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Zone of Inhibition of Ethanol and Ethyl Acetate Extracts of Forest Betel Leaves against *Escherichia Coli*

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Abstract: The betel plant is one of the medicinal plants known by the common people. This plant consists of more than 1000 species. One of the species is the betel forest. The part of the betel plant that is commonly used as medicine is the leaves. This study aims to determine the phytochemical content and inhibition of *Escherichia coli* in the ethanol and ethyl acetate extracts of forest betel leaves (*Piper aduncum* L.). The method used in the antibacterial test is the good diffusion method. The research showed that ethanol extract of forest betel leaves (*Piper aduncum* L.) leaves contains secondary metabolites, namely flavonoids, saponins, terpenoids, and tannins, while the ethyl acetate extract contains secondary metabolites, namely flavonoids, terpenoids, and tannins. The ethanol and ethyl acetate extracts of forest betel leaves (Piper aduncum L.) can inhibit the growth of *Escherichia coli* in the strong category.

Keywords: Antibacterial; *Escherichia coli; Piper aduncum* L; Secondary metabolites; Well diffusion method

Introduction

The use of natural materials as an alternative treatment has now been re-used by the community as traditional medicines, and some natural materials have even been produced by fabrication on a large scale. The proper use of medicinal plants does not cause side effects when compared to synthetic drugs. In addition, the use of medicinal plants to maintain health and prevent disease is relatively cheap and easy for many people to do (Ninin, 2016).

The betel plant is one of the medicinal plants known by the common people. This plant consists of more than 1000 species. One of the species is the betel forest. The part of the betel plant that is commonly used as medicine is the leaves. The main components of betel leaf show antiseptic, bactericidal, and antioxidant effects. Its chemical content is antiseptic because betel leaves contain essential oils. The antibacterial power of betel leaf essential oil is due to the content of phenolic compounds and their derivatives which can denature bacterial cell proteins (Novita, 2016). The forest betel leaves (*Piper aduncum* L.) contain alkaloids, flavonoids, saponins, steroids, polyphenols, tannins, and terpenoids (Nova, 2016). Forest betel leaf powder extract can inhibit the growth of the fungus *Ganoderma boninense* (Mahera et al., 2015). In addition, the research conducted by Elfina et al., (2015) showed that the administration of forest betel leaf powder extract was able to control anthracnose disease caused by the fungus *C. capsici*.

Forest betel leaf (*Piper aduncum* L.) has been known by the public and has properties in healing wounds, stopping vomiting, reducing nausea, improving digestion, as an antiseptic, and killing bacteria and fungi, and viruses. Forest betel leaf extract (*Piper aduncum* L.) can inhibit the growth of Staphylococcus aureus and *Escherichia coli* bacteria with an extract concentration of 60% (Hallianah et al., 2019).

Escherichia coli bacteria are normal opportunistic germs when found in the human large intestine, but *Escherichia coli* bacteria can also cause primary infections. This bacterium is pathogenic when it is outside the

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human large intestine. Organs that are often affected by *Escherichia coli* bacterial infections are the urinary tract, bile ducts, and other places in the abdominal cavity. If the resistance of the host's body is not strong, it can cause a local infection which clinically can reach the bloodstream and cause sepsis (Syahrinastiti et al., 2015).

Various ways can be done to overcome the problem of infection caused by bacteria. One of them by giving antibiotics. An antibiotic is a kind of compound both natural and synthetic, and has the function to press or stop a process of an organism's growth, particularly bacteria (Soleha, 2015). The high price of antibiotics is a major problem for low-income people to treat infectious diseases. In addition, the use of antibiotics can lead to resistance. The emergence of resistance adds to the list of unresolved problems, so the renewal or development of natural medicine is needed to kill bacteria and prevent resistance from occurring (Puteri and Milanda, 2017).

One of the areas that have a betel plant is Alor Island. The betel plants known in this area are green betel and forest betel. In general, people use the green betel plant specifically for the fruit and leaves to eat with areca nut. Apart from eating the leaves, they are also used to get rid of body odor, get rid of bad breath, treat itching, treat vaginal discharge and cure hemorrhoids or hemorrhoids. Meanwhile, forest betel leaves are used to heal wounds, and stop nausea and vomiting. However, the use of forest betel leaves has not been fully utilized by the community because forest betel plants grow wild in the forest so only a few people use these plants. Although a lot of research has been done on this plant, such as testing its chemical composition and bioactivity, chemotaxonomically the differences in the climate where a plant grows will affect the composition of the chemical compound content of a plant (Munte et al., 2016). And with the premise that people, in general, have not properly utilized forest betel leaves in the health sector. Therefore, this study aims to determine the phytochemical content and inhibition of *Escherichia coli* in the ethanol and ethyl acetate extracts of forest betel leaves (*Piper aduncum* L.).

Method

Materials

The materials used in this study were betel leaves, 96% ethanol, ethyl acetate, filter paper, H_2SO_4, Mayer's reagent, magnesium powder, HCl, $FeCl_3$, *Escherichia coli* bacterial isolates, MHA media (*Mueller Hinton Agar*), chloroform, antibiotic disks, physiological NaCl, DMSO (Dimethyl Sulfoxide), chloramphenicol, and distilled water. The tools used in this study were blenders, incubators, analytical balances, rotary evaporators, vials, test tubes, spatulas, test tube racks, micropipette, dropper pipette, volume pipette, Bunsen, wire loops, Erlenmeyer, volumetric flask, hot plate, magnetic stirrers, calipers, autoclaves, and Petri dishes.

Method

There is also a procedure for collecting data in this research in accordance with the chart in Figure 1.

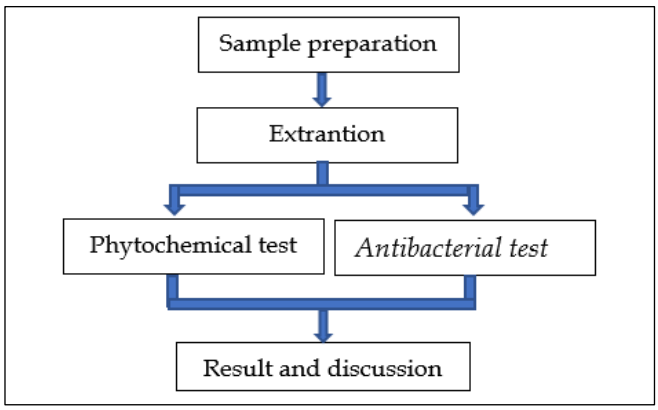

Figure 1. Step of research

Extraction

The betel leaf extract Forest sich leaves are washed and then air-dried, then mashed into powder. Forest betel leaf powder was macerated with ethanol and ethyl acetate solution for 1x24 hours. The filtrate obtained was then evaporated to obtain concentrated ethanol and ethyl acetate extracts of forest betel leaves. The extract obtained was then tested for its phytochemical content and anti-bacterial test.

Antibacterial test

Testing the antibacterial activity using a good method. As much as 5 mL of MHA and 1 mL of *Escherichia coli* bacterial suspension were put into a measuring cup and then poured into a petri dish until evenly distributed. The wells were made using solidified MHA media, which were perforated using wire loops (diameter 6 mm). In each petri dish, 3 wells or (3 quadrants) were made. The well was dripped with 0.1 mg/mL of forest betel leaf ethanol extract with a positive control (chloramphenicol) of 0.3 mg/mL and a negative control (equates) of 1 mL. Then incubated for 24 hours at 37^{o}C and observed the clear zone that formed around the well. The same method is used for the ethyl acetate extract of betel leaves.

Result and Discussion

Results

The results of the phytochemical test of the ethanol and ethyl acetate extracts of forest betel leaves can be seen in Table 1.

Table 1. The results of a phytochemical test of the ethanol and ethyl acetate extracts of forest betel leaves

Sample	Group compound	Conclusion
Ethanol extracts	Alkaloids	-
	Flavonoids	+
	Saponin	+
	Terpenoids	+
	Tannin	+
Ethyl acetate extracts	Alkaloids	-
	Flavonoids	+
	Saponin	-
	Terpenoids	+
	Tannin	+

The results of the antibacterial activity test of the ethanol and ethyl acetate extracts of forest betel leaves can be seen in Table 2 and the zone of inhibition can be seen in Figure 2.

Table 2. Inhibitory power of ethanol and ethyl acetate extracts of forest betel leaves

Sample	Concentration (%)	Inhibition zones (mm)
Ethanol extracts	25	11.9
	30	13.7
	35	15.6
Positive control		24.4
Negative control		0
Ethyl acetate extracts	25	12.2
	30	14.4
	35	16.7
Positive control		24.4
Negative control		0

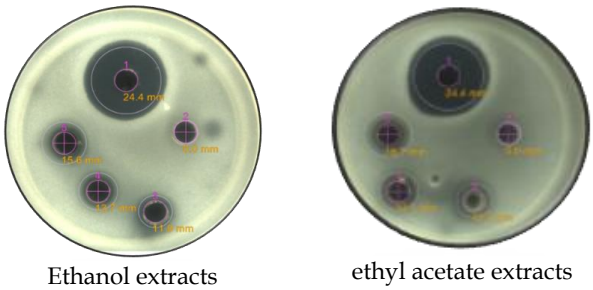

Figure 2. Zone of inhibition ethanol and ethyl acetate extracts of forest betel leaves

Discussion

The extraction of forest betel leaf extract (*Piper aduncum* L.) was carried out by maceration method using ethanol and ethyl acetate as solvents. The choice of solvent for the maceration process will provide high effectiveness by taking into account the solubility of natural product compounds in the solvent (Ningsih et al., 2016). The ethanol solvent is a universal solvent with a polarity index of 5.2 so that various compounds, both polar and nonpolar, such as alkaloids, flavonoids, saponins, tannins, as well as steroids, and terpenoids contained in betel leaf can be attracted to the solvent (Pangesti et al., 2017).

Phytochemical tests were carried out to determine the content of secondary metabolites contained in the ethanol and ethyl acetate extracts of betel leaves which have the potential as antibacterial agents. The use of ethanol as a solvent in the extraction process is widely used because ethanol is good for extracting antibacterial compounds such as tannins, phenols, and flavonoids. Ethanol is easier to penetrate cell membranes to extract intracellular materials from plant materials (Septiani et al., 2017). Ethyl acetate is used as a solvent because ethyl acetate can extract compounds that provide antibacterial activity, including polyhydroxy flavonoids and other phenols (Wardhani and Sulistyani., 2012).

Based on Table 1, the results of the phytochemical test of the ethanol extract of forest betel leaves has a group of secondary metabolite compounds namely flavonoids, saponins, terpenoids, and tannins, while the ethyl acetate extract leaves have a group of secondary metabolite compounds namely flavonoids, terpenoids, and tannins. Based on the alkaloid test results of the ethanol and ethyl acetate extracts of forest betel leaves, showed negative results. Negative results were characterized by the absence of white deposits in the extracts when using Mayer's reagent. The absence of white deposits indicates that a potassium-alkaloid complex is not formed.

The test results of flavonoid compounds in the ethanol and ethyl acetate extracts of forest betel leaves showed positive results which were indicated by a change in color to yellow. The addition of magnesium powder and hydrochloric acid will cause the reduction of existing flavonoid compounds, causing a color reaction that is characteristic of the presence of flavonoids. Flavonoid compounds will be oxidized by Mg^{2+} by forming complexes with magnesium ions (Wardana, 2016).

The test results of compounds saponin showed that the ethanol extract of betel leaf contains saponins because stable froth forms after shaking, while the ethyl acetate extract does not contain saponins because no froth forms. The difference in the presence or absence of foam produced by the ethanol and ethyl acetate extracts is because the saponin compounds are more perfectly extracted in ethanol solvent than in ethyl acetate solvent. The appearance of foam indicates the presence of glycosides which can form foam in water which is hydrolyzed into glucose and other compounds (Nugrahani et al., 2016).

The test results of terpenoid compounds in the ethanol and ethyl acetate extracts of forest betel leaves showed positive results. The principle of the reaction in the mechanism of the terpenoid test reaction is the

condensation or release of H_2O and combination with carbocations. A positive terpenoid result is indicated by the formation of a brown color when added with H_2SO_4. This color change occurs because acetic acid and sulfuric acid will bind to terpenoid compounds resulting in a reaction that appears as a color change (Sangi et al., 2012).

The test results of tannin compounds in the ethanol and ethyl acetate extracts of Siri Hutan leaves showed positive results which were marked with a black-green color. Once the addition of FeCl3 solution was done, it was estimated that this solution reacted with one of the hydroxyl groups present in tannin compounds. Tannins will form complex compounds with Fe^{3+} ions (Ergina et al., 2016).

The antibacterial activity test was carried out by the good diffusion method. This method was chosen because it is simple in determining the diameter of the bacterial inhibition zone. The diameter of the inhibition zone is determined by measuring the clear zone around the well. The clear zone was formed due to the activity of bacterial growth which was inhibited by the test sample (Bakhtra et al., 2018). The wider the clear zone, the greater the inhibitory activity of the antibacterial substance against the tested bacteria.

Dilution of the forest betel leaf extract using DMSO solvent. DMSO was chosen because DMSO can dissolve polar and nonpolar compounds. Chloramphenicol was used as a positive control because chloramphenicol could inhibit the growth of Gram-positive and Gram-negative bacteria (Bakhtra et al., 2018). The mechanism of chloramphenicol as an antibacterial is to inhibit protein synthesis in bacteria.

Based on Table 2, it can be seen that the higher the concentration of the ethanol and ethyl acetate extracts of forest betel leaves, the greater the inhibition of *Escherichia coli* bacteria. According to Susanto and Ruga (2012), the category of bacterial growth response based on the diameter of the inhibition zone is ≥ 21 mm which is categorized as very strong, 11-20 mm is categorized as strong, 6-10 mm is categorized as medium and < 5 mm is categorized as weak. The test results showed that at concentrations of 25, 30, and 35% ethanol extract, the inhibition power of Escherichia coli bacteria was 11.9; 13.7, and 15.6 mm while the ethyl acetate extract with a concentration of 25, 30, and 35% gave an inhibition of *Escherichia coli* bacteria of 12.3; 14.2, and 16.5 mm. The diameter of the inhibition zone of the ethanol and ethyl acetate extracts of forest betel leaves at each of these concentrations was categorized as having a strong inhibitory effect. The inhibition zone formed at each extract concentration indicated that there was an inhibition of *Escherichia coli* growth. The higher the concentration of the extract used, the wider the inhibition zone is formed. This is because the greater the concentration, the more bioactive compounds that work in inhibiting bacterial growth. The inhibition response that occurs in the ethanol and ethyl acetate extracts is caused by the presence of active ingredients or compounds possessed by the Forest betel leaf. The content in question is secondary metabolite compounds contained in forest betel leaves.

Based on the results of the phytochemical tests that have been carried out, the content of secondary metabolites in the ethanol extract are flavonoids, saponins, terpenoids, and tannins, while the secondary metabolites in the ethyl acetate extract are flavonoids, terpenoids, and tannins. These compounds have different antibacterial mechanisms.

The mechanism of action of flavonoids as antibacterials is to form complex compounds with extracellular and dissolved proteins so that they can damage the bacterial cell membrane followed by the release of intracellular compounds (Amalia, 2017). The mechanism of flavonoids in inhibiting the synthesis of nucleic acids is that they play an important role in the process of interaction or hydrogen bonding by forming nucleic acid bases that inhibit the formation of DNA and RNA (Carolia & Noventi., 2016). Flavonoids can also damage microbial membranes and can interfere with peptidoglycan transpeptidase activity so that cell wall formation is disrupted and causes bacterial lysis (Agustin et al., 2018).

The mechanism of action of saponins as antibacterials is by interfering with the surface tension of the cell walls (Karlina et al., 2013). Saponins will bind to lipopolysaccharides in the bacterial cell wall, resulting in increased permeability of the cell wall and lowering the surface tension of the cell wall so that when an interaction occurs the cell wall will break or undergo lysis and make the antibacterial substance enter the cell easily and will disrupt metabolism until it finally occurs. bacterial death (Sari et al., 2015).

Tannins are organic compounds that are active in inhibiting microbial growth by damaging the microbial cell walls (Sudira et al., 2011). The mechanism of inhibition of tannin compounds as an antibacterial agent is that they react with cell membranes, which results in inactivation of essential enzymes, and the destruction or inactivation of the function of genetic materia by inhibiting the reverse enzyme transcriptase and DNA topoisomerase so that the bacterial cells are not formed (Rijayanti, 2014). Tannins can also bind to one of the bacterial adhesion proteins used as a bacterial surface receptor, resulting in a decrease in bacterial adhesion and inhibition of protein synthesis for cell wall formation (Restiana et al., 2016).

Conclusion

The ethanol extract of forest betel leaves (*Piper aduncum* L.) has a group of secondary metabolite compounds namely, flavonoids, saponins, terpenoids, and tannins, while the ethyl acetate extract has a group of secondary metabolite compounds namely, flavonoids, terpenoids, and tannins. The ethanol and ethyl acetate extracts of forest betel leaves (*Piper aduncum* L.) can inhibit the growth of Escherichia coli in the strong category.

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