Comparison of Antimicrobial Activity of Different Extract of *Ocimum tenuiflorum* L. leaf and Stem

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**Authors’ contributions**

This work was carried out in collaboration between all authors. Author LM performed the study, statistical analysis and wrote the first draft of the manuscript. Author RMS designed and managed the study. Author VM who helped us to designed of the methods of extraction from these plants and prepared all material for antimicrobial test and author OMT who help us during this study. All authors read and approved the final manuscript.

**ABSTRACT**

**Aims:** We aim to evaluate and compare the antimicrobial activity of extracts from the leaves and stems of *Ocimum tenuiflorum* L. Various extractions in different solvents were compared to determine the extract with the best performance against bacteria, fungi, and yeasts.

**Study Design:** Prospective.

**Place and Duration of Study:** Food Technology Division, School of Industrial Technology Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia. This study was developed as prospective.

**Methodology:** Different extracts of non-polar and polar solvents were obtained from the macerate method from leaves and stems of *O. tenuiflorum*. The antimicrobial activities of
these extracts were evaluated against selected Gram-positive and Gram-negative bacteria and fungal and yeast strains via the following method: inhibition zone, minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and minimum bactericidal concentration (MBC).

**Results:** The antimicrobial activity tests were performed using common pathogenic bacteria (Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, and Listeria monocytogenes), yeast (Candida tropicalis and Saccharomyces cerevisiae), and fungus (Aspergillus niger and Penicillium sp.). *O. tenuiflorum* L. stem or leaf extracts were more or less active against most of the tested pathogenic strains. The inhibition zone ranged from ≥9 mm to ≤15 mm. The most susceptible bacterium and fungus were *L. monocytogenes* (IZ = ≥10–14 mm; MIC = 6.5 mg/ml) and *A. niger* (IZ = ≥15 mm; MFC = 3.12 mm), respectively.

**Conclusion:** The methanol and hexane extracts of *O. tenuiflorum* L. showed antimicrobial activities and exhibited significant potential as natural antimicrobial agents. By contrast, the water, ethyl acetate, and chloroform extracts from the leaves and stems of *O. tenuiflorum* L. can inhibit the microorganisms. All extracts from the leaves and stems failed to inhibit the *E. coli*, *S. typhimurium*, *S. aureus*, and *C. tropicalis*. However, further studies are still required.

**Keywords:** Antimicrobial plant extract activity; phytochemical, antimicrobial plant extract activity, medicinal plants.

1. **INTRODUCTION**

In the last century, the practice of herbalism has become important worldwide. Despite great advances in modern medicine, the use of plant products is still prevalent in health care [1]. The value of traditional medical systems, particularly those of Asian origin, is now being recognized. Medicinal plants with significant healing powers from indigenous pharmacopoeias have been identified. Medical plants are distributed worldwide, but they are most abundant in tropical countries [2]. *Ocimum* is among the important genera of family Lamiaceae which is widely used in traditional medicine practice.

*Ocimum tenuiflorum* L. is widely distributed in tropical countries. It is traditionally used as medicine and food in Malaysia. The sap and the extract of the plant have been reported to possess medicinal properties, such as anthelmintic, expectorant, diaphoretic, and stimulant effects. Infusion of the plant has been prescribed for arthritis, toothache, ringworm infections, and piles [3]. The search for components with antimicrobial activity has become increasingly important in recent times because of the alarming increase in the rate of infections caused by antibiotic-resistant microorganisms [4]. Hence, new and effective therapeutic agents are required. Many plant species have been used as traditional medicines, but there is a lack of scientific based evidence for the therapeutic properties of the plants. These botanical medicines may be used as resources for the development of effective drugs [5]. *O. tenuiflorum* L. is traditionally used as medicine and food in Malaysia.

The present study aimed to evaluate the antimicrobial activity of the *O. tenuiflorum* L. crude leaf and stem extracts on several pathogenic microorganisms that cause infectious diseases in humans.
2. MATERIALS AND METHODS

2.1 Plant Materials and Extraction

Fresh plants or plant parts were collected from Perak (Malaysia). Voucher specimens have been deposited at the Herbarium of the School of Biological Sciences, University Sains Malaysia (USM Herbarum number 11400). The plant materials were separated, washed and subsequently strained, dried, and ground to small pieces using a blender. Coarsely powdered dry leaves and stems were macerated and successively extracted with the following non-polar solvents: Hexane, chloroform, ethyl acetate, methanol, and water. In this method, 100g of dried powdered plant material was placed in the extraction chamber with 400mL of solvent for 7 days at room temperature after seven days the samples were wrapped properly in 24.0cm Whatman No. 1 filter paper and placed in the extraction chamber. At the end of the extraction process, the solvents containing the solutes in the round flask were concentrated using a rotary evaporator (EYELA, N1200B, 11013648) at 35ºC. Subsequently, the residue of the dried sample was allowed to dry off before being extracted subsequently with other organic solvents (chloroform, ethyl acetate, methanol, and water) with different polarity.

The dried extracts were then weighed using microbalance and were kept at 4ºC. Abbreviations for crude extract used in this study: HE I (hexane extract of leaf), CE I (chloroform extract of leaf), EAE I (ethyl acetate extract of leaf), ME I (methanol extract of leaf), WA I (water extract of leaf) HE II (hexane extract of stem), CE II (chloroform extract of stem) ME II (methanol extract of stem), WA II (water extract of stem).

2.2 Microorganisms

The microorganisms used in this study (Table 1) consisted of seven strains of bacteria, two yeasts and two fungi. The bacterial strains were grown and maintained on nutrient agar slants, while yeasts and fungi on Sabouraud glucose agar slants. The inoculated agar slants were incubated at 37ºC for bacteria and yeasts, and 30ºC for fungi.

2.3 Disc Diffusion Assay

Antimicrobial activity was determined by using Disc Diffusion following the method described by the National Committee for Clinical Laboratory Standar (NCCLS) (2000). All bacterial strains obtained from Microbiology Laboratory School of Industrial Technology University Sains Malaysia. The test bacteria was removed aseptically with an inoculating loop and transferred to a test tube containing 5mL of sterile distillate water. Sufficient inoculums were added until the turbidity equaled 0.5 McFarland \(10^8\text{cfu/mL}\) standards (bioMerieux, Marcy d'Etoile, France). The test tube suspension (1mL) was added to 15-20mL of nutrient agar or Sabouraud dextrose agar, before setting aside the seeded agar plate (9cm in diameter) to solidify for 15 min. three disks of Whatman's No. 1 filter paper, 6mm in diameter, were used to screen the antimicrobial activity. Each sterile disk was impregnated with 20µL of extract (corresponding to 100mg of crude extract/mL), Amoxicillin was used as positive control for bacteria and vancomycin for Streptococcus sp. Or miconazole nitrate (30µg/mL) as positive control for fungi, or pure methanol (v/v) (as negative control), before it was placed on the surface of the seeded plates. Then, the plates of bacteria and yeast were incubated at 37ºC overnight (18-24 hours) and 24-48 hours, respectively, where as the fungal plates were incubation at 30ºC for 24-72 hours. At the end of the incubation period the antimicrobial
activity was evaluated by measuring the clear inhibition zones formed around the discs (diameter of inhibition zone plus diameter of the disc). To calculate of percentage of antimicrobial activity, number of effective crude extract or number of tested microorganism were divided on total of selected microorganisms. We compared both part of plant extractions and between Gram-negative and Gram-positive bacteria.

2.4 Determination of the Minimum Inhibited Concentrations (MIC)

The method described by Sahin et al. was adapted with some modification for determined the MIC of the bacterial strains. Only those microorganisms which were found to be sensitive to leaf and stem extracts in disc diffusion assay were further analyzed for the MIC. In brief, the broth cultures activated for 18 hours were adjusted to 10^5 cfu/mL. All the extracts were dissolved in methanol 100mg/mL and were two-fold serially diluted (6 time: 3.12, 6.12, 12.5, 25, 50, and 100 mg/mL) to have a concentration series. For each dilution, 100μL of the extract was transferred into consecutive wells in a sterile flat bottomed 96-well plate. Each well was made to 200μL by adding 95 μL of nutrient broth and 5μL of each amoxicillin, vancomycin and miconazol nitrat standard were use as positive controls for bacteria and Streptococcus sp. and fungi, or pure methanol (v/v) (as negative control), plates were covered with sterile lids and incubated at 37ºC for 24 hours for bacteria and 30ºC for 48 hours for fungi. All the studied were performed in triplicates and at the end of the incubation period, 40μL of ethanolic (0.2mg/mL) ρ-iodonitrotetrazolium violet (INT)(Sigma-Aldrich, Germany) was dispensed in to each well and again incubated under same temperatures for another 30 min. Mic was recorded as the lowest concentration where pink color of INT is changes.

2.5 Determination of Minimum Bactericidal Concentration (MBC)

The MBC of the plant extracts on the clinical bacterial isolates was obtained via a previously described method [6]. One loop full of the inoculum from each of the agar plates was streaked onto the NA plate and incubated at 37ºC for 24 hours. The lowest concentration at which no visible growth was observed was designated as the MBC. The plates were examined for bacterial growth after incubation to determine the extract concentration that induced 99.9% mortality of bacterial isolates.

2.6 Minimum Fungicidal Concentration (MFC)

The samples (10μL) were removed from all wells of the standard MIC plates and were spotted onto rectangular dishes containing Sabouraud dextrose agar. The plates were incubated for 24 to 48 hours at 35ºC. MFC is defined as other concentration of antifungal agent at which the number of colony forming units is zero [6].

3. RESULTS AND DISCUSSION

The result of antimicrobial activity tests of crude extracts are shown in Table 1. It was found that ten crude extracts of leaf and stem of Ocimum tenuiflorum L. at 100mg/mL concentration exhibited various antibacterial and antimycotic and antifungal activity. A total of 11 microorganisms, which comprise of 7 bacteria, 2 yeasts, and 2 fungi, were used in antimicrobial tests. As shown in Table 1, the leaf and stem extracts showed higher effects on Gram-positive than on Gram-negative bacterial strains. Fungal strains were susceptible to methanol and hexane extracts. The degree of susceptibility varied depending on the
pathogenic species and on the crude extract of the plant. *Aspergillus niger* and *Penicillium* sp. showed higher susceptibility to methanol and hexane extracts issued from leaf and stem compared to other pathogens. These findings are in agreement with those obtained previously by Ibrahim and Osman [7], who showed that the antifungal activity of *O. tenuiflorum* L. is higher than its antibacterial activity.

*Listeria monocytogenes* and *Saccharomyces cerevisiae* showed high susceptibility to water and hexane extracts, respectively. However, a very small zone of growth inhibition (less than 9 mm in diameter) was obtained in this experiment.

The crude extracts of all solvents from both parts (leaf and stem) of *O. tenuiflorum* L. failed to control several important pathogens, such as *Escherichia coli*, *Salmonella typhimurium*, and *Candida tropicalis*.

The MFC and MIC values of the methanol leaf extract (3.12mg/mL) were found to be in the same range for *A. niger* and *S. cerevisiae*, and the MFC values of the hexane stem extract were found to be in the same range for these organisms too. The MIC values of the hexane and methanol leaf extracts (were found to be 6.12mg/mL) for *Penicillium sp.* and *L. monocytogenes*. Further investigation was performed to demonstrate the action of the extract on the pathogens at different concentrations (Table 2). The growth of pathogens decreased with increasing extract concentrations, and the growth of pathogens was completely inhibited at their corresponding MIC values.

MIC determination is important in selecting the appropriate and effective concentration of a therapeutic substance. The results from the inhibition zone tests showed that all the extracts from leaves and stems did not affect several important pathogens, such as *E. coli*, *S. typhimurium*, *Staphylococcus aureus*, and *C. tropicalis*.

Medicinal plants are raw sources of novel drug compounds, and the use of plant-derived medicines has largely contributed to the promotion of human health [8]. Traditional healers use water as solvent, but alcoholic extracts of medicinal plants show potent effects [9]. Active components may exhibit better solubility in organic solvents than in non-organic solvents [10]. These organic compounds extracted by organic solvents include alkaloids, tannins, and sterols. Extract obtained using such solvents might show antimicrobial activity [11]. It has been reported that bioactive compounds isolated from plant extracts had inhibitory effects on pathogenic strains [12].

According to Sharma et al. [10], the extracts of *O. tenuiflorum* were more or less active against most of the tested pathogenic strains. The extracts showed higher activity against Gram-positive than Gram-negative bacterial pathogens. Our experimental results prove the validity of these findings. The presence of active sites and the number of hydroxyl groups in the phenol components may have contributed to the increased toxicity of the extracts against the microorganisms [13]. The antimicrobial properties of tannins, complexity of polysaccharides, and the presence of enzymes and cell envelope transport proteins may be related to the ability of *O. tenuiflorum* extracts to inactivate microbial adhesion and to modify the morphology of microorganisms. It has been reported that bioactive compounds isolated from plant extracts have inhibitory effects on pathogenic strains [14-18].
Table 1. Antimicrobial activities of the various extracts of leaf and stems from Ocimum teuiflorum L.

| Type of Extraction/ Pathogenic microorganism | Leaves Extraction | Stem Extraction |
|---------------------------------------------|-------------------|-----------------|
|                                             | WA I | ME I | EA I | CH I | HE I | WA II | ME II | EA II | CH II | HE II |
| **Gram-negative:**                          |      |      |      |      |      |       |       |       |       |       |
| Escherichia coli                            |      |      |      |      |      |       |       |       |       |       |
| Salmonella typhimurium                      |      |      |      |      |      |       |       |       |       |       |
| Klebsiella pneumoniae                       |      |      |      |      |      |       |       |       |       |       |
| **Gram-positive:**                          |      |      |      |      |      |       |       |       |       |       |
| Bacillus cereus                             |      |      |      |      |      |       |       |       |       |       |
| B. subtilis                                 |      |      |      |      |      |       |       |       |       |       |
| Staphylococcus aureus                       |      |      |      |      |      |       |       |       |       |       |
| Listeria monocytogenes                      |      |      |      |      |      |       |       |       |       |       |
| **Yeast**                                   |      |      |      |      |      |       |       |       |       |       |
| Candida tropicalis                          |      |      |      |      |      |       |       |       |       |       |
| Saccharomyces cerevisiae                    |      |      |      |      |      |       |       |       |       |       |
| **Fungi**                                   |      |      |      |      |      |       |       |       |       |       |
| Aspergillus niger                           |      |      |      |      |      |       |       |       |       |       |
| Penicillium sp.                             |      |      |      |      |      |       |       |       |       |       |

Antimicrobial activity based on the diameter of inhibition zone (mm) follow this skim: +++ ≥15 mm, ++ ≥10-14 mm, + ≥9 ≤ mm. Non Detect

*Extraction showed reduced of inoculums but not hypha; WA I = water extract of leaf, ME I = methanol extract of leaf, EA I = Ethyl acetate extract of leaf, CH I = chloroform extract of leaf, HE I = hexane extract of leaf. WA II = water extract of stems, ME II = methanol extract of stems, EA II = Ethyl acetate extract of stems, CH II = chloroform extract of stems, HE II = hexane extract of stems
### Table 2. In vitro activity of pathogenic microbial for MFC, MIC and MBC (mg/mL)

| Crude plant extract | Bacteria                  | MIC/MFC value (mg/mL) | MBC value (mg/mL) |
|---------------------|---------------------------|-----------------------|-------------------|
| WAI                 | *Bacillus cereus*         | 25                    | 12.5              |
| MEI                 | *Bacillus cereus*         | 25                    | 50                |
| MEI                 | *Klebsiella pneumonia*    | 100                   | 3.12              |
| MEI                 | *Listeria monocytogenes*  | 25                    | 3.12              |
| MEI                 | *Saccharomyces cerevisiae*| 3.12                  | 3.12              |
| MEI                 | *Aspergillus niger*       | 3.12                  | -                 |
| EAI                 | *Bacillus cereus*         | 50                    | 3.12              |
| EAI                 | *Klebsiella pneumonia*    | 100                   | 100               |
| EAI                 | *Listeria monocytogenes*  | 6.12                  | 3.12              |
| CHI                 | *Bacillus cereus*         | 100                   | 3.12              |
| CHI                 | *Klebsiella pneumonia*    | 50                    | 100               |
| CHI                 | *Listeria monocytogenes*  | 50                    | 3.12              |
| HEI                 | *Saccharomyces cerevisiae*| 25                    | 6.5               |
| HEI                 | *Penicillium*             | 6.12                  | -                 |
| WAII                | *Klebsiella pneumonia*    | 100                   | 100               |
| WAII                | *Listeria monocytogenes*  | 100                   | 3.12              |
| MEII                | *Bacillus cereus*         | 12.5                  | 3.12              |
| MEII                | *Klebsiella pneumonia*    | 100                   | 6.5               |
| MEII                | *Listeria monocytogenes*  | 50                    | 3.12              |
| MEII                | *Saccharomyces cerevisiae*| 3.12                  | 3.12              |
| EAII                | *Bacillus cereus*         | 100                   | 6.12              |
| EAII                | *Klebsiella pneumonia*    | 100                   | 100               |
| EAII                | *Listeria monocytogenes*  | 25                    | 3.12              |
| HEII                | *Saccharomyces cerevisiae*| 3.12                  | 3.12              |
| HEII                | *Penicillium sp.*         | 3.12                  |                   |

WA I = water extract of leaf, ME I = methanol extract of leaf, EA I = Ethyl acetate extract of leaf, CH I = chloroform extract of leaf, HE I = hexane extract of leaf. WA II = water extract of stem, ME II = methanol extract of stem, EA II = Ethyl acetate extract of I stem, CH II = chloroform extract of stem, HE II = hexane extract of stem.
In vitro test results of the antimicrobial properties of *O. basilicum* showed that the ethanol, methanol, and hexane extracts failed to exhibit antifungal activities but exhibited anti-candidal and antibacterial effects. The hexane and methanol extracts of *O. basilicum* inhibited three strains of *C. albicans* [19].

The results of the present study support the use of traditional medicines with antimicrobial properties to produce new antibacterial drugs for the treatment of infectious diseases caused by different pathogens.

Regarding to some previous studies, methanol extract yielded higher antimicrobial activity than n-hexane and ethyl acetate [11]. Our results also confirmed their results due to high antimicrobial activity of methanol extract.

The present investigation confirms that there are antifungal properties in the crud extract of *Ocimum tenuiflorum* leaf and stem [12]. However, it is important to point out that further process of the crude to obtain pure compound(s) and/or active subfraction(s) are required to improved its antifungal activity.

4. CONCLUSION

In conclusion, *Ocimum tenuiflorum* could be suggested as a natural source of antimicrobial agent, especially its antifungal activity. However, the safety and toxicity issues should be addressed and further study on purification of the active compound(s) and/or fractionation of the extracts required.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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