4. DISCUSSION

4.1. Samples Preparation

In this research, *Z.officinale* Roscoe was brought from farmer in Salatiga. *Z.officinale* Roscoe is the only species of ginger than can be harvested at young age (3-4 months), which is usually used for pickles. In this research, 3, 4, and 5 months old of ginger were used. According to Ravindran & Nirmal (2005), ginger-growth stages consist of several phases. Three months old of ginger is in the germinating stage. This stage starts when the dormant bud begins to sprout to the opening of the first leaf. Growing stage occurs during 110-130 days, four months old of ginger is in this stage. This is the most crucial phase of growth because the plants show quick growth, many tillers arise and leaf number and area increase sharply, the rhizome branches producing fingers but their growth is slow. 5 months old of ginger is in the rhizome-expanding stage. At this time, the root quantity becomes stabilized, tillering speed decrease, and the leaf area reaches steady state. Three, four, and five months old of ginger are in the important growing stages, where the essential oils reach maximum level. After five months old of ginger, the essential oils will decrease due to evaporation, whereas the resin components will increase (Gersbenzon & Croteau, 1991). Therefore, 3, 4, and 5 months old were chosen for antibacterial activity test because the essential oil is the main components which play important role as antibacterial agent in ginger.

Fresh or dried plant material can be used as a source for secondary plant components, hence the comparison of antibacterial activity between fresh and powdered ginger should be done. Powdered ginger made by oven drying method. Based on Almasyuhrini *et al*., (2012), drying with oven is the best way to make powdered ginger compared to sun drying because it takes shorter time and can produce sufficient amount of essential oil and volatile compounds. Ginger is usually dried at temperature of 40-60°C. High temperature will damage the volatile compounds and essential oil (Fitriani *et al*., 2013). Suradikusumah (1989) stated that if the drying process approach 60°C, it will decrease some of the phenolic compounds because of the phenolic compound is sensitive to oxidation. The drying time depends on the moisture content of ginger. Drying ginger is

best done at moisture content until 7-12% to prevent the mold growing in the samples (Almasyuhrini *et al*., 2012).

4.2. Extraction Method

The method used in this research was solvent extraction and evaporation. Basic operation of extraction, such as washing, drying of plant materials or freeze drying, and grinding in order to obtain homogenous sample, improve the kinetics of analytic extraction, and also increase the contact of sample surface with the solvent system. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. A general principle in solvent extraction is "like dissolved like", which means that solvents only extracts those phytochemicals which have similar polarity with the solvents (Sasidharan, 2010). Research conducted by Jain *et al*, (2016) showed that antibacterial activity in *Z.officinale* Roscoe is higher when it dissolved in ethanol rather than ethyl acetate, methanol, and chloroform. Similar result was observed, when an ethanol extract of ginger showed the greatest effect on *Staphylococcus* aureus compared to water extract (Akintobi *et al*., 2011). Ethanol can be used to dissolve fat, protein denaturation, makes the cells become dehydrated, and affects the membranes of microbes. This solvent reported has ability to extract phenolic compound and terpenoids which is represent antibacterial agents on ginger (Larson & Morton, 1991). Hence, ethanol extraction was used in this research.

Extraction process followed by evaporation will produce oleoresin. Oleoresin contains volatile compounds (essential oil) and non-volatile compounds (resin). Evaporation process is very effective to separate most of the organic solvent during the extraction process but this process may cause loss of some components in essential oil (Rosevicka *et al*., 2007). According to Stahl (1973), essential oils produced by distillation process will only produce volatile compounds. While solvent extraction and evaporation process will produce essential oils (volatile compounds) and resin (non-volatile compounds). Study conducted by Nguanpuag *et al*, (2011) resulted that ginger oleoresin obtained by hydrodistillation is more effective to inhibit microorganisms than oleoresin obtained by solvent extract method.

After being evaporated, crude extract then dissolved in dimethylsulfoxide (DMSO) to make certain concentration before it is used for antibacterial activity test. DMSO often used to dissolve extract and rarely applied as an extraction solvent because it has high boiling point of 189℃, making it imposibble to remove at a low temperature (40℃) using rotary evaporator. While, excessive organic solvent will make the water extract impossible to freeze even at -40℃ thus, this extract should be used within a month (Thomas, 2010). DMSO polarity is 44.4 which is classified as semi polar solvent where it could dissolve both non-polar or polar molecules (Ian, 1996).

4.3. Antibacterial Activity

The content of secondary metabolites found in the ginger, especially the flavonoid, phenol, terpenoids and essential oils can inhibit the growth of pathogenic microorganisms (Nursal *et al*., 2006). This secondary metabolites produced by plants besides the primary biosynthetic and metabolic route of compounds for plant daily function used, this acts as protective functions against different forms of stress in plants (Veberic, 2010). *Z.officinale* Roscoe contains of 2 % essential oils, which are sesquiterpene hydrocarbon (zingiberene (35%), curcumen (18%), farnescene (10%), α-(7,84%) dan β- bisabolene (3,34%), limonen (1,48 – 5,08%)), and monoterpenoids (1,8-cineole, linalool, borneol, neral, and geraniol) (Felipe *et al*., 2008). Based on Table 1, it can be seen that inhibition zones produced by ginger extracts against five pathogenic bacteria indicated the potency of the bioactive components in ginger. The antimicrobial potency of ginger mainly caused by the presence of monoterpenes and sesquiterpenes, mostly zingiberene, and phenolic compounds (shogaol, gingerol) (Jain *et al*., 2016).

4.3.1. Effect of Harvesting Time on Antibacterial Activity

Essential oil of ginger rhizome which is harvested at young age, reported to be higher than the old harvested age. In old harvested ginger, the essential oils will decrease, whereas the levels of starch and fiber will increase. Concentration of terpenoids

compound is also greater in plants that still young. Therefore, young rhizome has higher antimicrobial activity than old rhizome (Gersbenzon & Croteau, 1991).

Table 1 showed that the increase of harvesting time (3, 4, and 5 months) will decrease the antibacterial activity. Three months old of ginger has highest antibacterial activity against five pathogenic bacteria. Indah *et al*., (2013) also study the antibacterial activity of fresh *Z.officinale* Roscoe where the inhibition zone against *S.aureus* is 0.223 cm and against *E.coli* is 0.25 cm. While in Table 1, the inhibition zone of 3, 4, 5 months old ginger against *S.aureus* is 0.46 cm, 0.317 cm, 0.268 cm, respectively and against *E.coli* is 0.45 cm, 0.255 cm, 0.321 cm, respectively.

Ekwenye & Elegalam (2005) studied the antibacterial activity of powdered ginger extract. DMSO also used to prepare extracts with different concentrations. In this study, 35.25 mg/ml of powdered ginger extract showed zero inhibition zone against *E.coli* and *S.thypimurium*. Meanwhile, in this research, at concentration of 30 mg/ml powdered ginger extract showed clear zone around the well against the entire pathogenic bacteria. It can be concluded that young rhizome reported to have higher antimicrobial activity than old rhizome.

4.3.2. Effect of Fresh and Powdered Ginger on Antibacterial Activity

Many studies usually use dried plant material or simplisia to test the secondary metabolites as an antibacterial agent. According to Burt (2004), secondary metabolites in ginger are affected by the form of ginger (fresh and dried form). Nada *et al*., (2014) reported that aquatic extract of powdered ginger has higher antibacterial activity than aquatic extract of fresh ginger. Based on Table 1, powdered ginger also showed higher antibacterial activity compared to fresh ginger. The inhibition zones of powdered ginger were 0.5 cm – 0.7 cm, however the inhibition zone of fresh ginger were 0.26 cm – 0.5 cm.

According to Widada (1993), grinding process will produce heat which causes polymerization of most components in essential oil. Polymerization causes the

formation of resin and polymers that have higher molecular weight. Formation of a resin will increase the number of phenolic components in ginger. These phenolic compounds are one of the secondary metabolites in ginger that can inhibit the growth of pathogenic microorganisms. Therefore, powdered ginger has higher antibacterial activity (Uhl, 2000).

4.3.3. *Z.officinale* Roscoe in Preventing Gram-Positive and Gram-Negative Bacteria

Sivasothy *et al*., (2011) stated that essential oil in ginger showed better result in preventing Gram-positive bacteria. Based on Table 1, the inhibition zones of three months old powdered ginger is higher against Gram-positive bacteria, which are *B.cereus*, *S.aureus*, and *L.monocytogenes* (0.580 cm, 0.670 cm, and 0.624 cm, respectively). In addition, the inhibition zones of Gram-negative bacteria is lower (0.581 cm against *S.thypimurium* and 0.501 cm against *E.coli*). However on the other samples, some inhibiton zones is higher against Gram-negative bacteria. Secondary metabolites in ginger can bind proteins and lipids in cell membranes so it is caused cell lysis. Cell lysis makes the transport of nutrients (compounds and ions) through the cell membrane disrupted so the enzyme activities decrease, and bacteria metabolism and reproduction become slower (Nursal *et al*., 2006).

Silhavy (2010) stated that there are three principal layers in the Gram-negative envelope, which are the outer membrane, the peptidoglycan cell wall and the cytoplasmic or inner membrane. The outer membrane is composed of lipopolysaccharides (LPS), lipoprotein (LP), and phospholipids. This organelle is essential as it acts as a protective barrier that prevents the entry of a variety of larger antibiotics included plant extract. To prevent these bacteria, antimicrobial compounds should penetrate the lipopolysaccharide (LPS) from the cell wall. Gram-positive bacteria do not have the LPS. Hence, the antimicrobial compound that is hydrophilic and hydrophobic (such as essential oils) can diffuse better into the Gram-positive cell (Ousallah *et al*., 2006).

Even though both microorganisms have different structures, which are the Gram positive bacteria do not have the outer membrane which is containing lipopolysaccharide and protein like Gram-negative bacteria, but the Gram-positive bacteria have the thicker peptidoglycan (main substance of bacteria cell wall) rather than Gram-negative bacteria. Therefore these microorganisms have the same ability to prevent the antibacterial properties to prevent and inhibit their growth (Keith *et al*., 2004). Based on Table 1, *Z.officinale* extracts can inhibit the growth of Gram-positive and Gram-negative bacteria, which means that the concentration of secondary metabolites in ginger is high enough to inhibit intracellular enzyme thus causing denaturation of bacterial proteins and lysis of the cell membrane (Maris, 1995).

4.3.4. Comparison of *Z.officinale* Roscoe and Amoxicillin as Antibacterial Agent

Further investigation conducted by comparing the effectiveness of *Z.officinale* Roscoe with commercial antibiotics (amoxicillin) as positive control, and dimethylsulfoxide (DMSO) as negative control. DMSO showed no clear zone for all of the bacteria. It means that the strength of inhibition had no influence from the DMSO as the solvent of *Z.officinale* Roscoe extract. The inhibition purely came from the secondary metabolites of *Z.officinale* Roscoe. Table 2 showed that although *Z.officinale* Roscoe has no antibacterial activity as strong as amoxicillin, but it is proved that *Z.officinale* Roscoe extracts still have ability to inhibit the growth of Gram-positive and Gram-negative bacteria.

Amoxicillin inhibit synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer. Bacteria cell walls composed of peptidoglycans and polysaccharides that are connected to each other in complex manner, responsible for maintaining the integrity of bacteria cell. Amoxicillin binds the protein which cause deactivation of enzyms involved in the transpeptidation reaction or cross linking of peptidoglycans. Inhibition of this cross linking reaction leads to loss of cell wall integrity, making the cell wall weak. When cell wall gets weak, the water enters the cell through the outside environment and causes cell lysis (Vincent *et al*., 2011).

On the other hand, secondary metabolites in ginger, especially the terpenoids and phenolic compounds involve in disruption of the cytoplasmic membrane and coagulation of the cell contents. Poeloengan (2011) stated that zingiberene precipitated the outer protein membranes, ruptured the cell wall, coagulated and caused loss of the cell contents and energy through the leakages of cell wall. Besides, the essential oils contained in the ginger extract were able to induce the leakage of ions and other cell contents. The extract, therefore, affected the bacterial cytoplasmic membrane and induced the loss of nucleic acid and ions which cause denaturation of proteins inside the cell.

4.4. Determination of MIC

MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC is used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints (Andrews, 2001). In this research, agar well diffusion was used to test the MIC. The least concentration of each extract showing a clear zone of inhibition was taken as the MIC (Okeke *et al*., 2001).

Based on Table 3, the concentration used for MIC test is 100 µg/ml, 50 µg/ml; 25 µg/ml; 12.5 µg/ml; 6.25 µg/ml; 3.12 µg/ml; 1.56 µg/ml 0.78 µg/ml. All of those concentrations produce clear zone around the well in inhibit Gram-positive and Gram-negative bacteria. Okeke *et al*., (2001) stated that the least concentration of extract showing a clear zone is taken as the MIC, but from Table 3, it can be concluded that the smallest concentration, which is 0.78 µg/ml still can inhibit the growth of the bacteria.

The negative control which is ethanol 96% also produce clear zone even though it is very small. Ethanol had been shown to be effective as antimicrobial agent. Ethanol affects the lipid cell membrane. It makes the lipids that are part of the outer protective cell membrane of each bacterium cell more soluble in water so that the cell membrane

begins to lose its structural integrity and fall apart. As the cell membrane disintegrates, Ethanol then enters the cell and denatures proteins within each bacterium. Ethanol is more effective against Gram-negative (with lipid outer membrane) than Gram-positive (thick peptidoglycan cell wall) bacteria for because of its strong effects on the membrane (Larson & Morton, 1991).

Table 3 showed that the inhibition zone of 3 months old fresh ginger against *E.coli*, 4 months old fresh ginger against *B.cereus*, and 5 months old powdered ginger against *L.monocytogenes* are smaller than the inhibition zone of ethanol. The clear zone which formed might be come from ethanol instead of components of ginger.

According to Klancnik *et al*., (2010) MIC values obtained by the agar dilution method were 3–20 times lower than MIC values obtained by the disk diffusion method, irrespective of the plant extract tested. The diffusion assay is not suited to natural antimicrobial compounds that are scarcely soluble or insoluble in water and thus their hydrophobic nature prevents uniform diffusion through the agar media. For quantitative determination of antibacterial activity, the agar dilution method is more appropriate, where antibacterial activity of plant extracts was shown at lower concentrations compared to the disk diffusion method.

4.5. Total Phenolic Compound

Oleoresin contains of 2-3% essential oil and resin. Essential oil in ginger or also called the volatile compounds is a component which produces the aroma and flavor in ginger. While, resin or known as non-volatile compound is a component which provides a pungent taste in ginger. Resin consists of phenolic compounds, which are gingerol, zingerone, shogaol, and paradol. Gingerol is a compound which is highly sensitive to heat. Heat treatments will make gingerol compound decrease and change into shogaol, which cause fresh ginger is most pungent than the other form of ginger (Raghavan, 2007). Those phenolic compounds are proven has antioxidant activity because it contains a benzene ring and a hydroxyl group (Merck Index, 1996).

Table 4 showed that the increasing of total phenolic compounds followed by harvesting time. According to Raghavan (2007), the volatile compounds reach maximum levels when rhizomes have more than 9 months period after planting, while the young harvested rhizomes will have less phenolic compounds. Table 4 also showed that powdered ginger has higher total phenolic compound compare to fresh ginger. According to Widada (1993), grinding process will produce heat which causes polymerization of most components in essential oil. Polymerization causes the formation of resin and polymers that have higher molecular weight. Uhl (2000) stated that resin consist of phenolic compounds such as gingerol, shogaol, and zingerone. Therefore, the formation of a resin compound will increase the number of phenolic components in oleoresin, resulting in higher phenolic compound in powdered ginger.

Jain *et al*., (2016) stated that phenolic compounds (shogaol, gingerol) has important role as antimicrobial agent in ginger. The roles of phenol on killing microorganism is by bind proteins and lipids in cell membranes so it is caused cell lysis, ruptured the cell wall, coagulated and caused loss of the cell contents and energy through the leakages of cell wall (Poeloengan, 2011). If the concentration of phenol is low, the cell constituents (nucleic acid and glutamic acid) are liberated in external media. But, when the concentration of phenol is high, the phenolic compound could inhibit intracellular enzyme thus causing denaturation of bacterial proteins and lysis of the cell membrane (Maris, 1995).