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Antibacterial activity of ethanolic extracts from *Zingiber zerumbet* rhizome against *Salmonella* spp.

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Abstract. *Rahayu ID, Widodo W, Prihartini I, Winaya A. 2019. Antibacterial activity of ethanolic extracts from* Zingiber zerumbet *rhizome against Salmonella spp. Biodiversitas 20: 3322-3327.* The study aimed at investigating the antibacterial activity of ethanolic extracts of the Lempuyang Gajah (*Zingiber zerumbet* (L.) Smith) rhizome against *Salmonella enteritidis* ATCC 31194 and *S. typhimurium* ATCC 23564. An experimental method was employed for testing the antibacterial activity, with a completely randomized design (CRD) of factorial patterns. Factor I, ethanol concentrations which consisted of 45%; 70% and 95%, while factor II was the concentration of extracts, i.e 0%; 2.5%; 5%; 7.5% and 10%. The phytochemical screening results showed that the *Z. zerumbet* extracts with 45% and 70% ethanol, contained alkaloids, tannins, terpenoids, and saponins; however, the-95%-ethanol did not result in any saponins, but flavonoids instead. Meanwhile, the chromatogram patterns of all extracts showed zerumbone is a dominant compound. Extraction of *Z. zerumbet* using 95% ethanol has higher antibacterial activity against *S. enteritidis* than *S. typhimurium.* The extract with 10% concentration gave the highest antibacterial activity than other concentrations. It can be concluded that the *Z. zerumbet* L. Smith extracts with 95% ethanol and 10% concentration has the best antibacterial activity against *S. enteritidis*. Whereas *S. typhimurium* is effectively inhibited by extracts with 45% ethanol and 7.5% extract concentration.

Keywords: Foodborne disease, phytobiotics, *Salmonella enteritidis*, *Salmonella typhimurium, Zingiber zerumbet*

INTRODUCTION

Salmonella enteritidis (*S. enteritidis*) and *Salmonella typhimurium* (*S. typhimurium*) are pathogen bacteria that commonly found in chicken and may cause contamination of chicken products (eggs or meat) either vertically or horizontally. It could lead to some foodborne-disease cases in various parts of the world including Indonesia, it may also become a carrier which causes several human diseases (Ariyanti and Supar 2008; Andino and Hanning 2015). *Salmonella enteritidis* infections have increased worldwide from the early 1970s and in 1990, this serovar replaced *S. typhimurium* as the leading cause of Salmonellosis in the world. In 1980 the *S. enteritidis* outbreak dramatically increased globally, posing a serious threat to the poultry industry and public health.

Meanwhile, the prevention of bacterial diseases using the addition of Antibiotic Growth Promoters (AGP) as a feed additive in livestock has been banned in Indonesia due to the negative impact. One of the most negative effects of the use of AGP is colonization-inhibition of some beneficial intestinal bacteria, such as *Lactobacillus*, *Bifidobacteria* (Vidanarachchi et al. 2005; Windisch et al. 2007), *Boostereroides*, and *Enterococci* (Panagiota et al. 2015). Thus, it may increase resistance against pathogenic bacteria, like *Salmonella* spp., including *S. enteritidis* and *S. typhimurium* which has proven could be found and isolated from broiler meat (Diaz-Sanchez et al. 2015; Thung et al. 2016).

On the other hand the use of AGP leaves antibiotic residues, such as sulfadiazine and oxytetracycline found in broiler meat (Diaz-Sanchez et al. 2015; Khatun et al. 2015), whereas, tetracycline, ampicillin, streptomycin, and an aminoglycoside are found in chicken kidney and liver (Kader et al. 2011; Sajid et al. 2016).

Phytobiotics generally consist of terpenoids (monoterpenoids, sesquiterpenes, and steroids), phenolics (tannins), glycosides, flavonoids alkaloids and glucosinolates, saponins, and zerumbone (Chang et al. 2012; Diaz-Sanchez et al. 2015). Rough extract of ethanol from the rhizome of *Z. zerumbet* can be used as an antibacterial agent to prevent from Gram-negative pathogenic bacterial diseases (Kader et al. 2011), including *Salmonella* spp.

Ethanol is a suitable solvent for active substances in *Z. zerumbet* in the form of alkaloids, flavonoids, tannins, and terpenoids (Pasril and Yuliasanti 2014), and is a versatile solvent that has the ability to extract with broad polarity, ranging from non-polar compounds to polar (Saifudin et al. 2011). Terpenoid compounds can inhibit the growth of microbes by interfering with the process of formation of membranes and/or cell walls, membranes or cell walls are not formed or formed imperfectly. Terpenoids can penetrate bacterial membranes and reach the inside of cells due to polysaccharide content and lipophilicity, so the cells are damaged (Hashemi and Davoodi 2010).

The use of various ethanol concentrations and *Z. zerumbet* extract concentration will result in the profile difference of the active compounds (phytochemicals) of

extracts, which then will affect the antibacterial activity of the extracts. This study emphasizes the development of medicinal plants, namely *Z. zerumbet* L. Smith as a natural antibacterial agent to *Salmonella* spp., so that it can be used as a substitute for AGP in poultry feed to ensure food safety.

MATERIALS AND METHODS

Plant materials and preparation

The *Z. zerumbet* L. Smith rhizome comes from UPT Materia Medica Batu, East Java, Indonesia, harvest age was 10 months. Complete determination shows that this plant includes *Plantae* kingdom, *Spermatophyta* division, *Angiosperms* subdivision, *Monocotyledonae* class, *Zingiberaceae* family, and *Zingiber* genus. Before the extraction process, the rhizomes are cleaned from attached soil, washed thoroughly, dried and thinly sliced, put into an oven (Hunan China XD-12) at 45ºC for 5 days. Furthermore, it was grounded by a machine (Hunan China CFSJ-250B), 0.5 kg of powder was produced from the rhizome wet weight of 5.2 kg. It takes 3 weeks for sample collection, from fresh rhizome to extract.

Extraction of *Zingiber zerumbet* rhizome

100 grams of rhizome powder that has been moistened with 45% ethanol solvent, put in a jar, flattened and added ethanol solvent until submerged as much as 1 L, close the jar tightly for 24 hours after it is shaken on a digital shaker with a speed of 50 rpm. Next filter the liquid extract using a filter cloth and collected it in the Erlenmeyer tube. The results of the liquid extract were evaporated using a rotary evaporator for 1 hour, then the extract was evaporated over a water bath for 2 hours. The process produces 90 mL of liquid extract. In the extraction of 100 grams of powder using 70% ethanol, 65 mL of liquid extract was produced, while the extraction results with 95% ethanol from 150 grams of powder produced 49 mL of extract. The specifications of the equipment used for extraction are rotary evaporator, "Buchi" (R-215), made in Switzerland, digital shaker, "Wise Shake" (SHO-2D), from Korea, while oven and Waterbath, "Memmert", made in Germany, then alcoholmeter, "Gay Lussac", from Paris.

Phytochemical screening

The chemicals compound obtained from extracting could be identified as alkaloids, flavonoids, saponins, terpenoids, and tannins. Alkaloid was tested by using three types of reagents, namely Meyer, Dragendrouf and Bouchardat, all three are produced by Merck, Germany. Positive reaction was based on the formation of white, orange and brown deposits on each reagent (Ditjen POM 1986; Sudjadi 1986; Nugroho 2017). Flavonoids were tested by adding 0.5g of Mg powder and three drops of concentrated HCl to the mixture of extract and hot aquadest. Red or pink color formation showed a positive reaction (Mojab et al. 2003). The saponins content was detected with shaking of the mixture of extract and hot aquadest strongly, permanent foaming formation for a minimum of 10 minutes as high as 1-10 cm mean positive reaction. The testing of steroids and triterpenoids based on the addition of Bouchardat reagents, the formation of a bluish-green color indicates steroids, meanwhile orange or brownish-orange color indicates triterpenoids. The tannin test was performed with the addition of FeCl3 1%. The existence of tannin is based on the formation of the color of blackish brown, blackish black, or dark green (Bintang 2010).

Gas Chromatography-Mass Spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS analysis of Z. zerumbet rhizome ethanolic extract was carried out using Perkin Elmer GC-MS (Perkin Elmer Clarus 680 GC-Clarus SQ 8T MS) which has an Elite-5 MS capillary column 30 m × 0.25 mm × 25 μm (5% diphenyl, 95% dimethylpolysiloxane, GC-MS was detected using an electron ionization system with 70 eV ionization energy. Gas carrier in the form of helium ultrapure was used with a constant flow rate of 1 mL/min. The ion source, mass transfer path, and injector temperature are respectively set at 230 ° C, 250 ° C, and 290 ° C. The oven temperature is set between 50 to 150 ° C at 3 ° C/min and in isothermal conditions for 10 minutes and subsequently raised to 250 ° C at 10 ° C/minute. Diluted samples (1/100, v/v in ethanol) from 1 wL were manually injected in split mode 120. Mass spectral scanning was in the range of 45-450 m/z with a solvent delay of 2 minutes. The extract components were identified based on comparison of the relative GC retention time and mass spectrum with components from NIST MS Search Library Software version 2.0. Cat 10 ° C/min (Valle et al. 2016).

Microorganism

The isolate used in this research was *Salmonella* spp., consisting of *S. enteritidis* serovar ATCC 31194 and *S typhimurium* serovar ATCC 23564. These isolates were obtained from the laboratory of microbiology, Universitas of Brawijaya, Malang, East Java.

Screening of the extracts for the antibacterial activity

To investigate the antibacterial activity of the extracts against *S. enteritidis*, an experimental method with a completely randomized design (RAL) of factorial patterns was utilized. Factor I was concentration of ethanol solvent: 45%, 70% and 95%, factor II was *Z. zerumbet* extract concentration: 0%; 2.5%; 5%, 7.5%, and 10%, replicated 10 times. The measured variable is the Minimal Inhibitory Concentration (MIC) employing microdilution and diffusion methods, which are sequentially used to obtain data on the effect of treatment on cell counts and inhibition zones of *Salmonella* spp. (Sutardi et al. 2015).

Statistical analysis

The phytochemical screening data were analyzed descriptively quantitatively, while the number and inhibitory zones of *Salmonella* spp.. were analyzed using ANOVA, differences among the treatment were tested by Duncan's Multiple Range Test (DMRT) method. The analysis was carried out through the SPSS program (Statistical Packages for Social Science).

Table 1. Chemical compounds of phytochemical screen of *Zingiber zerumbet* ethanolic extracts

Zingiber zerumbet extracts conc. (%)	Phytochemicals					
	Alkaloids	Flavonoids	Saponins	Terpenoids	Tannin	Steroids
45	+	-	+	+	+	-
70	+	-	+	+	+	-
95	+	+	-	+	+	-

Table 2. The influence of ethanol concentration on the cell number and inhibition zone of *Salmonella* spp.

Zingiber zerumbet extracts conc. (%)	Cell number of *Salmonella* spp (×10^8 CFU/mL)		Inhibition zone (mm)	
	S. enteritidis	*S. typhimurium*	*S. enteritidis*	*S. typhimurium*
45	2.31^c	3.97^b	2.41^a	1.90^c
70	2.41^b	4.52^c	3.40^c	1.79^b
95	1.67^a	2.34^a	2.65^b	0.00^a

Note: The number followed by different letters in the same column shows the difference in the 5% Duncan Test

RESULTS AND DISCUSSION

Phytochemical Screening

The results of phytochemical screening of the ethanolic extracts of *Z. zerumbet* are shown in Table 1. The phytochemical screening based on 3 types of reagents consist of Meyer, Dragendrouf and Bouchardat showed that *Z. zerumbet* extracts depict various beneficial chemical compounds, namely saponins, tannins, alkaloids, flavonoids, and triterpenoids. This findings showed that *Z zerumbet* has properties as phytobiotic, supported by previous theories, in which the positive effects of phytobiotics are mainly related to the plant constituents including terpenoids (monoterpenoids, sesquiterpenes, and steroids), phenolic (tannins), glycosides, alkaloids (as alcohols, aldehydes, ketones, esters, ether, and lactones), flavonoids and glucosinolate (Chang et al. 2012; Diaz-Sanchez et al. 2015). Extractions with the polar solvents water, ethanol, or methanol have identified phenolics, flavonoids, and sesquiterpenoids in *Z. zerumbet* (Ganapathy and Nair 2017).

Based on Table 1, the *Z. zerumbet* extract with ethanol concentrations of 45% and 70% contain saponins, tannins, and triterpenoids, while 95% of ethanol concentrations contain tannins, triterpenoids, and flavonoids. The results of this study are consistent with the statement by Pasril and Yuliasanti (2014) that ethanol is a suitable solvent for extracting active compounds from *Z. zerumbet*. The mechanism of active compounds as antibacterial is by disrupting the biochemical processes of bacterial cells, including inhibition of cell wall synthesis, cell membrane function, and protein synthesis. Alkaloids, terpenoids, and tannins have been successfully extracted from rhizome *Z. zerumbet* with ethanol at various concentrations (45%, 70% and 95%), this has an important meaning that the three compounds effective as antibacterials to suppress the growth of Gram-negative bacteria, because bacterial cell walls consist of polar lipopolysaccharides (LPS) (Huang et al. 2019).

The alkaloids that have been successfully extracted from the *Z. zerumbet* rhizome were suspected in the form of salt, as stated by Hanani (2014) and Harborne (2006), alkaloids are bound to organic acids found in plants, such as succinic acid, maleic, meconic, kinic, and is soluble in ethanol or water solvents. Ethanolic extract from *Z. zerumbet* does not contain steroids, so saponins that have been successfully extracted are likely to be triterpenes saponins, according to the statement by Leland (2006) and (Hanani 2014) that saponins are grouped into steroid and triterpenes, which are water-soluble, not soluble in ether. Reported by Sirait (2007) that triterpenes saponins are usually acidic because it contains one or two carbonyl groups in the aglycone, and it's an alcohol, aldehyde or carboxylic acid.

Flavonoids in *Z. zerumbet* successfully extracted with 95% ethanol solvent, it can be assumed that the flavonoids contained in the extract are in the form of glycosides, perhaps as flavonoids-O-glycosides or flavonoids-C-glycosides, bound to sugar. In line with the statements of Hanani (2014) and Harborne (2006), flavonoids are polyphenol compounds, present in two forms, namely aglycones, are less polar (isoflavones, flavanones, flavonoids and methylated flavones), soluble only in low polarity compounds, such as chloroform and ether, but can also be in the form of polar glycosides, so that they dissolve easily in polar solvents, such as ethanol. Several types of flavonoids contained in *Z. zerumbet* rhizome extract, including quercetin, kaempferol, catechin, and myricetin, whose levels are increasing according to increasing plant age (Ghasemzadeh et al. 2016).

Analysis of chemical compound components of the ethanolic extracts with GCMS

The qualitative data from the GCMS method in 45%, 70% and 95% ethanolic extracts of *Z. zerumbet* are displayed in Figure1, Figure 2, and Figure 3.

The most dominant compound contained in the extracts of *Z. zerumbet* with various concentrations of ethanol is zerumbone, in the 45%, 70%, and 95% ethanol, the zerumbone contents are as much as 91.011%, 75.422%, and 43.143% respectively (Figures 1, 2, 3). Moreover, other compounds, namely alpha-humulene, linalool, and humaladienone are also discovered in 95% of ethanolic extract, which becomes the most complete extract content of all active compounds. This result is in accordance with the results of previous studies, that zerumbone is the most common compound found in *Z. zerumbet* crude ethanolic extract (Yob et al. 2011), and belongs to the

sesquiterpenoid group, generally a component of essential oils (Dai et al. 2013 and Golam et al. 2010; Hanani 2014), with a level of 90.62% in the essential oil fraction obtained by steam and water distillation (Mulyani 2010). Sharifi-Rad et al. (2017) also reported that *Z. zerumbet* essential oil obtained by hydrodistillation from rhizomes containing zerumbone (69.9%), α humulene (12.9%), humulene epoxide II (2.5%), caryophyllene oxide (1.1%) and camphene (1.9%). Zerumbone has antimicrobial activity as a powerful antibacterial and anti-fungal (Bhuiyan et al. 2009). In addition to zerumbone, *Z. zerumbet* also contain bioactive compounds, namely humulene, and monoterpenes (Yob et al. 2011). *Z. zerumbet* contains various terpenoid types, including pinene, camphor, linalool, zerumbone, limonene, camphene, caryophyllene, 3-carene, 4-terpineol and eucalyptol (Bhuiyan et al. 2009). *Z. zerumbet* is also known to contain sesquiterpenes, zederon phenolics, saponins and terpenoids (Kader et al. 2011; Kader et al. 2010; Hashemi et al. 2008 and Yob et al. 2011).

In vitro antibacterial activity

The result of ANOVA shows that the ethanol concentration, the extract concentration and the interaction between both significantly influence the number of *Salmonella* spp. The DMRT test results of the effect of ethanol concentration on the cell number and inhibition zone of *Salmonella* spp. are shown in Table 2, while effect of *Z. zerumbet* extract concentration is shown in Table 3.

Based on Table 2 and Table 3, the study generally shows that ethanolic extract from *Z. zerumbet* has better antibacterial activity against *S. enteritidis* than *S. typhimurium.* The cell number of *S. enteritidis* is fewer and the inhibition zone of the extracts is wider than *S. typhimurium. Salmonella* spp. is a Gram-negative bacteria, with their cell walls containing LPS components; thus its sensitivity is low to Z. *zerumbet* extracts. It is proven that the cell number of *Salmonella* spp. is still high, between 1.67×10^8 CFU/mL to 4.52×10^8 CFU/mL and low inhibition zone, which is between 0.00 mm to 3.68 mm. It was reported that in general Gram-positive bacteria are more sensitive to antimicrobial compounds than Gram-negative bacteria, this is related to differences in the composition of cell walls, the Gram-negative structure consists of lipopolysaccharides which make the cell walls impermeable to lipophilic solutes, unlike bacteria Gram-positive which does not have this outer membrane (Kumar et al. 2013; Liu et al. 2013; Ghasemzadeh et al. 2016). Based on these reasons we suspect that *S. enteritidis* is more sensitive to ethanolic extracts from *Z. zerumbet* than *S. typhimurium* because the outer membrane is more hydrophilic, so the extract easily penetrates it. This is related to the content of several active compounds, such as flavonoids, alkaloids, tannins, and terpenoids (zerumbone) (Table 1, Figure 3) in *Z. zerumbet* ethanolic extracts. The action mechanisms of such active substances are in forms of inhibiting the cell wall synthesis, the cell membrane function and the protein synthesis (Bhuiyan et al. 2009; Pasril and Yuliasanti 2014).

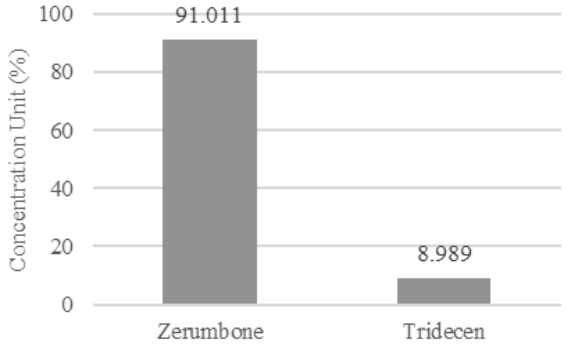

Figure 1. The compounds result of GCMS method in 45% ethanolic extract of *Zingiber zerumbet*

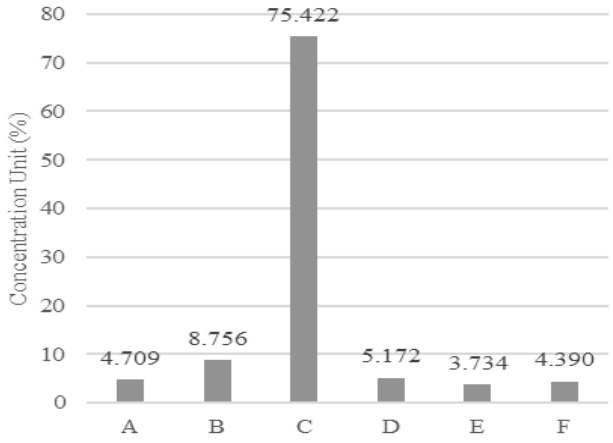

Figure 2. The compounds result in GCMS method in 70% ethanolic extract of *Zingiber zerumbet*. A: Tridecatretane, B: Humuladienone, C: Zerumbone, D: Patchulane, E: Pentadecanoic acid, F: octadecadienoic acid

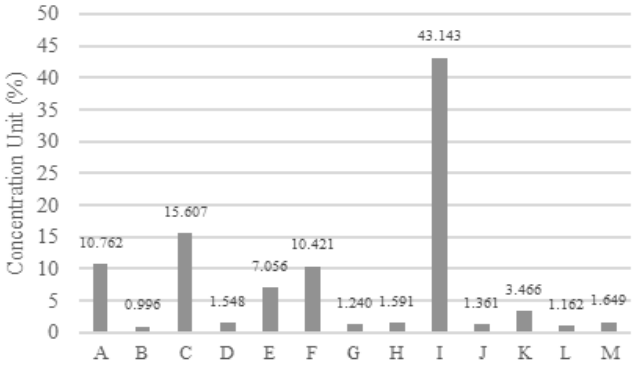

Figure 3. The compounds result in GCMS method in 95% ethanolic extract of *Zingiber zerumbet*. A: Linalool, B: Bicyclo [2.2.1] heptane-2-one, C: Alpha-Humulene, D: Patchulane, E: 7-Pentadecen-5-yne, F: Humuladienone, G: Curdione, H: Cis-1,7-Octadiene-3-yl acetate, I: Zerumbone, J: 1,5,9-Decatriene, K: Ledane, L: Pentadecanoic acid, M: Bicyclol (3,1,1 Heptane. 2,6,6-trimethyl

Table 3. The influence of *Zingiber zerumbet* extract concentration on the cell number and inhibition zone of *Salmonella* spp.

Zingiber zerumbet extracts conc. (%)	Cell number of *Salmonella* spp. ($\times 10^8$ CFU /mL)		Inhibition zone (mm)	
	S. enteritidis	*S. typhimurium*	*S. enteritidis*	*S. typhimurium*
2.5	2.30^{c}	3.70b	1.84^{a}	0.52^{a}
5.0	2.35^{cd}	3.56^{ab}	2.38^{b}	1.00^{b}
7.5	2.19^{b}	3.72^{bc}	3.37^{c}	1.88^{d}
10	1.68^{a}	3.46^{a}	3.68^{d}	1.52^{c}

Note: The number followed by different letters in the same column shows the difference in the 5% Duncan Test. Alphabet cd means there was no difference in the number of *S. enteritidis* cells in the use of extract with concentrations of 2.5% and 5%. Alphabet ab means there was no difference in the number of *S. typhimurium* cells in the use of extract with concentrations of 2.5% and 5%. Alphabet bc means there was no difference in the number of *S. typhimurium* cells in the use of extract with concentrations of 2.5% and 7.5%

Flavonoids are found only in 95% of ethanolic extracts of *Z. zerumbet*, they can inhibit phosphodiesterase, aldol reductase, monoamine oxidase, protein kinase, DNA polymerase as well as lipoxygenase (Linarti et al. 2011). The inhibitory action of tannins on microbes occurs in cell membranes, through cell aggregation and disruption of membranes and cell function, in general tannins cause protein deposition, but tannin's anti-microbial activity is specific, according to the chemical composition and structure of tannins. Condensed tannins isolated from several plants have been shown to have strong activity against Gram-negative bacteria, including *Salmonella*, *Shigella*, *Staphylococcus*, *Pseudomonas* and *Helicobacter pylori* (Liu et al. 2013; Wang et al. 2013). Tannins are known to have anti-inflammatory, astringent, and antiseptic activities, they inhibit the enzyme systems of microorganisms, tannic acid inhibits phosphatidylcholine liposomes which damage microorganism membranes, is also able to bind iron that is needed by microorganisms, resulting in malfunctioning of aerobic microorganisms (Linarti et al. 2011).

The alkaloid compound has an inhibitory mechanism by disrupting the peptidoglycan component of the bacterial cell, so that the cell wall layer is not formed completely or imperfectly and causes the cell's death (Juliantina et al. 2008; Retnowati et al. 2011). In alkaloids there are alkaline groups that contain nitrogen, alkaloid compounds will react with amino acids that make up the bacterial cell wall and bacterial DNA. Alkaloids are known as DNA intercalators and inhibitors of DNA synthesis, by inhibiting the topoisomerase enzyme (Gunawan 2008; Hashemi and Davoodi 2010). The hydrophilic nature of the alkaloids also makes it easier for the alkaloids to penetrate the additional layer (outer membrane) of the Gram-negative bacterial cell wall consisting of LPS. The hydrophilic side of the Gram-negative bacterial cell wall is a carboxyl group, an amino acid, and a hydroxyl group.

Saponins contained in ethanolic extract (45% and 70%) (Table 1) have antibacterial activity, as reported by Netala et al. (2015) that it can damage the hydrophobic bonds of cell membrane components, such as proteins and phospholipids, as well as the dissolution of hydrophilic components. Saponins also destroy the bivalent cationic bonds, including Ca^{2+} and Mg^{2+} with LPS in Gram-negative bacteria. Added by Arabski et al. (2009) and Ye et al. (2012) that saponins are also capable of interacting with cell membrane components, such as lipid A, which can cause increased permeability of the cell walls of Gram-negative bacteria. One type of saponins, namely camelliagenin, can interact with bacterial biofilm components, i.e., Mannitol Dehydrogenase (MDH) or extracellular DNA (eDNA) of *Escherichia coli* and *Staphylococcus aureus.* As stated by Aiello and Susan (2012), saponins may work as an antimicrobial; this compound can crush the cytoplasmic membrane and kill the cell.

Zerumbon has antimicrobial activity, as a strong antibacterial and anti-fungal (Bhuiyan et al. 2009; Kumar et al. 2013), because zerumbone has ketone groups on C-8 atoms so it is polar, which can be extracted using polar and semi-polar solvents (Kapitan et al. 2017). Polarity of antimicrobial compounds is an important chemical property, because compounds can dissolve in the water phase where microbes generally grow in the water phase.

It can be concluded that extraction of *Z. zerumbet* using 95% ethanol solvent has higher antibacterial activity against *S. enteritidis* than *S. typhimurium.* The extract with 10% concentration gave the highest antibacterial activity than other concentrations. At this concentration, the extract provides antibacterial activity against *S. enteritidis* which is higher than *S. typhimurium.* Whereas *S. typhimurium* is effectively inhibited by extracts with 45% ethanol and 7.5% extract concentration.

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