POTENTIAL OF HERBAL COMBINATION EXTRACTS AS GRAM POSITIVE AND GRAM NEGATIVE ANTIBACTERIAL COMPOUNDS

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Abstract. *Many medicinal plants that have antibacterial potential are found in Indonesia, including Moringa leaf (Moringa oleifera L.), breadfruit leaf (Artocarpus altilis F.), betel leaf (Piper betle L.), and wind wood (Usnea baileyi Z.). The active ingredients of these plants are flavonoids, saponins, tannins, essential oils, and phenolics. The study aimed to determine the antibacterial potential of the ethanol extract of the herbal combination against the growth of Staphylococcus aureus and Escherichia coli bacteria. Extraction using the maceration method with 70% ethanol as solvent. The extracts were made in series with concentrations of 2%, 4%, 6%, 8%, and 10% with positive control (clindamycin 2%) and negative control and tested against Staphylococcus aureus and Escherichia coli by disc diffusion method. The results obtained were analyzed using the One Way Anova test, Post Hoc LSD test, and Linear regression. The results showed that all concentrations of the herbal combination ethanol extract could inhibit the growth of bacteria against Staphylococcus aureus and Escherichia coli bacteria. The minimum inhibitory concentration against the second bacteria occurred at a concentration of 2% with clear areas of 3.45 mm and 3.48 mm, respectively. The results of the LSD test showed that there were significant differences in inhibition between Staphylococcus aureus and Escherichia coli bacteria at concentrations of 6% and 8%, while concentrations of 2%, 4%, and 10% were not significantly different. The effect of the concentration of the ethanol extract of the herbal combination on the diameter of the clear area in Staphylococcus aureus and Escherichia coli bacteria was 96.84% and 94.89% respectively, the rest was influenced by other factors. Conclusion The ethanolic extract of the herbal combination has potential as an antibacterial against Staphylococcus aureus and Escherichia coli bacteria.*

Keywords: *Antibacterial, herbal combination, Staphylococcus aureus and Escherichia coli*

INTRODUCTION

Plants are a source of several types of medicinal compounds. The utilization of plants as medicine has been used since ancient times and is a legacy of ancestors that has long been used in most parts of the world (Djauhariya & Hernani, 2004). Traditional medicine has long been used throughout the world, both in developing and developed countries. WHO says that 65% of the population in developed countries and 80% of the population in developing countries use herbal medicines as treatment (Sukandar, 2020).

In several developing countries such as Indonesia, traditional medicine is one of the primary treatment systems (Bhalodia et al., 2011). Traditional medicines are developed by considering economic factors because they are more affordable, easy to obtain, and have safety factors with few side effects (Vifta et al., 2017). Indonesia's tropical forests are a growing place for 80% of medicinal plants in the world consisting of 28,000 plant species, 1000 of which have been used as medicinal plants (Depkes, 2007). Compounds contained in medicinal plants are believed to be used to treat infections, one of which is an infection caused by bacteria. Bacteria based on the structure of their cell walls are grouped into two, namely gram-positive bacteria and gram-negative bacteria. *Staphylococcus aureus* is an example of gram-positive bacteria while *Escherichia coli* is an example of gram-negative bacteria.

Secondary metabolites contained in plants can be obtained by extraction. Several factors that need to be considered in extraction are the selection of the right solvent with a compound contained in secondary metabolites (Suhendra et al., 2019). The use of 70% ethanol as a solvent is because ethanol has neutral and polar properties and can attract flavonoids, tannins, and saponins. 70% ethanol solvent is difficult for fungi and molds to grow, non-toxic, and miscible with water in all ratios (Azis et al., 2014).

Purwoko et al. (2020) in their research said that the inhibitory activity of consortia of herbal plants was greater than that of single plants. Based on the description above, it is important to research the

antibacterial potential of the ethanol extract of the herbal combination of moringa leaves (*Moringa oleifera* L.), breadfruit leaves (*Artocarpus altilis* F.), green betel leaves (*Piper betle* L.) and angin wood (*Usnea baileyi* Z.) on the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria.

METHODS

This research is a type of quantitative experimental research using the disc diffusion method using a completely randomized design with 5 concentration treatments (2%, 4%, 6%, 8%, and 10%). Repetition was carried out 3 times with 2 test bacteria namely *Staphylococcus aureus* and *Escherichia coli*.

Moringa leaves, breadfruit leaves, and green betel leaves were taken randomly with the criteria that the leaves were still fresh green, not perforated, not rotten, and not attacked by pests obtained from Kabongan Kidul Village, Rembang District, Rembang Regency, Central Java. Angin wood is taken from trees or rocks in the mountainous area of Colo Village, Dawe District, Kudus Regency, Central Java with the criteria of being fresh and free from pests, bacteria culture *Staphylococcus aureus* ATCC 4205 and *Escherichia coli* ATCC 6310 from Medis Sarana Medika Laboratory Semarang.

The herbal combination powder weighed 37.5 grams each (1:1:1:1 ratio) for a total of 150 grams. Each powder was mixed and macerated with 70% ethanol solvent as much as 600 mL for 1 x 24 hours with 3x maceration in a closed container and protected from light while occasionally stirring. The Maserati obtained is filtered through a flannel cloth and stored in a closed Erlenmeyer (Fauzi et al., 2020). Maserati was collected and thickened in a water bath at 40ºC until a thick extract was obtained.

The ethanol-free test was carried out by heating 0.1 grams of herbal combination extract and then adding 5 drops of acetic acid (CH_3COOH) and 2 drops of concentrated sulfuric acid (H_2SO_4). Extracts that are free from 70% ethanol solvent are indicated by the absence of a distinctive ester odor from alcohol in the esterification test (Sayuti, 2015). Phytochemical screening test using the color change method for the content of secondary metabolites such as flavonoids, saponins, tannins, essential oils, and phenolics. Preparation of bacterial suspension by taking one dose of bacterial colonies that have been incubated in 10 mL of Nutrient Broth then incubated in an incubator at 37°C for 18 hours. Bacterial cell density was measured using a spectrophotometer with a wavelength of 625 nm (Rosmania & Yanti, 2020). The absorbance value obtained is between 0.08-0.1 which is equivalent to the 0.5 Mc Farland standard (1.5x10^8 CFU/mL) (Sureshjani et al., 2014). The antibacterial test used the Pour plate method and was carried out near a bunsen in a Laminar Air Flow (LAF). A bacterial suspension of 100 µl with a bacterial cell density of 10^8 CFU/mL was poured into an empty sterile petri dish. The Nutrient Agar media which had cooled slightly and had not hardened was then poured into a petri dish as much as 25 mL. The petri dish containing the bacterial suspension and NA media was rotated in Figure 8 so that it could be mixed homogeneously. Eight paper discs for each treatment were soaked in combination extracts with concentrations of 2%, 4%, 6%, 8%, 10%, 2% clindamycin (positive control), 1% DMSO (negative control) and sterile distilled water until all absorbed in the disc paper and wait for it to dry by aerating. Eight paper discs were divided into two Petri dishes, each petri dish filled with 4 paper discs and then placed on the surface of the Nutrient Agar medium and incubated at 37˚C for 18 hours. This test was repeated 3 times and then the presence or absence of a clear zone was observed around the disc paper and the diameter was measured with a vernier caliper. The data obtained were processed using the computer software SPSS 20.0 (Statistics Program for Social Science) using the Shapiro-Wilk test, One Way Anova, Post Hoc LSD, and linear regression.

RESULTS AND DISCUSSION

Herbal Combination Extract Manufacturing

The results of viscous extracts, percentage (%) yield, and organoleptic tests of the ethanol extracts of the herbal combination (moringa leaves, breadfruit leaves, green betel leaves, angin wood) can be seen in Table 1.

Table 1. Result Extraction of Herbal combination

Description	Result
Powder Weight	150 g
Consortium moisture content	7.21%
Extract	42 g
% yield	28%
Organoleptic	The extract is blackish brown, sticky, and has a characteristic odor

The results of maceration of a combination of moringa leaves, breadfruit leaves, green betel leaves, and angin wood obtained a viscous extract of dark brown-black color, with a sticky texture and a characteristic odor. The amount of extract obtained is 42 grams with a yield value of 28%, which means the yield is good because the solvent can attract good active compounds with a yield value > 10% (Wardaningrum, 2019).

The herbal combination's ethanol condensed extract was subjected to an ethanol-free test using alcohol esterification. Alcohol esterification aims to determine whether the extract of the herbal combination is completely free of 70% alcohol with no distinct ester odor.

The results of the ethanol-free test show that the herbal combination extract is ethanol-free from the solvent, namely 70% ethanol, as indicated by the absence of a distinctive ester odor from ethanol. The purpose of the ethanol-free test is that the herbal combination extract must be completely free of 70% ethanol because ethanol can have antibacterial and antifungal properties so it will cause false positives for the presence of clear areas (Kurniawati, 2017).

Phytochemical screening was carried out to determine the content of secondary metabolites in herbal combination extracts which included flavonoids, saponins, tannins, essential oils, and phenolics. The results of the phytochemical screening of the ethanol extract of the herbal combination can be seen in Table 2.

Table 2. Phytochemical herbal combination extract

No.	Secondary metabolic	Color change	Results test	Conclusion
1.	Flavonoid			
	a. Wilstater test	Red/orange	Formed orange color	+
	b. Bath-smith test	red	Formed red color	+
	c. NaOH test	orange	Formed orange color	+
2.	Saponin	Stable foam	Formed stable foam	+
3.	Tannin	Greenish brown/dark blue	Formed greenish brown	+
4	Essential oil	Residual odor	Formed residual odor	+
5.	Phenolic	Blackish green	Formed blackish green	+

Note: (+) positive for the compound.

The Flavonoid test was carried out using the Wilstater test, Bate-Smith test, and 10% NaOH test. Testing for flavonoids using the Wilstater, NaOH and Bate-Smith methods, the ethanol extract of the herbal combination was positive for containing flavonoids indicated by the formation of orange or red color. The addition of Mg powder and concentrated HCl aims to reduce the benzopyrene nucleus contained in the flavonoid structure to red or orange flavilium salts (Ergina et al., 2014). Flavonoid compounds are abundant in plants with 2 classes of flavonoids, namely flavonoid aglycones, and glycosides.

The saponin test of the ethanol extract of the herbal combination showed positive results which were indicated by the formation of stable froth or froth. The addition of HCl was able to make the foam more steady and stable. The foam that arises is caused by saponin compounds containing compounds that are partially soluble in water (hydrophilic) and compounds that dissolve in nonpolar solvents (hydrophobic) as surfactants which can reduce surface tension (Harborne, 1998). Polar and non-polar compounds are surface active, saponins when shaken with water will form micelles. Polar compounds face outward, while non-polar compounds face inward to form foam (Sangi et al., 2008). In addition, because saponins are polar and dissolve easily in water and ethanol, they are attracted to thick ethanol extracts (Firawati & Pratama, 2018).

The tannin test of the herbal combination's ethanol extract showed positive results due to a change in color to greenish brown. In the phytochemical screening of tannins, FeCl3 was used to determine the presence of phenol groups which formed a greenish-black color because tannins will form complex compounds with Fe3+ ions. (Harborne, 1987).

Testing the essential oil content of the ethanol extract of the herbal combination showed positive results as indicated by the characteristic odor produced by the residue. Essential oils are oils from plants whose components are generally volatile, so many call them flying oils. Essential oils are also called ethereal oils or etheric oils because they are ether-like. Essential oils are commonly called essential oils (essen oils) because they are distinctive as a giver of aroma/smell (essence). In a fresh and pure state, essential oils are generally colorless, but on prolonged storage, the color changes to a darker color (Ketaren, 1985).

The phenolic test of the ethanol extract of the herbal combination showed positive results due to a change in color to blackish green. Phenolic compounds are compounds that have hydroxyl groups and are found in many plants. These compounds have structural uniformity, from simple phenols to complex phenols and polymerized components (Balasundram et al., 2006). The characteristics of phenolics include the ability to form chelate compounds with metals, are easily oxidized, and form polymers that give rise to dark colors. The emergence of dark color on the cut or dead plant parts is caused by this reaction, this also inhibits plant growth (Pratt & Hudson, 1990).

The ethanol extract of the herbal combination showed positive results for containing flavonoids, saponins, tannins, essential oils, and phenolic compounds. Flavonoids, saponins, tannins, essential oils, and phenolics contained in the ethanol extract of the herbal combination have an important role in inhibiting bacterial growth.

Antibacterial potency of ethanol extract of herbal combination against *Staphylococcus aureus* and *Escherichia coli* bacteria

The ethanol extract of the herbal combination containing flavonoids, saponins, tannins, essential oils, and phenolic compounds was tested for potential as an antibacterial against *Staphylococcus aureus* and *Escherichia coli* bacteria using the disc diffusion method. The absorbance value of *Staphylococcus aureus* after being measured using a UV-Vis spectrophotometer was 0.091 and that of *Escherichia coli* was 0.092. The absorbance results obtained were between 0.08-0.1, which was equivalent to the standard 0.5 Mc Farland (1.5×10^8 CFU/mL) (Sureshjani et al, 2014). The cell density was measured so that the density of the resulting bacterial cells was measurable and uniform. The wavelength used is 625 mm which is included in visible or visible light. Visible light has a wavelength between 380-780 nm (Depkes RI, 1979). These wavelengths also do not have a bactericidal effect.

The results of measuring the diameter of the clear area of *Staphylococcus aureus* and *Escherichia coli* at various concentrations of the ethanol extract of the herbal combination can be seen in Table 3.

Table 3 Diameter of the Clear area of the Herbalcombination's Ethanol Extract against *Staphylococcus aureus* and *Escherichia coli*

Concentration	Diameter of clear area *Staphylococcus aureus*	Diameter of clear area *Escherichia coli*
Extract of herbal combination 2%	3,45	3,48*
Extract of herbal combination 4%	4,45	4,03*
Extract of herbal combination 6%	6,42	4,97
Extract of herbal combination 8%	7,90*	6,07
Extract of the herbal combination 10%	8,37*	8,03
Clindamycin 2% (positive control)	24,63	10,40
Extract of Moringa leaves 10%	1,57	-
Extract of Breadfruit leaves 10%	2,35	1,6
Extract of Green betel leaves 10%	3,2	2,65
Extract of Angin wood 10%	5,97	-

Source: Primer date (*2022*)

Note : (*) = Not significant (Sig. in level 0.05)

In *Staphylococcus aureus* bacteria, the extract concentration of 2% is the minimum inhibitory concentration with a clear area diameter of 3.45 mm. The optimal inhibition concentration was shown at an extract concentration of 8% with a clear area diameter of 7.90 mm (moderate category). The positive control (2% clindamycin) had a clear area diameter of 24.63 mm (very strong category). In *Escherichia coli* bacteria, the extract concentration of 2% is the minimum inhibitory concentration with a clear area diameter of 3.48 mm (weak category). The optimal inhibition concentration was at an extract concentration of 10% with a clear area diameter of 8.03 mm (medium category). The positive control (2% clindamycin) had a clear area diameter of 10.40 mm (strong category). These results indicate that the antibacterial potential of the herbal combination extract is far below that of clindamycin (positive control).

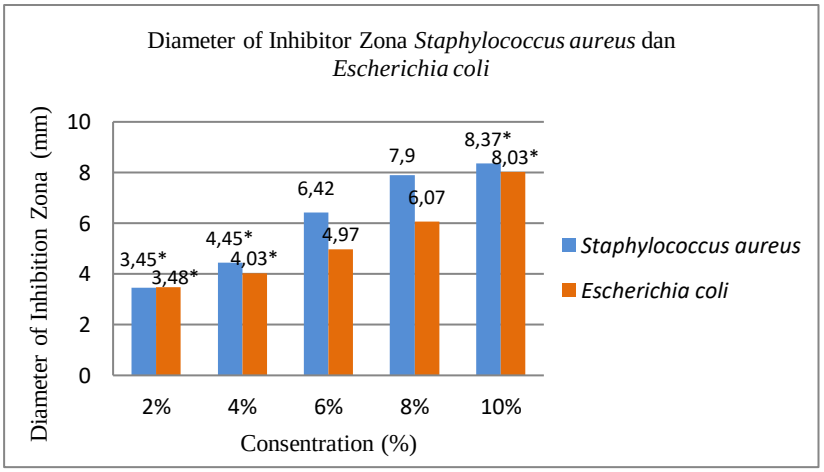
From the results of measuring the clear area of each single extract at a concentration of 10%, it was found that the clear area was low or could not exceed the clear area in the herbal combination extract at a concentration of 10% against *Staphylococcus aureus* and *Escherichia coli* bacteria. The conclusion is that the herbal combination is higher in inhibiting the diameter of the clear area of *Staphylococcus aureus* and *Escherichia coli* than a single compound with the same concentration of 10%. The herbal combination provides an adaptive effect, namely adding.

Based on the results of the antibacterial activity test showed that the ethanol extract of the herbal combination could inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria. This is due to the content of compounds in the extract that act as antibacterial including flavonoids, saponins, and tannins. Each of these compounds inhibits the growth of bacteria in the flavonoids which have an acidic nature called ascorbic acid. Phenol can damage cell membranes and denature proteins. As a result, the transport of nutrients through the cell membrane is disrupted, resulting in growth experiencing a lack of the necessary nutrients (Virgianti & Purwati, 2015).

The release of proteins and enzymes from the cell can be caused by saponins. The surface tension of the bacterial cell wall decreases and impairs the permeability of the membrane. The survival of bacteria will be threatened by damage to the cell membrane. Saponins diffuse through the outer membrane and vulnerable cell walls and bind to the cytoplasmic membrane thereby disrupting and reducing the stability of the cell membrane (Virgianti & Purwati, 2015).

The content of other chemical compounds from the ethanol extract of the herbal combination is tannin. Cells cannot carry out living activities so growth will be hampered and even lyse or die. Protein-bound to tannins can interfere with the growth of protein synthesis and bacteria (Sulastrianah et al., 2007).

The test results for differences in the diameter of the clear area of herbal combination extracts between *Staphylococcus aureus* and *Escherichia coli* bacteria, namely the diameter of the clear area for *Staphylococcus aureus* bacteria is greater than *Escherichia coli* bacteria. At a concentration of 2%, the clear area of *Staphylococcus aureus* and *Escherichia coli* bacteria were not significantly different with a sig value of 0.939. The results of the different tests can be seen in Figure 1.



Note: (*) Indicates no significant difference

Figure 1. Differences in the inhibition of *Staphylococcus aureus* and *Escherichia coli* bacteria

The results of measuring the diameter of the clear area showed that the ethanol extract of the herbal combination had a greater inhibitory effect on *Staphylococcus aureus* bacteria than *Escherichia coli* bacteria. The results of data analysis in Figure 1 show that there are significant differences between *Staphylococcus aureus* bacteria and *Escherichia coli* bacteria at concentrations of 6% and 8%.

Figure 2
Diameter Inhibition Zona of Herbalcombination Extract againt

Figure 3
Diameter Inhibition Zona of Herbalcombination Extract againt *Escherichia coli*

The effect of the concentration of the ethanol extract of the herbal combination on the diameter of the clear area of *Staphylococcus aureus* with a value of R^2 = 0.9684 showed an effect of 96.84%. The equation of the linear regression line between the concentration of the ethanol extract of the herbal combination and the diameter of the *Staphylococcus aureus* clear area is y = 0.6645x + 2.131. The results of the linear regression analysis between the concentration of the ethanol extract of the herbal combination and the diameter of the clear area can be seen in Figure 4.

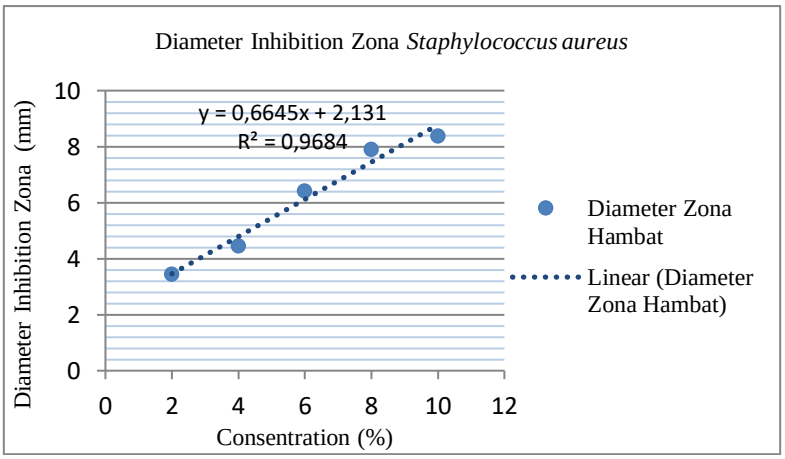


Figure 4 Graph of the effect of the concentration of the extract of the herbal combinationon the inhibition of *Staphylococcus aureus*.

Based on the results of the linear regression test in Figure 4, the value of Y = 0.6645x + 2.131 means that every 1% increase in concentration can increase the diameter of the clear area by 0.6645 mm with an initial diameter of 2.131 mm. The diameter of the bacterial clear area was affected by the extract concentration of 96.84% (R^2= 0.9684). The remaining 3.16% is influenced by other factors such as temperature, radiation, light, air, humidity, pH, and others.

The effect of the concentration of the ethanol extract of the herbal combination on the diameter of the clear area of *Escherichia coli* bacteria with a value of R^2 = 0.9489 showed an effect of 94.89%. The equation of the linear regression line between the concentration of the ethanol extract of the herbal combination and the diameter of the *Escherichia coli* clear area is Y = 0.557x + 1.974. The results of the linear regression analysis between the concentrations of the ethanol extract of the herbal combination and the diameter of the clear area can be seen in Figure 5.

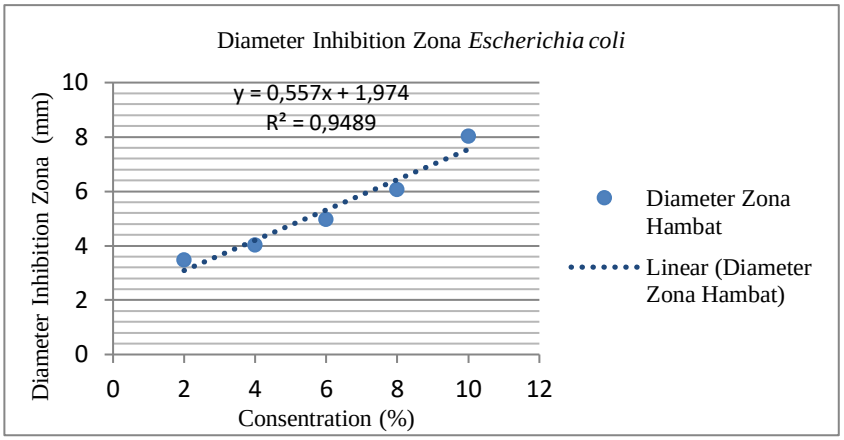


Figure 5 Graph of the effect of the concentration of the extract of the herbal combination on the inhibition of *Escherichia coli*

The effect of the concentration of the ethanol extract of the herbal combination can be determined if the results of the correlation test show a strong relationship. In this study, the correlation value between the ethanol extract of the herbal combination and the inhibition of bacteria was very strong so it could be followed by a linear regression analysis.

Based on the results of the linear regression test in Figure 5, the value of Y = 0.557x + 1.974 means that every 1% increase in concentration can increase the diameter of the clear area by 0.557 mm with an initial diameter of 1.974 mm. The diameter of the bacterial clear area was affected by the extract concentration of 94.89% (R^2= 0.9489). The remaining 5.11% is influenced by other factors such as temperature, radiation, light, air, humidity, pH, and others.

CONCLUSION

The ethanol extract of the herbal combination has antibacterial potential against *Staphylococcus aureus* and *Escherichia coli* bacteria. The minimum inhibitory concentration of the ethanol extract of the herbal combination on both bacteria is at a concentration of 2% in the weak category and the concentration of the extract that is close to the positive control is 10% in the medium category. Significant differences in inhibition of the ethanol extract of the herbal combination between *Staphylococcus aureus* and *Escherichia coli* were found at concentrations of 6% and 8%, while at concentrations of 2%, 4%, and 10% there were no significant differences. The effect of the concentration of the ethanol extract of the herbal combination on the diameter of the clear area in *Staphylococcus aureus* and *Escherichia coli* bacteria was 96.84% and 94.89% respectively, the rest was influenced by other factors.

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