EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 7
Issue: 1
353-364
Year: 2020

THE EFFECT OF TOTAL PHENOL AND FLAVONOID TOTAL COMPOUNDS OF THE N-BUTANOL AND ETHYL ACETATE FRACTION OF *ACORUS* SP. TO ANTIBACTERIAL ACTIVITIESIN STREPTOCOCCUS AUREUS AND ESCHERICIA COLI

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Article Received on 29/10/2019 **Article Revised on 19/11/2019** **Article Accepted on 09/12/2019**

ABSTRACT
Background: Red Jeringau or Acorus sp. is a wild plant that lives in wet and moist soil. The red jeringau rhizome is empirically used by the Dayaks as a treatment for dengue and according to Saman et al. (2013) states that it is traditionally used as a stomach medicine and skin disease. The aims of this study to determine the activity of antibacterial against Staphylococcus aureus and Eschericia coli bacteria from the n-butanol and ethyl acetate fraction of red jeringau rhizomes and the levels of total phenols and total flavonoids of n-butanol fraction and ethyl acetate red rhizome which give antibacterial activity. **Method**: The study was conducted by measuring total phenol levels and total flavonoids as well as the diameter of inhibition zones of Staphylococcus aureus and Eschericia coli in the n-butanol and ethyl acetate fractions with positive control using ciprofloxacin. The results of inhibition zone diameter data were analyzed using SPPS One Way ANOVA analysis. **Result:** The results obtained total phenol levels for n-butanol fraction of 10.32% and total flavonoid levels of 0.688% had significant differences with the positive control group at a concentration of 100% (10.44 ± 0.32) against Staphylococcus aureus and at a concentration of 50% (12.14 ± 0.48) and 100% concentration (12.11 ± 0.16) against Eschericia coli. In the ethyl acetate fraction with phenol levels of 43.26% and flavonoid levels of 1.49% the 100% concentration group (12.43 ± 1.29) was significantly different from the positive control group against Eschericia coli bacteria.

KEYWORDS: Red jeringau, total flavonoids, total phenol, antibacterial, n-butanol fraction and ethyl acetate.

BACKGROUND
Indonesia is a tropical country, where infection can be contributor to morbidity and mortality. This is supported by Indonesia which is a developing country that has a large number of middle-to-lower class people, with a low level of education that supports the handling factors of hygiene and lack of a healthy lifestyle.

The use of antibacterial in killing and weakening bacteria and fungi in medicine uses many synthetic drugs. The problem that arises in the use of antibacterial drugs today is the emergence of resistance due to the many inappropriate use of antibiotics in the community. Resistance to antibiotics is especially common in the beta lactam antibiotic class.

The use of plants as a way to treat various diseases has been carried down for generations by people in Indonesia. This can be due to the presence of secondary metabolites produced by plants as a result of the metabolic process. Secondary metabolites produced by plants have the ability to overcome various diseases, due to the synergistic effect on secondary metabolites produced by plants that can provide complementary effects in treating diseases. In addition, secondary metabolite compounds have polivalent activity, making it possible to overcome various diseases.[35]

One of the nutritious plants that can be used as medicine is red jeringau (Acorus sp.) Which is a plant that lives on wet and moist soil. The red jeringau rhizome is empirically used as a treatment for dengue fever by the Dayaks. Red jeringau has anti-infectious, anti-inflammatory (inflammatory), anti-toxic, heat-lowering, urine laxative, anti-leprosy, anti-syphilis, antioxidant properties, brain repair, nerve protection and hypertension as a stomach medicine and skin disease.[39]

Staphylococcus aureus and Escherichia coli are bacteria that often cause infections in humans.[5] Staphylococcus aureus is a gram-positive spherical cell which is the most common cause of skin infections. Escherichia coli is a gram-negative rod-shaped bacterium that is a member of the family Enterobacteriaceae and normal intestinal flora

that has contributed to the normal function of intestines and nutrients.

Several studies conducted by Hartati (2012) showed that jeringau rhizome extract (Acorus calamus L.) has biological activity against microbes such as Salmonella typhosa bacteria, Candida albicans fungus, viruses and nematodes as well as against pest insects and pathogen vectors that harm humans, animals and plants. The activity is related to compounds contained in red jeringau rhizome (Acorus calamus L.) from terpenes, alcohols, aldehydes, and phenols such as carvacrol, eugenol, thymol, cinnamaldehyde, cinnamic acid, and perialdehyde.[12] In a study conducted Anisah et al. (2014) Jeringau is included in a spice that has long been known by the people of Indonesia. This plant contains essential oils called calamus oil (calamus oil). In Vietnam, calamus oil can be used to extend the shelf life of shelled corn, and can be used as an antibacterial and antifungal agent.[37]

The content of compounds can affect the activity of a plant, including the form of the tested preparations. The fraction carried out on an extract will give effect to the reduction in the number of metabolites to be simplified by being influenced by the nature of fractions classified as polar or semi-polar and non-polar. The nature of the fraction will affect the compound content, which in the polar fraction will have different metabolite content with semi-polar and non-polar. The difference in the content of these compounds has an effect on the activity of bacterial inhibition in the zone of inhibition of discs on solid media overgrown with Staphylococcus aureus and Eschericia coli.

METHOD

Raw materials of tradisional medicine

The plant used in this study was red jeringau (Acorus sp.), The part used was the rhizome. Red jeringau (Acorus sp.) Was obtained in the area of Parit Village in Surabaya, Sungai Ambawang District, Kubu Raya Regency, West Kalimantan Province. Red jeringau rhizome (Acorus sp.) used with a wet weight of 4 kg.

Cultivation

The red jeringau plant that has been taken is ripe with long ribbon-shaped leaves that are dark green and the rhizomes are red. Plants are taken before 12 am and are taken whole by cutting off the roots. The part used is only part of the rhizome, not the whole plant.

Extraction and fractination

Extraction using maceration method using ethanol 96%. Fractionation is carried out successively using hexane solvents; ethyl acetate; n-butanol; dichloromethane and distilled water.

Thin layer chromatography

The stationary phase used by the KLT GF254 plate was then put into the chamber and eluted with a n-Butanol-Acetate acid-Water mobile phase in a ratio of 4: 1: 5 to the mark.

Determination of total phenol and flavonoid levels

Determination of phenol levels using the Folin-Ciaocalteu method and determination of total flavonoid levels using the colorimetric method.

Bacterial rejuvenation

One ose bacteria of E.coli and S. aureus were inoculated into the TSA agar medium separately and aseptically by placing the cultured ose needle on the base of the agar slope and pulled by zig-zag motions. Two ose E.coli and S. aureus bacteria were inoculated into separate TSB media. Then each incubated at 37ºC for 24 hours. Rejuvenation is done every week.

Preparation of test solutions

The fractions of n-butanol and ethyl acetate of rhizome are made in concentrations of 25%, 50%, 75% and 100% v / v by taking 2.5 g, 5 g, 7.5 g and 10 g extracts respectively dissolved in 10 ml of CMC-Na (0.5% w / v).

Testing antibacterial activity

Antibacterial activity tests were carried out on two types of bacteria namely E.coli and S.aureus. Antibacterial testing was carried out using agar diffusion method using Mueller-Hinton media. Sterile paper discs are inserted into vials containing fractions of n-butanol and ethyl acetate red jeringau rhizomes with concentrations of 25%, 50%, 75% and 100% with positive control using ciprofloxacin.

RESULT

Extraction and fractination

Herbs or intact plants from red jeringau obtained are as much as 4 kg. The herbs are then separated and part of the rhizome is taken, the red jeringau rhizome is obtained with a total mass of 1.7518 kg or 43.795%. The rhizome is then formed into dried simplicia powder and the simplicia weight is 531.18 grams or 30.332%. Drying red jeringau rhizome is done by using an oven with a temperature of 80oC to obtain a dry simplicia with dark brown characteristics and when squeezed by hand it will be easily broken.

The extraction method used in this study is maceration. Maceration is done by dissolving 300 grams of red jeringau simplisia powder with 96% technical ethanol as much as 200 mL per day. Maceration is stopped when the maserat looks clear on the 10th day of maceration. The acquisition of thick extract from the results of the maserat was 33.87 grams of 300 grams of macerated simplicia, so that a percentage of 11, 29% was obtained.

Fractionation was carried out after technical ethanol extract 96% red jeringau was obtained by dissolving the extract into a polar solvent namely aquadest. A total of 27.67 grams of the extract was dissolved into 250 mL aquadest and then technical n-hexane was added.

Fractionation is continued by replacing n-hexane solvents with solvents whose polarity is higher than n-hexane in this case technical dichloromethane. The fractionation method is continued by dissolving the water fraction that has been separated from the organic solvent fraction until saturated or looks clear with organic solvents whose polarity is higher, in this case after dichloromethane is continued by dissolving the water fraction with technical ethyl acetate until saturated, then separated again and added the separated water fraction with technical butanol solvent until it is saturated or has a clear color.

The acquisition of the butanol fraction from the red jeringau ethanol extract was 5.8 grams from a total of 27.67 grams of extract, so that the butanol fraction obtained was 20.961%. The yield of ethyl acetate fraction obtained was 0.75 grams or 2.71%.

Thin Layer Chromatography

a. n-butanol fraction

The stationary phase used is the top phase of butanol: acetic acid: water with a ratio of 6: 1: 3 as much as 1 mL added with methanol as much as 4 mL. The red jeringau butanol fraction was dissolved in a methanol: water (1: 1) solvent then each of the fractions was bottled to the GF_{254} silica gel stationary phase.

The elution process that has been completed is then continued at the spraying stage of special reagents to identify the presence or absence of flavonoid and phenol compounds. Specific reagents used to identify flavonoid compounds are 1% $AlCl_3$, while for phenolic compounds 1% $FeCl_3$ is used.

The results showed that the water fraction and butanol fraction showed the presence of a flavonoid compound after being sprayed with a 1% $AlCl_3$ spot viewer because there were yellow spots that could be seen visibly in visible light. When sprayed with 1% $FeCl_3$ patches to determine the presence or absence of phenol compounds in the two fractions, visible black patches showing the presence of phenolic compounds.

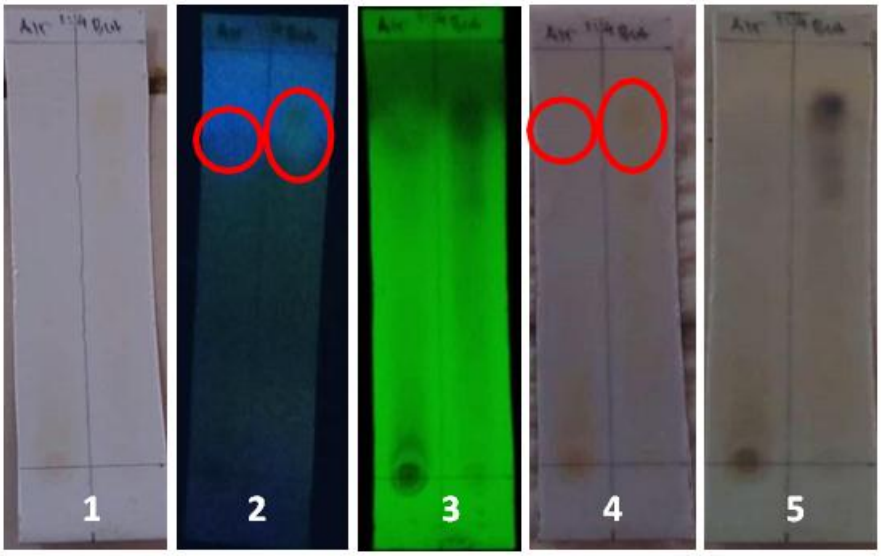

Figure 1: TLC profiles of phenols and flavonoids of the n-butanol fraction of the Red Jeringau Rhizome.
Note: Before Reagent Sprayed Visually (1), at UV_{366} nm (2) and UV_{254} nm (3), after Spraying $AlCl_3$ (4) and $FeCl_3$ Visible Light (5)

b. Ethyl acetate fraction

Identification of flavonoid compounds using AlCl3-spotted appearance reagents, with the mobile phase used is butanol: acetic acid: water (BAA) eluent in a ratio of 6: 1: 3. The elution result is then sprayed with the AlCl3 reagent. The identification results show a very striking orange patches.

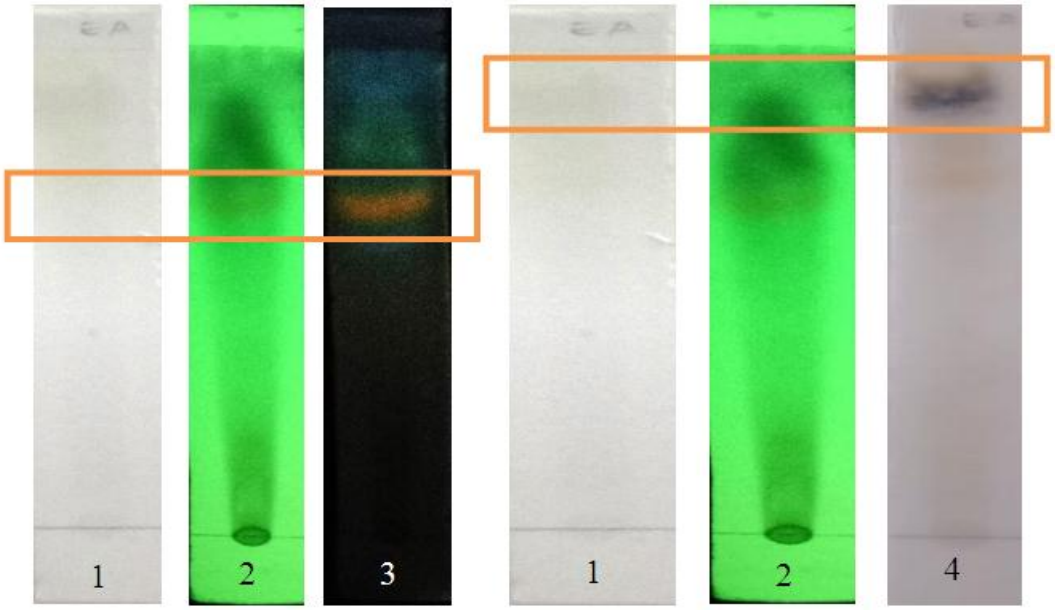

Figure 2: TLC profile of phenol and flavonoids of ethyl acetate fraction of Red Jeringau Rhizome.
Note: Before Reagent Sprayed Visually (1), at UV_{254} nm (2) and UV_{366} nm (3), after Spraying $AlCl_3$ (4) and $FeCl_3$ Visible Light (5).

Judging from the flavonoid and phenol chromatograms that have been done, the orange stain that is in the identification of flavonoids is known not to be a flavonoid compound. Flavonoids are polyphenol compounds that should be sprayed with FeCl3 reagents also cause black stains. Observed from both chromatograms, orange stain on UV light 366 nm did not give a black color after being sprayed with FeCl3, so it can be concluded that the orange stain is not a flavonoid compound but another phenolic compound.

Total phenol levels

Quantitative measurement of phenolic compounds using the Folin Ciocalteau method. This study uses gallic acid as a comparison compound. Total phenol levels in this study will be measured in mg GAE units / gram sample, where GAE is Gallic Acid Equivalent or gallic acid equivalence.

The maximum wavelength of gallic acid measured was 757.5 nm so that measurements of the sample n-butanol fraction and the ethyl acetate fraction of the red jeringau rhizome to determine equality were also carried out at that wavelength.

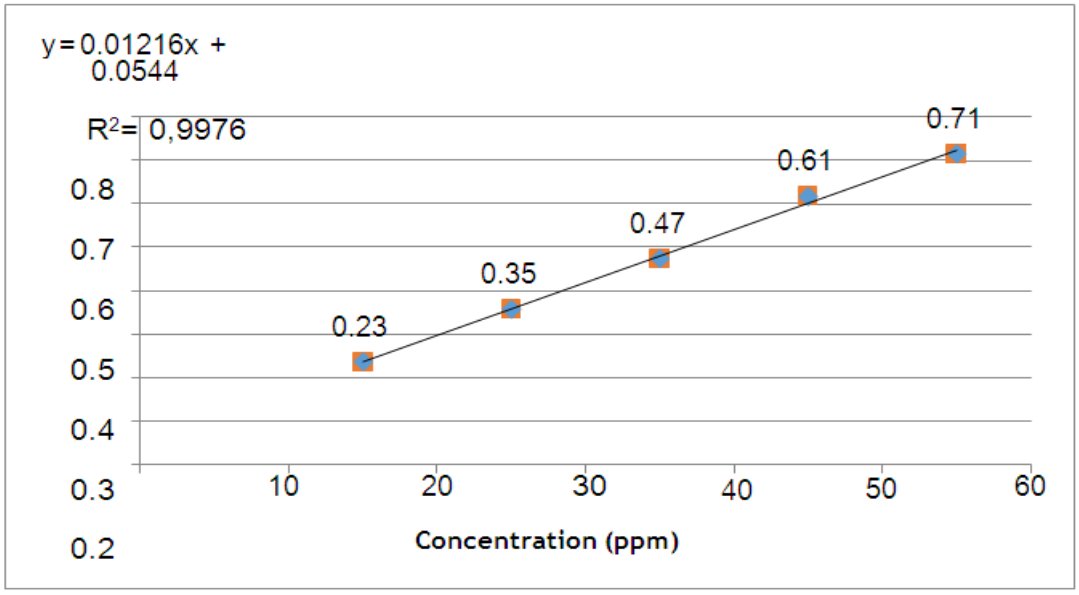

Figure 3: Gallic acid curve.

a. n-butanol fraction

Table 1: Table Total Phenol Levels of Red Jeringau Rhizome n-butanol Fraction.

Replication	Total Phenol Levels (mg GAE/g sample)	Average± SD
1	101,1034	103,1468± 2,2188
2	102,8300	
3	105,5070	

The results of the measurement of total phenol levels based on the equivalence of gallic acid in the n-butanol fraction from the ethanol extract of red rhizome rhizome were 103,146 ± 2,219 mg GAE / gram of butanol fraction, it can also be said that the percentage of total phenol in the butanol fraction was 10.315%.

b. Ethyl acetate fraction

Table 2: Table Total Phenol Levels of Ethyl Acetate Fraction in Red Jeringau Rhizome.

Replication	Total Phenol Levels (mg GAE/g sample)	Average ± SD
1	445,9918	432,5710 ± 12.0119
2	422,8279	
3	428,8934	

The results of the measurement of the total phenol fraction of ethyl acetate red rhizome at a concentration of 100 µg / mL was 432.5710 ± 12.0119 mg GAE / gram fraction or equivalent to 43.2571% of the sample.

The acquisition of total phenol levels in the red jeringau rhizome ethyl acetate fraction was the highest compared to the other fractions. This is illustrated by the research conducted by Srividya (2014), which examined the total phenolic content in various fractions, namely petroleum ether, chloroform, ethyl acetate, acetone and water in the rhizome of white jeringau plants (Acorus calamus). The results of the study stated that the ethyl acetate fraction had the highest total phenol content compared to other fractions.

Total flavonoid levels

The measurement of flavonoid compounds content in the ethanol fraction of red jeringau rhizome ethanol extract was carried out according to the Chang method which has the principle of measuring total flavonoid levels based on the reaction of complexation between the flavonoid compound with $AlCl_3$ which forms a yellow color so that the wavelength shifts from UV to visible. Quercetin is used as a standard compound to determine the equivalent content of quercetin, which is one example of flavonoids that are often used as raw compounds.

The maximum wavelength of quercetin compound obtained was 433.5 nm, so the measurement of n-butanol and ethyl acetate fractions of red jeringau rhizome to determine the equivalent level of quercetin equality was also performed using these wavelengths.

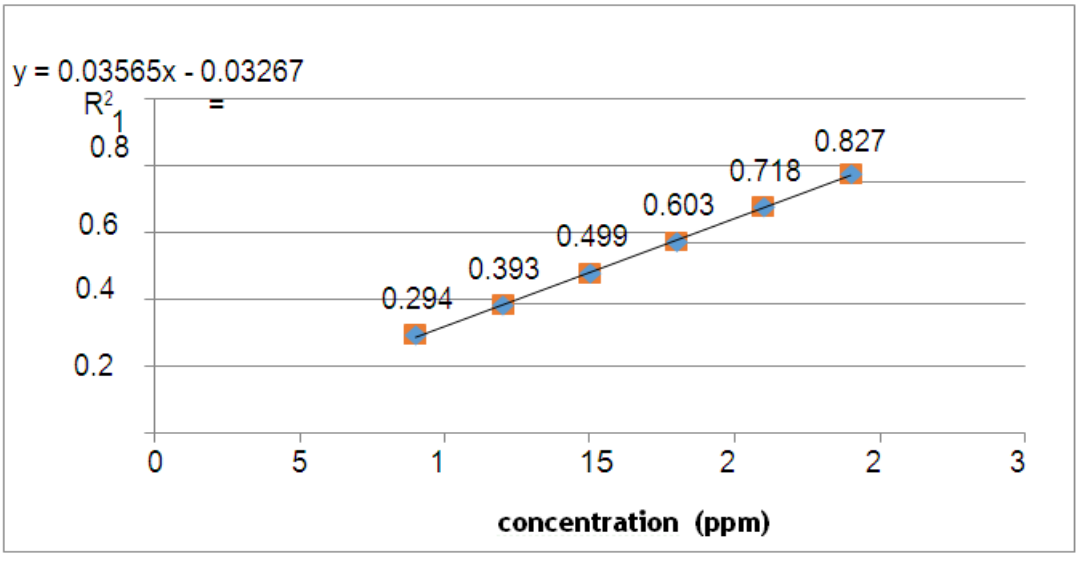

Figure 4: Quercetin curve.

a. n-butanol fraction

Table 3: Table Total Flavonoid Levels of n-butanol fraction of Red Jeringau Rhizome.

Replication	Total Flavonoids Levels (mg QE/g sampel)	Average ± SD
1	6,9071	6,8771± 0,1287
2	6,9881	
3	6,7361	

The result of total flavonoid measurement of n-butanol fraction sample from ethanol extract of red jeringau rhizome was 6.880 ± 0.129 mg QE / gram. It can also be said that the percentage of flavonoid compound content in the butanol fraction is 0.688%.

b. Ethyl acetate fraction

Table 4: Table Total Flavonoid Levels of ethyl acetate fraction of Red Jeringau Rhizome.

Replication	Total flavonoids levels (mg QE/g sampel)	Average ± SD
1	15,2918	14,8836 ± 0,3590
2	14,6168	
3	14,7423	

The results of measurement of total flavonoid levels in the sample of ethyl acetate fraction of red jeringau rhizome at a concentration of 500 µg / mL were 14.8836 ± 0.3590 mg QE / gram of fraction or equivalent to 1.4884% of the sample portion.

Antibacterial activity test

Testing antibacterial activity using the agar diffusion method by measuring the inhibition zone formed from the diameter or around the clear zone of the sample being tested. Tests were carried out on the bacteria Staphylococcus aureus and Eschericia coli.

Staphylococcus aureus bacteria are gram-positive bacteria, some of which are normal flora on the skin, respiratory tract, and food digestive tract in humans. Infection by S. aureus is characterized by tissue damage accompanied by festering abscesses. Some infectious diseases caused by S. aureus are ulcers, acne, impetigo, and wound infections.

Escherichia coli is a Gram negative bacteria that is facultative anaerobic or aerobic, not spherical, in the form of short rods. Eschericihia coli generally colonizes the large intestine as normal flora and becomes a pathogen if the number of these bacteria in the digestive tract increases or is outside the intestine. Escherichia coli produces enterotoxins which cause several cases of diarrhea, associated with enteropathogenic production of enterotoxins in epithelial cells.

a. n-butanol fraction

a.1. Staphylococcus aureus

The results of inhibition zone measurements in the n-butanol fraction obtained the largest inhibition zone diameter obtained in the n-butanol fraction with concentrations of 75% and 100%.

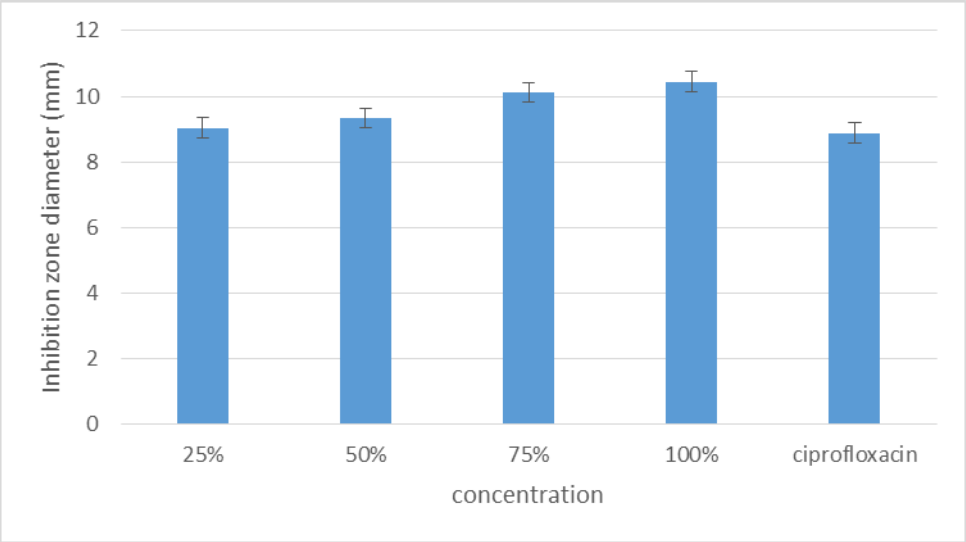

Figure 5: Inhibition zone diameter of the effect of n-butanol fraction on the growth of Staphylococcus aureus bacteria.

The analysis using One way ANOVA showed that the n-butanol fraction group with a concentration of 25%, 50% and 75% were not significantly different (P> 0.05) to the positive control group (ciprofloxacin), which indicated that the inhibitory zone activity was at concentration it has the same inhibitory power as ciprofloxacin. In contrast to the n-butanol fraction group with a concentration of 100% had a significant difference (P <0.05) from the positive control group, which indicates that the inhibitory activity at the concentration of n-butanol fraction with a concentration of 100% was greater than the positive control group.

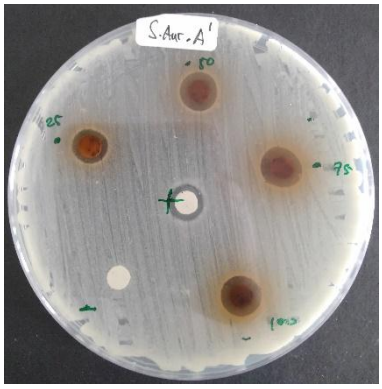

Figure 6: Effect of n-butanol fraction on the growth of Staphylococcus aureus bacteria.

a.2. Eschericia coli

The results of inhibition zone measurements in the n-butanol fraction obtained the largest inhibition zone diameter obtained in the n-butanol fraction with concentrations of 50% and 100%.

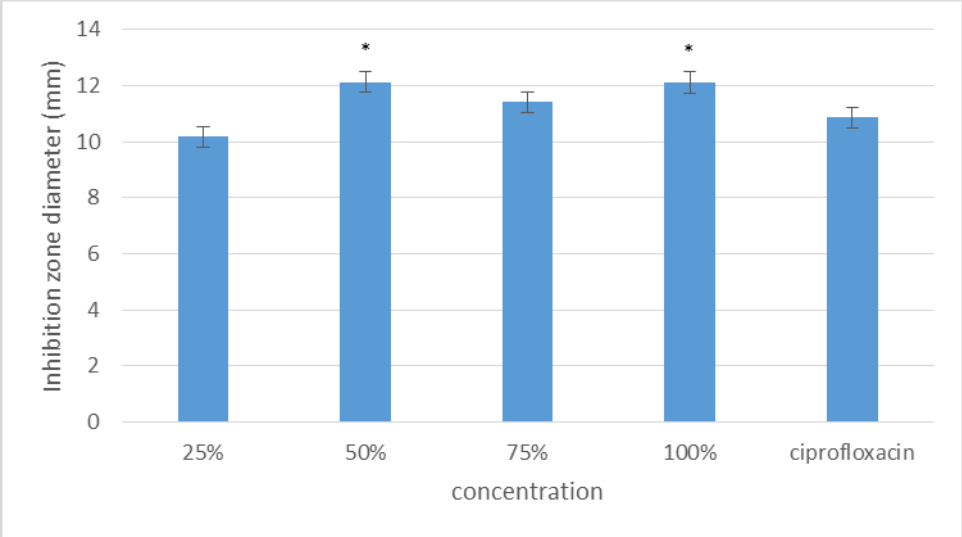

Figure 7: Inhibition zone diameter of the effect of n-butanol fraction on the growth of Eschericia coli bacteria.
Note: * = significantly different from the positive control group (ciprofloxacin)

The analysis using One way ANOVA showed that the n-butanol fraction group with a concentration of 25% and 75% were not significantly different (P> 0.05) to the positive control group (ciprofloxacin), which indicated that the inhibitory zone activity at that concentration had power the same inhibitors as ciprofloxacin. In contrast, the 50% and 100% concentration groups had a significant difference (P <0.05) from the positive control group, which indicates that the inhibitory activity at the concentration of n-butanol fraction with concentrations of 50% and 100% was greater than the positive control group.

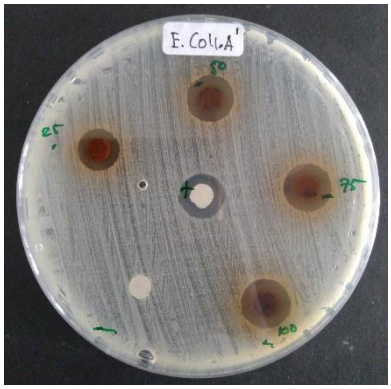

Figure 8: Effect of n-butanol fraction on the growth of Eschericia coli bacteria.

b. Ethyl acetate fraction

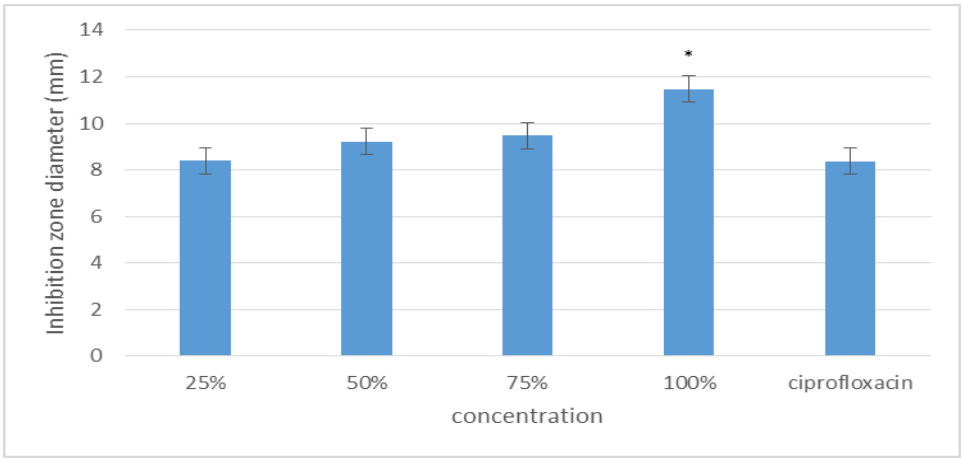

Figure 9: Inhibition zone diameter of the effect of ethyl acetate fraction on the growth of Staphylococcus aureus bacteria.

Note: * = significantly different from the positive control group (ciprofloxacin)

The analysis using One way ANOVA showed that the ethyl acetate fraction group with a concentration of 25%; 50%; 75% and 100% were not significantly different (P> 0.05) to the positive control group (ciprofloxacin), which indicates that the inhibitory zone activity at these concentrations had the same inhibitory power as ciprofloxacin.

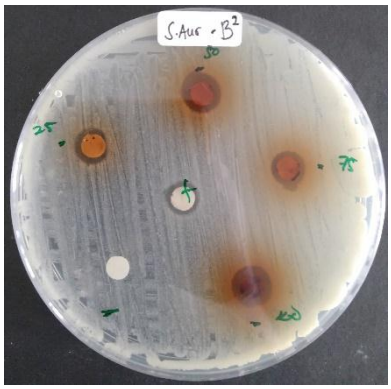

Figure 10: Effect of n-butanol fraction on the growth of Staphylococcus aureus bacteria.

b.1. Staphylococcus aureus

Inhibition zone measurement results in the ethyl acetate fraction obtained the largest inhibition zone diameter obtained in the fraction n-butanol with a concentration of 100%.

b.2. Eschericia coli

Inhibition zone measurement results in the ethyl acetate fraction obtained the largest inhibition zone diameter obtained in the fraction n-butanol with a concentration of 100%.

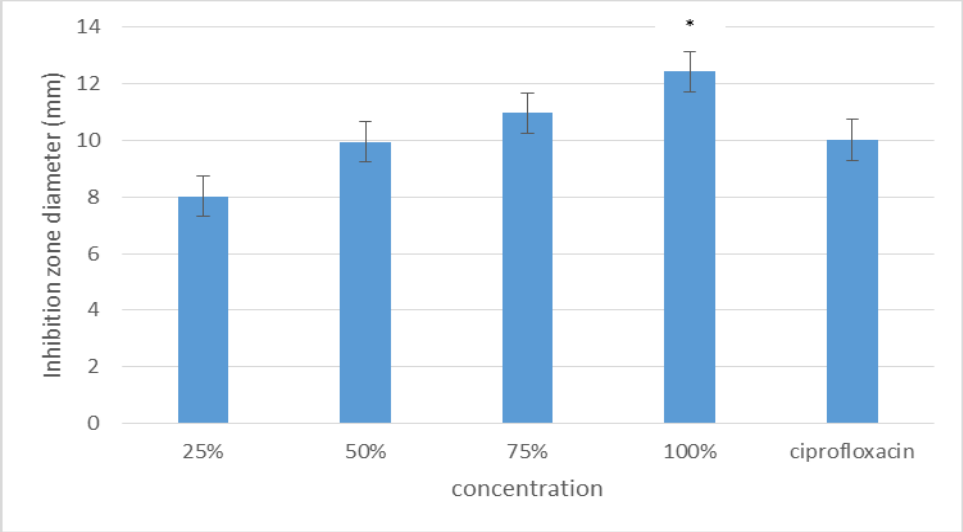

Figure 11: Inhibition zone diameter of the effect of ethyl acetate fraction on the growth of Eschericia coli bacteria.
Note: * = significantly different from the positive control group (ciprofloxacin)

The analysis using One way ANOVA showed that the n-butanol fraction group with a concentration of 25%; 50% and 75% were not significantly different (P> 0.05) to the positive control group (ciprofloxacin), which indicates that inhibitory zone activity at these concentrations had the same inhibitory power as ciprofloxacin. In contrast, the 100% concentration group had a significant difference (P <0.05) from the positive control group, which indicates that the inhibitory activity at the concentration with a 100% concentration was greater than the positive control group.

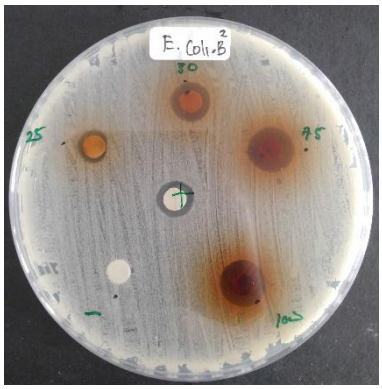

Figure 12: Effect of n-butanol fraction on the growth of Eschericia coli bacteria.

DISCUSSION

Ciprofloxacin is a third-generation fluoroquinolone antibiotic. This class of antibiotics tend to be toxic, so their use must be used appropriately. The mechanism of the fluoroquinolone group is inhibiting topoisomerase II (DNA gyrase) and topoisomerase IV which is needed in the replication process, with this mechanism the bacteria can be inhibited its growth due to inhibited the replication process.

The largest total flavonoid and total phenol content in the ethyl acetate fraction. In the research results obtained in the n-butanol fraction and ethyl acetate fraction the greatest inhibition was obtained in the test of Eschericia coli bacteria in the n-butanol fraction test with a concentration of 50% and 100% in the Eschericia coli bacterium the inhibitory values were respectively 12.14 ± 0.48 mm and 12.11 ± 0.16 mm. Inhibition on the ethyl acetate fraction against Eschericia coli bacteria obtained the greatest inhibition at a concentration of 100% with a diameter of 12.43 ± 1.29 mm. In the test of Staphylococcus aureus bacteria in the n-butanol fraction the largest inhibition zone diameter was obtained at a concentration of 75% and 100% with each inhibition zone diameter of 10.17 ± 0.47 and 10.44 ± 0.32, while in the fraction ethyl acetate obtained the largest inhibition zone diameter at a concentration of 100% with an inhibition zone diameter of 11.47 ± 0.74. Based on the test results on inhibitory zones of Eschericia coli and Staphylococcus aureus bacteria, it can be seen that with large levels of total flavonoids and total phenols, the diameter of inhibition zones against bacteria is also large. This can be seen in the ethyl acetate fraction which has greater total flavonoid and total phenol levels compared to the n-butanol fraction obtained by inhibition zone diameters in the bacteria Eschericia coli and Staphylococcus aureus which are greater than the n-butanol fraction.

Strain differences can cause differences in inhibition zones obtained, this is seen in bacteria Eschericia coli inhibition zone diameter is greater than the bacteria Staphylococcus aureus. This is caused by differences in enzymes and substances produced by bacteria, and cell walls in Staphylococcus aureus which are gram-positive much thicker and there are peptidoglycan in the upper membrane and there are three other polymer layers, namely: lipoprotein, outer membrane and lipopolysaccharide compared to bacteria Escherichia coli

which is gram-negative with a thin layer of lippopolisaccharide with peptidoglycan being above the upper membrane and lower membrane that affects the passage of molecules or compounds from drugs or compounds in natural materials. In addition, the inhibition zone is also influenced by the speed of diffusion, the stability of the bacterial material, the size of the molecule, the nature of the agar media used, the rate of bacterial growth, the concentration of chemicals and the conditions during incubation. Some of the factors above affect the activity of the test and the size of the inhibition zone diameter for the test results of the material being tested.

Increased levels of total flavonoids and total phenols are directly proportional to the increase in inhibition zone diameter in the bacteria Eschericia coli and Staphylococcus aureus. The mechanism of flavonoids inhibits bacterial metabolism by inhibiting oxygen and inhibiting cytochrome C reductase. Flavonoids can also inhibit nucleic acid synthesis and cell membrane function. The function of cell membranes is inhibited by forming complex compounds with extracellular proteins and dissolved so that it damages bacterial cell membranes, besides that flavonoids cause damage to cell walls also cause damage to microsomes and lysosomes which are the result of interactions between flavonoids and bacterial DNA. Phenol compounds have antibacterial activity by killing microorganisms through denaturation of cell proteins. Denaturation of cell proteins occurs through hydrogen bonds between phenol compounds and proteins that affect the permeability of cell walls and cytoplasmic membrane bacteria whose components are proteins. The imbalance causes macromolecular and ion imbalances which can cause lysis. Natural flavonoids are mostly found in the form of glucose, in humans flavonoids can act as antioxidants in cancer prevention, in some cases flavonoids can act as antibiotics by disrupting the function of microorganisms such as viruses and bacteria.

CONCLUSION

1. Total phenol levels for n-butanol fraction of 10.32% and total flavonoid levels of 0.688% had significant differences with the positive control group at a concentration of 100% (10.44 ± 0.32) against Staphylococcus aureus and at a concentration of 50 % (12.14 ± 0.48) and 100% concentration (12.11 ± 0.16) against Eschericia coli.
2. Ethyl acetate fraction with phenol levels of 43.26% and flavonoid levels of 1.49% the 100% concentration group (12.43 ± 1.29) was significantly different from the positive control group against Eschericia coli bacteria.

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