Antibacterial activity of *Vitex trifolia* methanol extract against pathogenic bacteria

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**Abstract.** *Vitex trifolia* (Legundi plants) is a plant that is widely used as an ingredient of traditional medicine in Lombok Island for a variety of microbial infections. In this study, a qualitative test of *V. trifolia* methanol extract was shown to contain secondary metabolites, such as steroids, saponins, alkaloids, flavonoids, terpenoids, and tannins. The inhibitory test of methanol extract of roots, stems and leaves of *V. trifolia* against pathogenic bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Klebsiella pneumoniae*) has been carried out. The experimental design used was a Completely Randomized Design. The treatment factor was the concentration of methanol extract with 4 levels (C1 = 7.5%, C2 = 15%, C3 = 30%, C4 = 60%). The extraction method used was methanol maceration, and the well diffusion method was used in antibacterial activity assay. Data were analyzed by using Anova followed by DMRT test. Anova analysis results showed that differences in the concentration of methanol extract of leaf have a significant effect in inhibiting the growth of all tested bacteria. The DMRT showed that the best treatment was with 60% methanol extract. In this study, *S. aureus* was the most sensitive bacterium to *V. trifolia* methanol extract, compared to other test bacteria. It can be concluded that the methanol extract of *V. trifolia* is an effective antibacterial source, especially against *S. aureus*.

1. Introduction

Plants have been used as a source of medicine in traditional or folk medicine from time immemorial due to their magical power to cure diseases [1]. Nowadays, researchers have given more attention to discover new antimicrobial drugs of plant origin because most of the available synthetic antibiotics are losing their capacity to inhibit the growth of microorganisms [2]. This is mainly due to the ability of microorganisms to develop resistance against the continuously using antibiotics.

*Vitex trifolia* (legundi plant) is a plant that has long been known as a traditional medicinal ingredient. Parts of plants that can be used as medicine are all parts of the leaves, stems, roots, flowers and seeds. The leaves of Vitex trifolia were used as a remedy for rheumatism, gout, sinus, hydrocele and hemorrhoids [3]. *V. trifolia* leaf extract also has anti-inflammatory activity in Wistar rats by inhibiting prostaglandin synthesis and inhibiting increased vascular permeability [4]. The hydro-alcoholic extract of Vitex trifolia leaves also showed significant results on anti-inflammatory activity in acute and sub-acute inflammation which were tested on Wistar rats [5].
Furthermore, *V. trifolia* in several studies showed inhibitory activity against pathogenic microbes. The methanol extract of *V. trifolia* leaves gave significant results in inhibiting gram-positive bacteria such as *B. cereus* and *B. pumilus* with the inhibition zones of 15.49 mm and 14.82 mm respectively and gram-negative bacteria, *Salmonella flexneri* and *Shigella sonnei* with inhibition zones of 12, 25 mm and 12.93 mm, respectively [6]. Ethanol, methanol and ethyl acetate extracts from *V. trifolia* leaves at a concentration of 25 mg/ml gave significant results on the antibacterial activity in *E. coli*, *Shigella flexneri*, *Proteus mirabilis*, *Pseudomonas diminuta*, *Enterobacter cloacae*, and *Staphylococcus aureus* [7]. Other studies report that acetone, ethanol and aqueous extracts of *Vitex trifolia* leaves show antibacterial activity against *Bacillus subtilis* (0.5 cm, 0.75 cm and 0.1 cm inhibitory zones) and *E. coli* (successive inhibition zones also 0.15 cm, 0.25 cm and 0.35 cm) [8].

In addition to being useful as a medicinal and antimicrobial ingredient, *V. trifolia* is also useful as larvicide. The methanol extract of *V. trifolia* leaves showed the highest larvicidal activity (*LC*₅₀ = 41.41 ppm) compared to *V. peduncularis* (*LC*₅₀ = 76.22 ppm), *V. altissima* (*LC*₅₀ = 128.04 ppm) [9]. The ethanol extract of *V. trifolia* leaves also showed activity as a repellent against *Aedes aegypti* with an ED₅₀ value of 14.809% and an ED₉₀ of 41.442% [10].

The compound content in Vitex trifolia has been reported in several studies. The ethanol extract of *V. trifolia* leaves contained secondary metabolite compounds including alkaloids, saponins, flavonoids, and glycosides [11]. A qualitative analysis of the leaves of *V. trifolia* with standard methods using petrollium ether, benzene, acetone, ethanol and water, indicating the presence of alkaloids, saponins, tannins, phenols, terpenoids, flavonoids, and steroids [8].

In this study, we evaluate the presence of secondary metabolites of *V. trifolia* form Lombok Island, qualitatively, and evaluate the antimicrobial activity against two species of Gram positive bacteria and two species of Gram negative bacteria.

2. **Material and methods**

This study was an experimental laboratory study with treatment factors for variations in the concentration of legundi plant leaves namely concentrations of 7.5%, 15%, 30% and 60%, against clinical isolates of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*. The experiment was done in a completely randomized design (CRD) with one factor consisting of four levels of concentration treatment (*C₁* = 7.5%, *C₂* = 15%, *C₃* = 30%, *C₄* = 60%). Each treatment was repeated 3 times.

2.1. **Preparation of *V. trifolia* methanol extract**

Legundi plants includes the roots, stems and leaves of legundi plants. The location of the sampling of legundi plants is in the rice field area of Lingsar Village, Narmada District, West Lombok Regency. Methanol extraction by maceration method from roots, stems and legundi leaves was carried out in the Chemistry Laboratory FKIP Unram.

Legundi plant extraction is done by maceration using methanol as a solvent. The extraction procedure was carried out in accordance with the Natheer procedure [7]. Plant samples were dried aerated and blended. Subsequently, 1 kg of each plant part, root, stem, and leaf were incubated at room temperature for 48 hours in 3 liter of methanol. Filtering is carried out to obtain the filtrate. The filtrate is then concentrated with a vacuum rotary evaporator, and the extracts were then weighed. Fifty percent of the extract was used for phytochemical testing and the rest was used for antibacterial assay. Four variations of the extract concentration (7.5%, 15%, 30%, and 60%) were made.

2.2. **Phytochemical test**

Phytochemical tests to determine the content of secondary metabolites produced by *V. trifolia* plant was carried out using the standard procedure of Harborne [12]. a). **Steroid test.** The extract was added 10 drops of CH₃COOH and 2 drops of H₂SO₄. The solution is shaken slowly and left for several minutes. The change in color to blue or green indicates the presence of steroids; b). **Saponin test.** The extract is boiled with 10 ml of water in a water bath, the filtrate is shaken and allowed to stand for 1 minute.
Added 2 drops of HCl 1 N. The formation of a stable foam indicates the presence of saponins; c). *Alkaloid test*. The extract was mixed with 2 ml of chloroform and 2 ml of ammonia then heated, shaken and filtered. 3-5 drops of 2 N sulfuric acid are added to each filtrate, then shake and settle. The top of each filtrate was then taken and tested by Meyer, Wagner, and Dragendorf reagents. The formation of orange, brown and white deposits indicates the presence of alkaloids; d). *Flavonoid test*. The extract was added with hot water and boil for 5 minutes then filtered. To the 5 ml of filtrate, 0.05 g of Mg and 1 ml of concentrated HCl were then added, and shaken vigorously. The formation of red indicates the presence of flavonoids; e). *Triterpenoid test*. The extract was mixed with 2 ml of chloroform and 3 ml of concentrated sulfuric acid. The formation of a brownish red color between the surfaces shows the presence of triterpenoids. f). *Tannin test*. The extract is boiled with 20 ml of water and then filtered. A few drops of 1% ferrochloride were added and the formation of a greenish-brown or black-blue color indicates the presence of tannins.

2.3. Rejuvenation of tested bacteria

Test bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsella pneumonia*) were rejuvenated by inoculating as much as 1 ose on NA aseptic media aseptically and incubated for 18-24 hours at 37°C. Test bacteria were taken from NA medium, then suspended into a test tube containing 5 ml of 0.9% physiological salt. Addition of bacteria continued until the same turbidity was obtained with the standard solution Mc. Farland on a scale of 0.5 [13].

2.4. Antibacterial activity assay

The Mueller Hinton Agar (MHA) plate was made for antibacterial testing. This media is made by means of 38 grams of Mueller Hinton Agar (MHA) media suspended with 1 liter of distilled water. The solution is then heated on a hot plate and stirred with a magnetic stirrer until it is homogeneous. Media were sterilized by autoclaving at 121°C for 15 minutes. Then the media were poured into petri dishes ± 10 mL each [14].

Inhibitory activity of extracts of leaves of Vitex plants was done by using the well diffusion method. The culture suspension was spread evenly on the MHA media plate with a sterile cotton swab and allowed to stand for 10 minutes in an incubator so that the microbial suspension permeated the culture media. The wells are made by perforating the solid agar media which has been inoculated with a test bacteria with a diameter of 6 mm. The size of the wellbore has been adjusted to the standard of wellbore for sensitivity testing, which is between 6-8 mm. The pit is then injected with the extracts in 4 variations of the concentration to be tested. The amount of extract injected is 50 µl. The media were then incubated for 24 hours. Antimicrobial activity in the form of clear zone is then measured in mm [15].

2.5. Data analysis

Data were analyzed by using Anova, and DMRT at α = 0.05% using SPSS 23.

3. Results and discussion

3.1. Qualitative test of methanol extract

The results of extraction by methanol maceration showed that all groups of tested secondary metabolites were detected, except for steroids was not detected in the roots and stems (Table 1).
Table 1. Phytochemical test results for several secondary metabolites of *V. trifolia* from roots, stems and leaves.

| No | Secondary metabolite group | Root | Stem | Leaf |
|----|---------------------------|------|------|------|
| 1  | Steroid                   | -    | -    | +    |
| 2  | Saponin                   | +    | +    | +    |
| 3  | Alkaloid                  | +    | +    | +    |
| 4  | Flavonoid                 | +    | +    | +    |
| 5  | Triterpenoid              | +    | +    | +    |
| 6  | Tannin                    | +    | +    | +    |

3.2. *Antibacterial activity*

The average of the clear zone diameter data as a result of differences in concentration treatment on the growth of test bacteria from 3 replications is presented in Figure 1.

![Figure 1. The average diameter of inhibition zone of leaf methanol extract against *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumonia*.](image)

The effect of these concentrations was analyzed by using ANOVA and showed significant different at $\alpha = 0.05$ (Table 2).
Table 2. Anova test results regarding the effect of the concentration of methanol extract of V. trifolia leaves on the growth of test bacteria.

| Tested bacteria | Sum of Squares | df | Mean Square | F   | Sig. |
|----------------|---------------|----|-------------|-----|------|
| S.aureus       |               |    |             |     |      |
| Between Groups | 288.483       | 3  | 96.161      | 29.377 | .000 |
| Within Groups  | 26.187        | 8  | 3.273       |       |      |
| Total          | 314.669       | 11 |             |       |      |
| S.epidermidis  |               |    |             |     |      |
| Between Groups | 53.476        | 3  | 17.825      | 14.906 | .001 |
| Within Groups  | 9.567         | 8  | 1.196       |       |      |
| Total          | 63.043        | 11 |             |       |      |
| E. coli        |               |    |             |     |      |
| Between Groups | 58.189        | 3  | 19.396      | 10.011 | .004 |
| Within Groups  | 15.500        | 8  | 1.938       |       |      |
| Total          | 73.689        | 11 |             |       |      |
| K. pneumoniae  |               |    |             |     |      |
| Between Groups | 68.543        | 3  | 22.848      | 11.813 | .003 |
| Within Groups  | 15.473        | 8  | 1.934       |       |      |
| Total          | 84.017        | 11 |             |       |      |

ANOVA test results (Table 2) showed that the treatment of different extract concentrations on the growth of the four test bacteria showed a significant difference (Sig. <0.05) based on the average diameter of the inhibitory zone in the growth of the test bacteria.

Further tests were specifically conducted to know the significant differences of the average value among the treatments in S. aureus. The DMRT results (not shown) showed that all treatments (7.5%, 15%, 30% and 60%) were different significantly from each other, where treatment with 60% of methanol extract of V. trifolia leaves gave the best effect by being able to achieve an average inhibition zone against S. aureus at the value of 15.1 mm. The content of secondary metabolites in the form of steroids, saponins, alkaloids, flavonoids, triterpenoids and tannins detected as shown in Table 1, has more inhibitory effect on Gram positive bacteria, in this case S. aureus and S. epidermidis, compared to Gram negative bacteria, E. coli and K. pneumoniae. The use of crude extracts of plants parts and phytochemicals of known antimicrobial properties can be of great significance in the therapeutic treatments. In fact, many plants have been used due to their antimicrobial properties which are actually the secondary metabolites synthesize by the plants.

The present study has shown that methanolic extract of V. trifolia leaf has promising antibacterial activity. This is probably why the plant is widely used in traditional medicine. It is also used as anti-inflammatory, antipyretic agent. They are also used as sedative for rheumatism, headache and common cold in some countries. Vitex species used in traditional medicine to treat ailments like wounds, allergies, asthma and body pains [16]. There were reports that methanol extract demonstrated inhibitory effects to S. aureus but not E. coli [17]. Several research activities on antibacterial activities of crude extracts have implicated the methanol extract for being more active than the other solvents extracts [18].

The activity of the plant against both Gram positive and Gram negative bacteria can be indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant. The antimicrobial activity of the extracts of V. trifolia may be due to the presence of flavonoids, triterpenoids, and tannins in the plant extract [6-8].

In the present study Gram positive bacteria were found to be more susceptible to the plant extract than Gram negative bacteria which corroborated the previous reports that plant extracts are more active against Gram positive. Gram-negative bacteria are considered to be more resistant due to their outer membrane which acting as a barrier to many environmental substances including antibiotics [19]. This outer membrane includes the asymmetric distribution of the lipids with phospholipids and lipopolysaccharide located in the inner and outer leaflets, respectively. This characteristic that is absent
in the Gram-positive bacteria might have acted as the additional barrier that hinders the movement of foreign substance into the cell.

4. Conclusion
V. trifolia methanol extract contains 6 types of secondary metabolites, such as steroids, saponins, alkaloids, flavonoids, triterpenoids and tannins. This study shows that the methanolic extract of Vitex trifolia leaves exhibited appreciable antibacterial properties inhibiting growth of Gram positive bacteria, especially to S. aureus. It could serve as useful source for new antimicrobial agents in the future.

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