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Phytochemical Screening of Active Secondary Metabolites and Antibacterial Activity Kaffir Lime Leaf (*Citrus hystrix* and Turmeric Leaf (*Curcuma longa* Linn.) Against E. coli

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Abstract. This study aims to determine the content of active compounds of secondary metabolites and antibacterial activity of secondary metabolites from a maceration of turmeric leaf (*Curcuma longa* Linn.) and kaffir lime leaf (*Citrus hystrix*) against Escherichia coli (*E. coli*). Each sample of kaffir lime leaf and turmeric leaf was extracted by maceration with ethanol 96%. The phytochemical screening test was carried out by qualitative method using chemical reagents. The antibacterial activity test of the extract of the combination of turmeric leaves (*Curcuma longa* Linn.) and kaffir lime leaves (*Citrus hystrix*) used the Punch Hole Diffusion method with various concentrations of 20%, 40%, 60%, 80%, and 100%. The results showed that the combined extract of turmeric leaf (*Curcuma longa* Linn.) and kaffir lime leaf (*Citrus hystrix*) contained secondary metabolites in the form of flavonoids, alkaloids, steroids, tannins, and saponins. The optimum zone of inhibition to inhibit the growth of E. coli produced was 9.73 ± 0.78 mm. Overall, it can be concluded that the combined extract of turmeric leaf (*Curcuma longa* Linn.) and kaffir lime leaf (*Citrus hystrix*) has potential as an alternative to antibacterial active compounds.

Keywords : Antibacterial, combination extract , *Escherichia coli*, phytochemical screening

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Introduction

The wealth of biological resources that spread from Sabang to Merauke is a matter of pride for Indonesia. In it, there are various types of plants, animals both living on land and in the ocean, and microbes also become an important part of maintaining the balance of the sustainability of the universe including human existence [1]. The utilization of biodiversity such as plants as medicines [2] cannot be separated from the influence of the pharmacological activities contained in these plants [3]. Bioactive is known to come from the secondary metabolite biosynthetic pathway [4]. The presence of these compounds is important and distinctive [5]. The use of plants as ingredients for traditional medicine has long been used by the people of Indonesia, partly based on hereditary experience and partly developed through scientific research [6]. One of the plants that can be used as medicine is turmeric (*Curcuma longa* Linn.) [7]. Turmeric has a variety of benefits [8], all parts of this plant can be used as traditional medicine, especially on the leaves. Generally, utilization of turmeric leaves has not been maximally used by the community due to lack of knowledge so turmeric leaves are only used as waste by the community.

This is very unfortunate considering that several active compounds can be used as ingredients in the manufacture of drugs. Turmeric leaf extract has a strong essential oil aroma so that to minimize the aroma it can be combined with other plants. One of the plants that can be used is extracted from kaffir lime leaves with a unique aroma [9]. Similar to turmeric leaves, kaffir lime leaves are also rarely used by the community as an ingredient in making medicine, [3] people only know kaffir lime leaves only as an ingredient in cooking even though in kaffir lime leaves many compounds can be used as medicine [10]. The results of Research showed that ethanol extract and essential oil of kaffir lime peel and leaves had antibacterial activity against several species of salmonella and enterobacteria that we're able to inhibit the cultivation of 5 strains of Propionibacterium acnes [11]. Kaffir lime (*Citrus hystrix*) contains tannins, steroids, triterpenoids, and essential oils that can be used as antiseptics and antioxidants [12]. Alkaloid compounds, flavonoids, and tannins contained in kaffir lime leaves function as antibacterial [9]. Based on research conducted the informed, kaffir lime (*Citrus hystrix*) contains chemicals such as essential oils and phenols that are bactericidal with the ability to inhibit the growth of Staphylococcus aureus and E. coli at concentrations of 0.625% v/v and 1.25% v/v [13], the higher the concentration of kaffir lime (*Citrus hystrix*) the better the inhibition.

Experimental

Chemical

Maceration extractions were used ethanol 96% purity (Merck). Solvents were used n-hexane (Merck) and methanol (Merck). Reagents were used Wagner (Sigma-Aldrich), Mayer (Sigma-Aldrich), Dragendorff (Sigma-Aldrich), Liebermann -Burchard (Sigma-Aldrich), Mg powder (Merck), chloride acid (HCl) (Merck), ferric chloride ($FeCl_3$) (Merck), and aquadest. Antibacterial assay was used amoxicillin (Dexa Medica 500 mg) as positive control and Muller Hinton agar (Merck) as medium bacteria growth.

Preparation of Samples

The fresh, turmeric leaf and kaffir lime leaf were collected in Pagesangan, Mataram, West Nusa Tenggara. Both turmeric leaf and kaffir lime leaf were prepared by each 2 kg which has been sorted by light green color and is still fresh. Then the sample was washed using clean running water. After cleaning, all the leaves were cut into pieces using a knife and air-dried (37 °C) until completely dry for 3 days without being exposed to the sun directly [14]. Once dry enough, the leaves were blended until smooth and sieved using a 100-mesh sieve to obtain a uniform powder then stored until ready to be used in further analysis.

Maceration

Weighed each turmeric leaf and kaffir lime leaf that had been mashed using a ratio (1:1), i.e. 100 g of turmeric leaf and 100 g of kaffir lime leaf. Then put the combination of turmeric leaves and kaffir lime leaves into a 1 L beaker glass and add 96% ethanol solvent until the sample is completely submerged and tightly closed or in a ratio of 1:5 (w/v). After the simplicia was completely submerged, it was stirred every two to three hours and then allowed to stand for 3 x 24 hours. The results of the soak are filtered every 1 x 24

hours and separated between the results of the macerate and the pulp The sample dregs will be soaked again using a new solvent using the same maceration procedure as before. The total macerate yields are combined and concentrated using a rotary evaporator using a temperature of 50-70°C. Solvent evaporation is carried out until no more solvent drips. The sample extract was stored in a desiccator before being used for further analysis.

Phytochemical Screening (qualitative analysis)

Alkaloid Test [15]

Samples weighing 0.1 g in the form of a combination of extracts were added, with 10 ml of chloroform solvent and a few drops of ammonia solution. The chloroform fraction formed was taken and acidified using 1-2 drops of concentrated H_2SO_4. The acid fraction was used and divided into different test tubes and marked. Dragendroff, Mayer, and Wagner reagents were added to each tube. The content of alkaloids present in the sample is indicated by the formation of a white precipitate when reacted with Mayer's reagent, a red precipitate is formed when Dragendorff's reagent is added and a brown precipitate is formed when Wagner's reagent is added.

Flavonoid Test [16]

A total of 1 g of sample was dissolved in hot methanol solvent and added 0.01 g of Mg powder and 3-5 drops of concentrated HCl. The formation of color changes to orange, brick red, pink, and dark red indicates the presence of flavonoids in the sample.

Triterpenoid-Steroid Test [17]

A total of 1 g of sample was dissolved in 0.5 ml of chloroform solvent and given Liebermann-Burchard reagent. The presence of terpenoids is indicated by a red or purple color, while the presence of steroid compounds is indicated by the formation of green color.

Saponin Test

The saponin test on the sample was carried out by dissolving 1 g into 4 ml of hot distilled water, then cooled and filtered. The obtained filtrate is shaken vigorously for 15 to 20 seconds [18]. Next, add 1-3 drops of concentrated HCl to the foam formed. If the foam remains, the sample is indicated to contain saponins.

Tannin Test [17]

A total of 1 g of sample was added with 10 ml of distilled water and then boiled in a water bath. The cooled filtrate was added as much as 5 ml of 1% (w/v) $FeCl_3$. The formation of a color change to dark blue, blue-black, dark brown, or green-black indicates that the sample contains tannins.

Antibacterial Test

Testing the antibacterial activity of the combination of turmeric leaf extract (*Curcuma longa* Linn.) and kaffir lime leaf extract (*Citrus hystrix*) against Escherichia coli with various concentrations (20%, 40%, 60%, 80%, 100%) and positive control amoxicillin is used with concentration 100%. The test was carried out by the agar diffusion method with a well (Punch Hole Diffusion) against *Escherichia coli*. This method was followed by inoculating well-grown E. coli on the Muller Hinton agar plate. A 100 µL of the combined extract was properly added in wells with a diameter of 5 mm and kept in the incubator for 24 h [19]. The zones were measured using vernier calipers for the antibacterial effect of the combination extract.

Results and Discussion

The results of phytochemical screening (Table 1) show that the combined extract sample of turmeric leaf (*Curcuma longa* Linn.) and kaffir lime leaf extract (*Citrus hystrix*) contains several active compounds of secondary metabolites. The extract obtained has physical properties in the form of a very thick precipitate weighing 20 g. Extraction by maceration was a simple method done using 96 % ethanol with a temperature was maintained not exceeding 70 °C to avoid degradation of the targeted compound. Then this method is a traditional process that the plant was in contact with liquid solvent at a set temperature and time directly [20].

The result of the combination extract has a dark green color (Figure 1) and a very unique aroma. Based on Table 1, shows that the sample contains several secondary metabolites in the

form of flavonoid compounds, alkaloids, steroids, tannins, and saponins. Phytochemical screening as qualitative analysis provides a quick potential bioactivities plant [4].

Figure 1. Combination extract

Flavonoid Test

Showed positive results because of the color change formed, namely a red-orange color after the addition of magnesium powder (Mg) and a few drops of concentrated HCl. The color change formed can occur due to a reduction reaction by concentrated hydrochloric acid and magnesium metal powder. The possible reactions that occur are shown in Figure 2 [16].

Alkaloid Test

Furthermore, the results of the alkaloid test showed positive results when chemical reagents were added in the form of Mayer, Wagner, and Dragendorf reagents. The resulting color changes are white precipitate, brown precipitate, and dark orange-red precipitate. These precipitates can be formed due to the reaction that occurs between the nitrogen atom which has a lone pair of electrons with the metal cation potassium (K^+) in the reagent used. One of the alleged reactions of alkaloid compounds with Mayer's reagent is shown in Figure 3 [16].

Mayer's reagent contains chemical compounds in the form of a complex compound of dipotassium tetraiodomercurate (II). The presence of lone pair of electrons in the alkaloid structure will allow the formation of a coordinating covalent bond between the nitrogen atom and the metal cation potassium (K^+) so that a precipitate is formed in the form of potassium-alkaloid. The positive result of testing for alkaloid compounds using Wagner's reagent is due to the reaction that occurs between the electron pair on the nitrogen atom belonging to the alkaloid compound and the metal cation potassium (K^+) which are bonded to each other through coordinate covalent bonds. The test reaction with Wagner's reagent is shown in Figure 4 [16].

The next test for alkaloids is using Dragendorff's reagent which can be prepared from bismuth nitrate compounds. A positive result of the presence of alkaloid compounds using this reagent indicates the formation of a brownish or yellowish precipitate. The test reaction using Dragendorff's reagent is shown in Figure 5 [16].

Saponin Test

The test results for the presence of saponin compounds showed positive results, this indicated the presence of saponin compounds in the sample. The permanent foam formed indicates the presence of a glycoside which can produce

Table 1. Phytochemical screening result of combination extract

Phytochemical	Test	Observation	Combination extract
Alkaloid	Mayer's test	White precipitate	+++
	Wagner's test	Brown precipitate	+++
	Dragendorff's test	Red precipitate	+++
Flavonoid	Mg / HCl	Dark red	+++
Saponin	HCl	Foam	++
Tannin	Ferric chloride test	Dark brown	++
Steroid	Liebermann-Burchard's test	Light green	+
Terpenoid		absence	-

Note: negative sign (-) indicates absence, positive sign (+) indicate presence

foam if there is water which will hydrolyze the glycoside bonds in glucose. The test reaction is shown in Figure 6 [16].

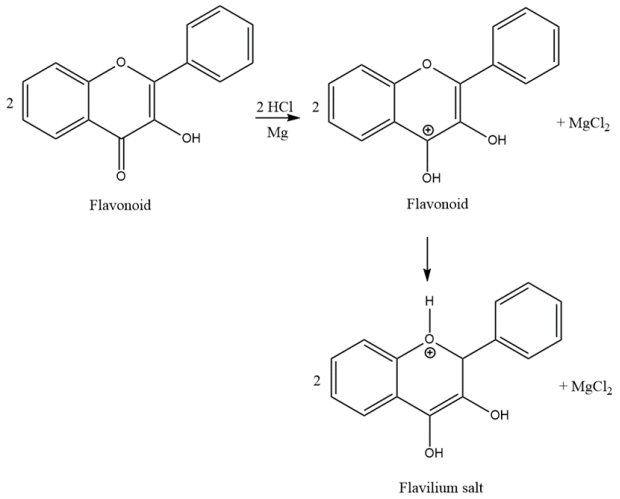

Figure 2. Flavonoid test [16]

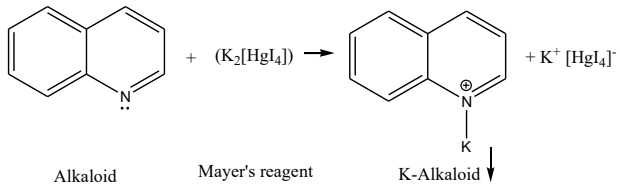

Figure 3. Alkaloid test by Mayer Reagent [16]

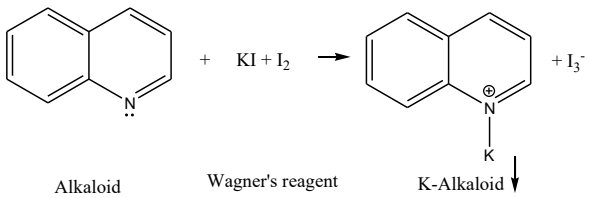

Figure 4. Alkaloid Test by Wagner reagent [16]

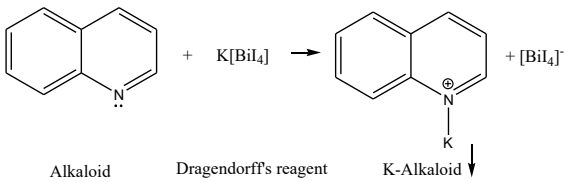

Figure 5. Alkaloid test by Dragendorff reagent [16]

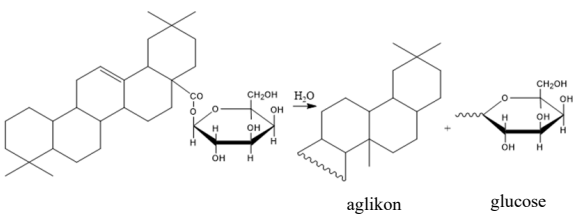

Figure 6. Saponin test [16]

Tannin Test

The results of the tannin test showed a dark brown color change which indicated a reaction between the tannin compound and $FeCl_3$ reagent. The reactions that may occur are shown in Figure 7 [21].

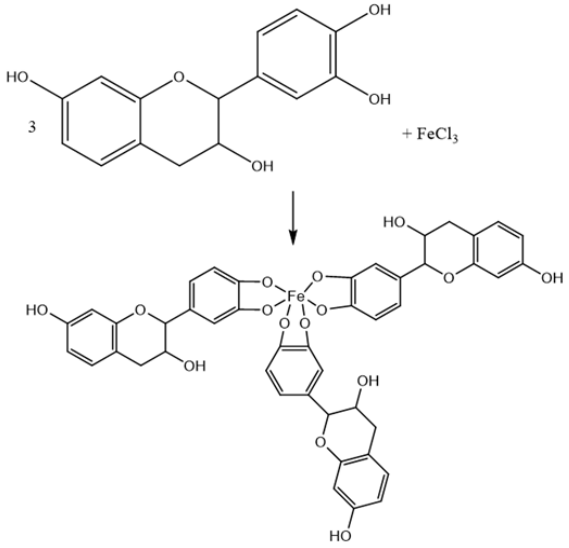

Figure 7. Tannin test [21]

Terpenoid-Steroid Test

The results of the steroid test were positive, which was indicated by the formation of green color when the Liebermann-Burchard reagent was added [22]. One example of a steroid compound is cholesterol which can react with the Liebermann-Burchard reagent which consists of acetic anhydride which is used as a solvent and dehydrating agent and sulfuric acid which acts as a dehydrating and oxidizing agent. The test reaction is shown in Figure 8 [23].

Antibacterial activity

The diameter of the inhibition zone for the growth of E. coli is shown in Figure 9 and Table 2. The higher the concentration of the given sample, the greater the diameter of the resulting inhibition zone. While the large diameter of the inhibition zone was the positive control in the form of amoxicillin. If grouped based on the diameter of the inhibition zone obtained, the combined extract samples with a concentration of 20% to 100% were included in the medium category. In contrast to the inhibition zone obtained in

the positive control, the inhibitory power was categorized as strong. As for the response range of the inhibitory power of a sample in inhibiting bacterial growth, if the diameter of the inhibition zone 5 mm is in a low category; 5-10 mm in the medium category; 10-20 mm in the strong category; ≥20 is in the very strong category.

The ability of the combined extract samples to inhibit the growth of *E. coli* may occur due to the presence of several secondary metabolites which have the potential as antibacterial active compounds. Turmeric extract that combined has been reported as effective against *E. coli* [24].

These phytochemical compounds play an important role in the antibacterial ability of a plant. The mechanism of action of flavonoids as an antibacterial is by inhibiting the function of cell membranes and energy metabolism of bacteria while inhibiting function cell membranes, flavonoids form complex compounds with extracellular proteins that can damage bacterial cell membranes, then followed by the release of these bacterial intracellular compounds [1]. In addition, flavonoids as part of phenolic compounds that can interfere with bacterial growth [25], especially with the prenyl group as hydrophobic substituents [26] then interactions of flavonoids with Gram-

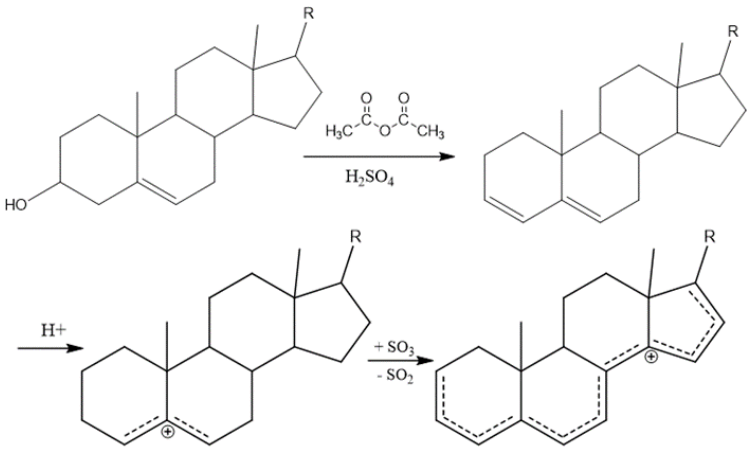

Figure 8. Steroid test [23]

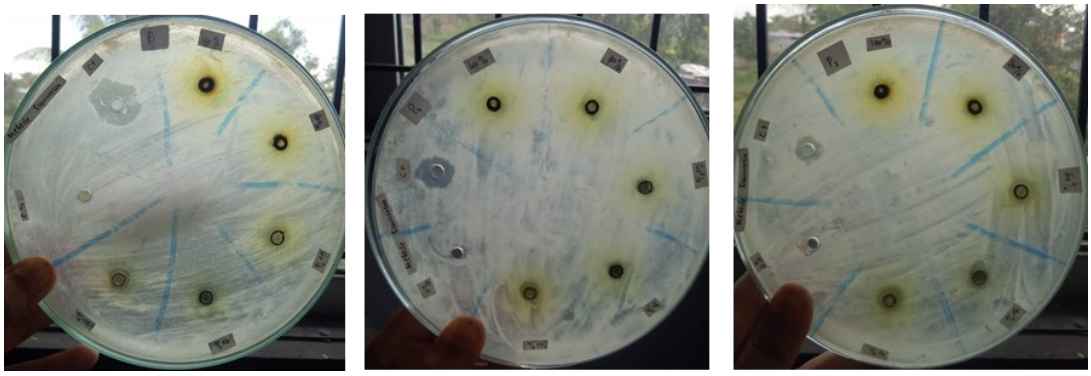

Figure 9. Antibacterial test (from left to right for repetition: I, II, III)

Table 2. Zone of inhibition

[Sample]	Sample repeat			Inhibition zone
%	I	II	III	(mm) + SD
20	8,20	8,80	8,80	8,60 ± 0,35
40	8,80	9,30	9,30	8,93 ± 0,64
60	9,20	8,90	8,40	8,83 ± 0,40
80	10,60	9,10	8,50	9,40 ± 1,08
100	11,10	8,70	9,40	9,73 ± 1,23
Control +	15,20	13,70	14,10	14,33 ± 0,00
Control -	0,00	0,00	0,00	0,00 ± 0,00

negative bacteria by two mechanisms. First by associated with the non-polar compound in the hydrophobic interior of the membrane, second by a form hydrogen bond with polar and hydrophilic at the interface membrane [27]. The phenol is acidic alcohol that can denature proteins and damage bacterial cell membranes. The mechanism of action of steroids as antibacterial in inhibiting bacterial growth is related to membrane lipids and sensitivity to steroid components that cause leakage in bacterial liposomes. The mechanism of action of saponins as an antibacterial is by denaturing proteins [28]. The surface-active substances of saponins are similar to detergents, saponins can be used as antibacterial where the surface tension of the bacterial cell wall will be lowered and the permeability of the bacterial membrane is damaged [29]. Tannins were reported can be used as an antibacterial by precipitating protein and making nutrition unavailable [18].

Conclusion

The results of the phytochemical screening of extracts from the combination of turmeric leaves (*Curcuma longa* Linn.) and kaffir lime leaves (*Citrus hystrix*) contain metabolites in the form of flavonoid compounds, alkaloids, steroids, tannins, and saponins. The antibacterial activity of the combined extract of turmeric (*Curcuma longa* Linn.) and kaffir lime leaves (*Citrus hystrix*) leaves resulted in an optimum growth inhibition zone of 9.73 ± 0.78 mm against *Escherichia coli* with a concentration of 100%.

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Author Contributions

All authors mentioned contributed to conception, acquisition, analysis, interpretation of data and agree to be responsible for the work.

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