%, nutrient agar (NA), nutrient broth (NB), HCl 0.1 N, NaOH 0.1 N, pH 4 and 7 buffer solution, and isolates of *Staphylococcus aureus* (Gram positive bacteria), *Escherichia coli* (Gram negative bacteria, and *Bacillus cereus* (spore bacteria).

Sample Preparation Phase Methods. Sample preparation steps that were performed to obtain star anise powder, included size reduction (35 mesh), conducting proximate analysis, and determining optimum time of tested bacteria growth using bacterial growth curve. Total colony used for well diffusion test in this research were 10^8 CFU mL^{-1}.

Phase I Methods. Phase I research (Fig 1) started with maceration process of star anise powder (1:10; 20-25 °C) using ethanol and acetone for 3 days. Extraction was executed by constant shaking at 150 rpm. Filtration of the filtrate (using Whatman No.1 and Buchner vacuum) and evaporation (40 °C; 35 rpm; 1 hour) were carried out in order to produce antibacterial compound extract. Extract was then diluted to 5 levels of concentration (10, 20, 30, 40, and 50% (w/v)). Antimicrobial activity test was conducted using well diffusion method in order to determine selected extract which was the best extract concentration in this research.

Phase II Methods. In phase II (Fig 1), the stability test was performed by setting the extract into different temperature (60, 70, 80, and 90 °C) with different heating time (15 and 30 minutes), and pH (4, 5, 6, and 7); MIC and MBC test; component analysis using GC-MS; and toxicity test (Sangi 2012) were also performed toward selected extract from phase I research.

Analysis. Proximate analysis in phase I research was used to determine the water content of dry star anise powder (AOAC 2005). MIC and MBC in Phase II research were used to analyze the minimum value needed to inhibit bacterial growth and kill 90% of tested bacteria (Bloomfield 1991). In addition, to test the cytotoxicity of extract, we used BSLT method (Sangi 2012), whilst GC-MS test was 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 five levels (10% [A_1], 20% [A_2], 30% [A_3], 40%, [A_4]), and 50% [A_5]) with three repetition. Experimental design used in phase II research for stability against heating temperature and time was Completely Randomized Design with two factors and three repetition. Temperature factor used four levels which were: 60 (A_1), 70 (A_2), 80 (A_3), and 90°C (A_4); while heating time used two levels which were: 15 minutes (B_1), and 30 minutes (B_2). Stability test against pH value was performed using Completely Randomized Design with one factor and three repetition. Levels of pH were: 4 (A_1), 5 (A_2), 6 (A_3), and 7 (A_4).

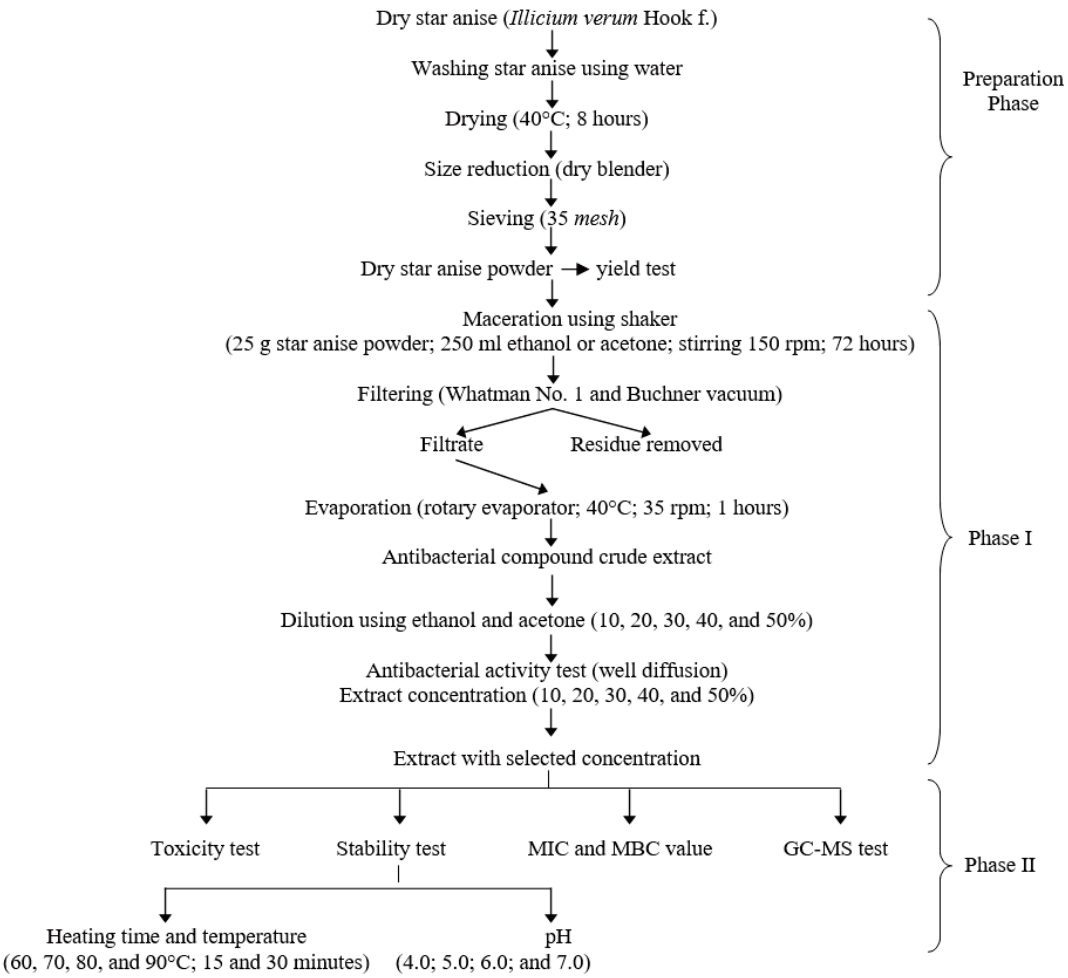

Fig 1 Flowchart of star anise antibacterial activity research.
Source: Modification from Ahmad and Youssef (2015); Badal and Degoda (2017); Nam *et al.* (2017); Shete and Chitanand (2014); Wei *et al*. (2014)

RESULTS

Phase I. The yield of 72 hours maceration extraction using ethanol was 15.19 ± 0.35% and 12.45±0.37% using acetone. Ethanol is a polar solvent, so it is easy to attract most of the polar in star annise.

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 *S. aureus*, *E. coli*, and *B. cereus* used in test were $1.9x10^8$ CFU mL^{-1}, $1.8x10^8$ CFU mLl^{-1}, and$2.5x10^8$ CFU mL^{-1}.

Inhibition Diameter Based on Extract Concentration. Table 1 showed that extract concentration significantly affecting inhibition diameter ($p<0.05$). The bigger extract concentration would produce larger inhibition diameter. Table 1 also showed that ethanol was proven to be effective in inhibiting the growth of tested bacteria. At a concentration of 30%, ethanol was able to inhibit the bacteria with more than 6 mm inhibition diameter, it was minimal inhibition according to Khalaphallah (2015); thus, the extract concentration used for the next phase was 30%.

Phase II. Phase II Research was done in order to determine the stability against heating temperature (60, 70, 80, and 90 °C) and heating time (15 and 30 minutes), and pH (4, 5, 6, and 7). In this phase, MIC and MBC test, toxicity test, and component analysis using GC-MS were also done towards selected extract from phase I.

Extract Stability Based on Heating Temperature and Time. Statistical test results (Table 2) showed that stability of antibacterial activity of extract on *S. aureus* and *E. coli* was affected by heating temperature and time ($p<0.05$), but there was no interaction between these two factors; when tested on *B. cereus* (Table 3), stability of antibacterial activity of extract was affected by both factors (heating temperature and time) interactively ($p<0.05$). Higher

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

Concentration (%)	Solvent	Inhibition diameter (mm)		
		S. aureus	*E. coli*	*B. cereus*
10	Ethanol	4.25 ± 0.15^{a}	4.83 ± 0.19^{l}	4.89 ± 0.16^{v}
	Acetone	4.30 ± 0.18^{a}	3.43 ± 0.16^{k}	3.25 ± 0.05^{t}
20	Ethanol	4.88 ± 0.21^{b}	5.88 ± 0.26^{mn}	4.95 ± 0.20^{v}
	Acetone	5.19 ± 0.21^{bc}	5.09 ± 0.24^{l}	4.39 ± 0.16^{u}
30	Ethanol	6.65 ± 0.04^{d}	6.49 ± 0.28^{o}	6.04 ± 0.08^{x}
	Acetone	5.43 ± 0.19^{c}	5.72 ± 0.28^{m}	5.06 ± 0.14^{vw}
40	Ethanol	7.35 ± 0.34^{e}	7.07 ± 0.17^{p}	6.15 ± 0.24^{x}
	Acetone	6.42 ± 0.29^{d}	6.18 ± 0.30^{no}	5.40 ± 0.20^{w}
50	Ethanol	7.91 ± 0.31^{f}	8.04 ± 0.27^{q}	6.89 ± 0.33^{z}
	Acetone	7.14 ± 0.29^{e}	6.49 ± 0.20^{o}	6.51 ± 0.28^{y}

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 on *Staphylococcus aureus* and *Escherichia coli*)

	Inhibition diameter (mm) based on heating temperature					Inhibition diameter (mm) based on heating time (minutes)		
	Control	60°C	70°C	80°C	90°C	Control	15	30
S. aureus	6.36 ± 0.26^{b}	7.03 ± 0.28^{a}	5.66 ± 0.28^{c}	4.61 ± 0.17^{d}	3.97 ± 0.22^{e}	6.36 ± 0.26^{h}	5.46 ± 0.35^{hi}	5.18 ± 0.36^{i}
E. coli	6.53 ± 0.18^{k}	6.39 ± 0.33^{k}	5.39 ± 0.26^{l}	4.44 ± 0.32^{m}	4.42 ± 0.23^{m}	6.53 ± 0.18^{p}	5.38 ± 0.25^{q}	4.94 ± 0.25^{q}

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

Table 3 Phase II analysis results (extract stability based on heating temperature and time on *Bacillus cereus*)

Heating temperature (°C)	Heating time (minutes)	Inhibition diameter (mm) *B. cereus*
Control (no heat treatment) (~25°C, 0 minute)		6.68 ± 0.12^{a}
60	15	6.43 ± 0.24^{a}
	30	6.00 ± 0.17^{b}
70	15	5.43 ± 0.21^{c}
	30	5.29 ± 0.19^{c}
80	15	4.11 ± 0.08^{de}
	30	4.30 ± 0.16^{d}
90	15	4.22 ± 0.15^{de}
	30	3.95 ± 0.14^{e}

Different notation showed that there was significant difference ($p<0.05$)

Table 4 Phase II analysis results (extract stability based on pH

pH	Inhibition diameter (mm)		
	S. aureus	*E. coli*	*B. cereus*
Control (pH ~5.66)	6.63 ± 0.20^{a}	6.59 ± 0.23^{n}	6.38 ± 0.29^{a}
4.0	6.24 ± 0.14^{b}	6.83 ± 0.25^{m}	6.54 ± 0.27^{a}
5.0	6.22 ± 0.10^{b}	6.67 ± 0.32^{l}	6.40 ± 0.29^{a}
6.0	6.10 ± 0.28^{b}	5.86 ± 0.18^{k}	5.59 ± 0.25^{b}
7.0	3.42 ± 0.16^{a}	2.44 ± 0.12^{k}	2.13 ± 0.10^{c}

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

heating temperature and time tend to produce smaller inhibition diameter. When heated for more than 60°C for 15 minutes heating period, the extract was not showing active antibacterial activity (inhibition diameter <6 mm).

Extract Stability Based on pH. Changes in pH affected the inhibition diameter ($p<0.05$). The addition of HCl 0.1 M as the acid regulator and NaOH 0.1 M as base regulator on extract gave significant difference compared to control extract with pH ~5.66 (the extract without any treatment). Table 4 showed that pH escalation in extract towards neutral produced smaller inhibition diameter, and antibacterial compound in star anise extract was more stable at pH 4-5.

MIC and MBC. MIC and MBC value were determined based on selected extract's (30%

concentration) inhibition zone. MIC value was the minimum concentration needed to inhibit 90% growth of tested bacteria, while MBC value was the minimum concentration needed to kill 90% of tested bacteria (Zadrazilova *et al.* 2015). MIC and MBC test results were 1.59% and 6.36% for *S. aureus*, 1.04% and 4.18% for *E. coli*, and 0.59% and 2.39% for *B. cereus* (Table 1).

Toxicity. Selected star anise extract was categorized as low toxic compound (LC_{50} = 212.09 ppm) in its function as antibacterial compound that contains as follows (in the order of the best antibacterial potential).

GC-MS. Star anise extract contained major antibacterial compounds in the form of terpenoids (anethole, β-caryophyllene, β-bisabolene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-2-norpinene, β-Linalool, p-allylanisole, Trans-γ-bisabolene); and minor antibacterial compounds in the form of fatty acids (6-octadecenoic acid, hexadecanoic acid, stearic acid) and benzaldehyde (methoxy acetophenone, 4-anisaldehyde, 3-propenylphenol).

DISCUSSION

Star anise extract made using ethanol for 3 days extraction time at 30% concentration effectively exerted best inhibition toward three food spoiling bacteria (*Staphylococcus aureus*, *Escherichia coli*, and *Bacillus cereus*). According to Baldosano *et al*. (2015), most of the compounds in star anise tend to be polar, thus the amount extracted using ethanol solvent was greater than acetone solvent. More active compounds found in an extract would produce larger inhibition diameter (Khasanah 2014). According to Khalaphallah (2015), antimicrobial compound was categorized as active if the inhibition diameter is more than 6 mm. Antibacterial activity test result of star anise extract using ethanol was proven to be effective in inhibiting the growth of tested bacteria. Tannin and phenolic compounds were antimicrobial agent that tend to be polar, thus both were extracted more in ethanol extract and resulting greater inhibition zone than acetone extract (Iloki-Assanga *et al*. 2015; Wijayanti, 2016; Medini *et al*. 2014; Chouksey *et al*. 2013).

MIC and MBC value against the three tested bacteria respectively were: 1.59% and 6.36% (*S. aureus*), 1.04% and 4.18% (*E. coli*), and 0.59% and 2.39% (*B. cereus*). The more susceptible the bacteria to anethole (Mohammed 2009). Different structure and outer cell membrane component caused different MIC and MBC value in *S. aureus* (Gram positive) and *E. coli* (Gram negative), this was also affected by membrane sensitivity against specific antimicrobial compounds. Gram negative bacteria have more complex cell wall which contain thin peptidoglycan (10-50%). Antimicrobial compounds destroy outer cell membrane through porins (hydrophilic pathway) then separate phospholipids and lipopolysaccharides. Gram positive bacteria don't have outer cell membrane, but it's composed with thicker peptidoglycan (90%), thus generally harder to be penetrated by antimicrobial compounds.

Star anise extract was unstable toward heating treatment and changes in pH value; but heating treatment at 60 °C for 15 minutes and pH 4-5 was able to exert closest result in inhibiting bacterial growth when compared to control extract. Generally as the heating temperature and time escalated, the antibacterial compounds lost its stability (thermolabile) because chemical structure of the compounds was degraded during heating and the antimicrobial activity decreased (Turek and Stintzing 2013; Sant'Anna *et al.* 2012; and Zhang *et al.* 2014). Ardiansyah (2002) reported that in certain specific condition, antibacterial activity could also increase as the temperature increases. Degradation of extract compounds formed new compounds which also have the potential to inhibit microorganism growth. Oxidized anethole compound would form 4-methoxy benzaldehyde (4-anisaldehyde) and anisketone which act as antimicrobial compound (Okamoto *et al.* 2002; Fahlbusch *et al.* 2003; Kosalec *et al.* 2005).

Escalation in pH also affected extract stability. The presence of fatty acids, such as hexadecanoic acid, 6-octadecenoic acid, and stearic acid in extract made the extract more stable at pH 4-5. pH can affect the absorption of fatty acids in bacteria. As the pH increase, effectiveness of fatty acid absorption would decrease, thus the antibacterial activity also decreased (McGaw *et al.* 2002). Purbowati *et al.* (2016) and Pan *et al.* (2014) added that phenolic compounds in the extract are damaged by increased pH and causing the antibacterial activity became less effective. In reverse, a decrease in pH (an increase in H^+ ions) causes bacterial cytoplasm became less stable and it needs more energy to restore the cell's internal pH to normal state. Cell metabolism will be disturbed as more energy required to keep the normal state of cell's internal pH, then the bacteria cells will die over time (Naufalin *et al.* 2006). The decrease in pH also increases the stability and effectiveness of phenolic compounds which are more hydrophobic in acidic conditions thereby facilitating the dissolution of

bacterial membrane fat (Purbowati *et al.* 2016; Pan *et al.* 2014).

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 amount of compound needed to kill 50% of shrimp larvae, then the compound will be categorized as more non-toxic. Juniarti *et al*. (2009) divides the toxicity level of LC_{50} into three categories which are: LC_{50}>1000 ppm as non-toxic, 30<LC_{50}<1000 ppm as low toxic, and LC_{50}<1000 ppm as toxic. Test result showed that star anise extract had LC_{50} of 212.09 ppm (low toxic). Nakamura (1996) reported that veranisatin A, B, and C were toxic compound which contributed in star anise toxicity test, but the concentration of veranisatin A, B, and C in dry star anise were low, respectively, 0.00016%, 0.00010%, and 0.000015% and it's categorized as safe to use in medicine and food products. FDA already classified *Illicium verum* Hook. f. as *generally recognized as safe* (IOFI 2017). Dewi *et al*. (2012), Soetan and Oyewole (2009), and Yunilla (2016) added that the natural antinutrient bioactive components formed by plant metabolism can also be toxic, such as glycosides, tannins, lignin, triterpenoids, oxalates, and amino acids. Low toxicity is considered safe and harmless if consumed as herbal intake at the right dose (Yunilla 2016). Further in vivo research is needed as dose reference for the application in food products that are safe for humans (BPOM 2014).

The major antibacterial compound in star anise extract, including terpenoids (anethole, β-caryophyllene, β-bisabolene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-2-norpinene, β-Linalool, p-allylanisole, Trans-γ-bisabolene); and minor antibacterial compound, including fatty acids (6-octadecenoic acid, hexadecanoic acid, stearic acid) and benzaldehyde (methoxy acetophenone, 4-anisaldehyde, 3-propenylphenol). All component were categorized as essential oil that could act as antibacterial agent (Attokaran, 2017; Aly *et al*. 2016; Taniguchi *et al.* 2014; Herman *et al.* 2016; Andrade *et al.* 2015; Okatomo *et al.* 2002; Fahlbusch *et al.* 2003; Chong *et al.* 2015; Carneiro *et al.* 2017; Dahham *et al.* 2015; McWilliams, 2006; Courtois *et al.* 2012; Rai *et al.* 2011; Marteau *et al.* 2016; Karimi *et al.* 2015; Uitterhaegen *et al.* 2016). Okamoto *et al.* (2002), Tisserand and Young (2014), and Kosalec *et al.* (2005) added that oxidized anethole will form anisketone which also acts as antimicrobial agent. In the recent study of Alhajj *et al.* (2019) showed that star anise alcoholic extract have antibacterial and antifungal activities and could be used as natural antimicrobial agent. The stability of star anise volatile essential oil and its antibacterial activity can be significantly enhanced by using HPCD encapsulation (Zhang *et al.* 2018).

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