Evaluation of antibacterial properties of some essential oils from Vietnam

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Abstract. Natural products have attracted attention from scientists because of their potential to replace antibiotics in the near future. Thanks to Vietnam’s richness in biodiversity, this study aimed at evaluating antibacterial activity of eleven essential oils from various plants. These essential oils were collected from various plants across Vietnam such as corn mint, clove, clove basil, eugenol, tea tree, terpinen-4-ol, kaffir lime, Mexican mint, rosemary, Homalomena occulta, and betel. By chemical reaction and fractional vacuum distillation method, eugenol and terpinen-4-ol was isolated from clove basil and tea tree oils with the content reaching up to 97.0% and 95.8%, respectively. The antibacterial activity against Staphylococcus aureus, Bacillus subtilis, Escherichia coli of essential oils was determined by disk diffusion and broth dilution method. As a result, Mexican mint oil and eugenol exhibited high antibacterial properties with low MICs and MBCs (62.5 to 125 µL/L), whereas other essential oils (clove, clove basil, betel, tea tree, terpinen-4-ol) had higher MIC and MBC values ranged from 125 to 500 µL/L.

1. Introduction

From ancient times to the modern era, mankind has been able to adapt and thrive with precious help from plants. Of all six kingdoms on earth, plants, which belong to Plantae, can produce secondary metabolites. These compounds help plants survive in their environment by acting as attractants of pollinators. Chemical defenses against micro-organisms, insects, and predators [1-2] take advantage of this secret weapon from plants; human collects these phytochemicals in the form of essential oils through hydro-distillation. Essential oils possess various biological activities from antimicrobial, antibiotic, insecticidal properties to highly important pharmaceutical activities [3]. They are a major source of antibiotics, perfumes, cosmetics as well as food additives. Fortunately, Vietnam is a wonderful place to cultivate various medicinal plants such as Ocimum gratissimum L, Syzygium aromaticum, Rosmarinus officinalis, M. arvensis, Citrus hystrix, Piper betle, Homalomena occulta (Lour.) Schott, Melaleuca...
*Alternifolia, Coleus amboinicus*. They are well-known for their antibacterial and anti-inflammatory properties and are traditionally used to treat oral diseases [4].

Oral diseases are one of the most harmful diseases globally. According to the Global Burden of Disease (GBD) 2010 study, around 3.9 billion people worldwide live with dental conditions, such as dental caries (tooth decay), periodontal (gum) disease, and oral cancer. Untreated caries in permanent teeth were the most common (35% for all ages combined), whereas severe periodontitis and untreated caries in deciduous teeth were the 6th and 10th most prevalent conditions, 11% and 9%. Severe tooth loss was the 36th most prevalent condition, with a global estimate of 2% [5]. These oral diseases have substantially reduced the life quality of patients due to chronic pain and economic burden. Oral disease affects different life stages of humans from children, adults to older people. Due to a lack of awareness and healthcare service, a large group of people in developing countries have suffered from these dental conditions. Particularly in Vietnam, there is a high number of patients who experience dental caries [6].

Therefore, essential oils extracted from plants are a valuable source to solve this problem. These active ingredients can be added to oral caring products like mouthwashes and toothpaste to treat oral diseases. The purpose of the research is to determine the antibacterial properties of some essential oils in Vietnam and to orient their application to mouthwash products.

2. Materials and Methods

2.1 Source of Essential Oil

Essential oils were collected from various plants like *Ocimum gratissimum L.*, *Syzygium aromaticum*, *Rosmarinus officinalis*, *M. arvensis*, *Citrus hystrix*, *Piper betle*, *Homalomena occulta* (Lour.) Schott, *Melaleuca alternifolia*, *Coleus amboinicus*. These plants were cultivated in Vietnam.

2.2 Eugenol and terpinen-4-ol isolation

Eugenol isolation procedure: 50 g of clove basil oil was added into the mixture of saturated KOH and alcohol solution (pH = 14). The volume ratio of oil and KOH was 1:1. The reaction was carried out at 45°C and stirred for 30 minutes. After that, the reaction mixture was then inserted separating funnel and allowed to stand for a while to form two layers. The bottom layer was K-eugenolat (aqueous layer) and the top was the other compounds. K-eugenolat was then acidified with HCl (36.5%) until pH = 4. The eugenol was formed at the bottom layer. The oil was then purified by hydro-distillation using the Clevenger-type apparatus. The yield of eugenol was 69.24 %. The percentage of eugenol was analyzed by GC/MS: from 81.1% (from clove basil oil) up to 97.0%.

To isolate terpinen-4-ol from tea tree oil, a vacuum fractional distillation method was applied. This method was suitable for this experiment as it was used for separating elements which had different boiling points. Furthermore, the system was operated at vacuum condition, which allowed the terpinen-4-ol to evaporate at low temperature and prevent it from decomposition. The system was operated at 60 mmHg and 115 °C (top pressure) with 300 mm Hempel column. With 100 mL tea tree oil (39.4% terpinen-4-ol), 31.1 g terpinen-4-ol was obtained. GC-MS analysis revealed the percentage of purified terpinen-4-ol up to 95.8%.

2.3 GC-MS analysis

The analysis of essential oils was performed using a GC-MS G1530A-serial US00002778. An electron ionization system with an ionization energy of 70 eV was used. The column was maintained at 40 °C for 1 min and then programmed to 280 °C at 10 °C/min and held for 3 min. The injector was 250 °C. The carrier gas was helium introduced at a rate of 1.0 ml/min. A volume of 0.1 μl of essential oil was injected manually.

2.4 Tested micro-organism and culture

The essential oils were individually tested against a panel of micro-organisms, including Gram-positive *Bacillus subtilis*, *Staphylococcus aureus*, and Gram-negative *Escherichia coli*. The reference strains of
bacteria were maintained on nutrient slants at 4 °C. All the strains were activated in nutrient agar at 37 °C for 24 h.

The products were weighed in each experiment to calculate the yield and underwent chromatographic analysis to investigate the chemical composition and the 1,8-cineole content. The following formulas calculated the yield of each fraction and the overall 1,8-cineole recovery:

\[\text{Yield} = \frac{\text{Weight of fraction}}{\text{Weight of total}}\]
\[\text{1,8-cineole recovery} = \frac{\text{Weight of 1,8-cineole}}{\text{Weight of total}}\]

2.5 Disk-diffusion assay

The essential oils were dissolved in DMSO (dimethylsulfoxide) (the ratio of DMSO: essential oil = 4:1). The antibacterial tests were then carried out by disk diffusion method [7] with bacterial density = 108 CFU/mL. The discs (6 mm in diameter) were impregnated with 10 μL of essential oil placed on the inoculated agar. Negative controls were prepared using DMSO. Gentamicin diluted with DMSO (10 μg per disc) was used as the positive reference standard to determine the sensitivity of bacterial species tested. The inoculated plates were incubated at 37 °C for 16-14 hours. The antibacterial activity was evaluated by measuring the zone of inhibition.

2.6 MIC and MBC determination.

Based on the results of the antimicrobial activity of essential oils in the disk-diffusion assay, different concentrations of essential oils were investigated to determine MIC and MBC values by broth dilution method [8]. Essential oils with high bacterial activity at susceptibility test were collected to evaluate the MIC and MBC values. The bacterial strains B. subtilis, E. coli, S. aureus grew in nutrient broth, and they were incubated at 37°C for 48 hours. The bacterial density was adjusted at 5x105 CFU/mL. Each essential oil was diluted with tween 80 (the ratio of tween 80 and essential oil = 2:1) and water at an initial concentration of 1000 µL/L. Then the solution was diluted with nutrient broth at the concentration of 500, 250, 125, 62.5 (µL/L). Bacterial culture (0.1 mL) was added to each tube containing different concentrations of essential oils. The tube was then incubated at 37 °C for 18 - 24 hours. After that, the turbidity of each tube was observed to recognize the MIC values. The dish spread with 50 µL of bacterial culture in each tube was incubated at 37 °C for 24 hours. After that, the dish without bacterial growth was considered to be MBC value.

3. Results and discussion

3.1. Chemical constituents of nine essential oils

A total of nine essential oils was introduced in this part. They were extracted from various plants like corn mint, kaffir lime, clove, clove basil, Mexican mint, rosemary, tea tree, Homalomena occulta, and betel. The major chemical constituents of these essential oils were analyzed by GC-MS (Table 1).

| Essential oils     | Major components        | Content (%) |
|--------------------|-------------------------|-------------|
| S. aromaticum      | Eugenol                 | 70.7        |
|                    | Aceteugenol             | 17.8        |
|                    | Caryophyllene           | 10.4        |
| O. gratissimum     | Eugenol                 | 81.1        |
| M. alternifolia    | Terpinen-4-o1           | 39.4        |
|                    | γ-terpinene             | 25.8        |
|                    | α-terpinene             | 15.1        |
| C. amboinicus      | Carvacrol               | 51.6        |
| Essential Oil | Component                        | Percentage |
|--------------|----------------------------------|------------|
| 3-carene     | p-methyl cumene                  | 15.7       |
|              | Caryophyllene                     | 11.3       |
| C. hystrix   | β-pinene                         | 33.5       |
|              | Limonene                         | 25.6       |
|              | Sabinene                         | 15.5       |
| P. betle     | Chavibetol                       | 64.4       |
|              | Limonene                         | 11.2       |
| M. arvensis  | Menthol                          | 72.6       |
| H. occulta   | Linalool                         | 65.9       |
| S. rosmarinus| 1R-a-pinene                      | 23.6       |
|              | Eucalyptol                       | 14.8       |
|              | (-)-Verbenone                    | 13.5       |

3.2. Disk diffusion assay.

Antibacterial susceptibility of essential oils against B. subtilis, E. coli, and S. aureus was presented in figure 1. Of all eleven essential oils extracted, it was clear that C. ambionicus, eugenol and O. gratissimum oil (inhibition diameters ranged from 15–20.5 mm), inhibited significantly the growth of all the micro-organisms tested when compared with positive control (Gentamicin). M. alternifolia, S. aromaticum and P. betle oil showed moderate antibacterial activity. On the other hand, S. rosmarinus oil and H. occulta, M. arvensis and C. hystrix oil were weakly effective against these bacterial strains. Therefore, these 4 essential oils were eliminated in further assays.

Moreover, eugenol and terpinen-4-ol, isolated from O. gratissimum and M. alternifolia oil respectively, exhibited higher zones of inhibition in their purified forms. While O. gratissimum oil (eugenol content: 81.1%) had inhibition zones ranged from 10.5-14.0 mm, eugenol (97%) had slightly higher values from 15.0 to 16.3 mm. Similar to eugenol, zone diameters of terpinen-4-ol (87%) were around 1.2 times as many as M. alternifolia oil (terpinen-4-ol content: 39.4%). These data revealed that antibacterial properties of clove basil and tea tree depended on compounds with significant structures like eugenol and terpinen-4-ol.

![Figure 1](image_url)

Figure 1. Inhibition zone diameters of essential oils against B. subtilis, E. coli, S. aureus
These plant extracts from the steam distillation process show antibacterial activity at a different level, which results from the chemical structures presented in each essential oil. As defense mechanisms against predators, plants have the ability to synthesize aromatic compounds that fortunately serve as natural antibiotics to humans. These secondary metabolites contain mostly phenols or their oxygen-substituted derivatives. GC-MS analysis revealed the major constituents in C. amboinicus to be dominated by phenolic compounds, specifically carvacrol with 51.58%, which alters the cell wall or disrupt the cell membrane of bacteria [9]. Thus, its structure is probably responsible for the strong growth inhibitory effect observed. P. betle oil also contained 64.4% chavibetol, another phenolic compound. Likewise, eugenol, a major component in O. gratissimum (81.1%) and S. aromaticum (70.67%) has its hydroxyl group on phenol ring, which is thought to get bind to proteins, preventing enzyme action [10]. Beside phenolic compounds, monoterpenoid like terpinen-4-ol found in M. alternifolia (39.4%) contributed to antibacterial activity of this essential oil. However, the selected bacterial strains are almost resistant to S. rosmarinus, H. occulta, M. arvensis and C. hystrix oil as these four essential oils have a few to no phenols.

Even though Gram-positive and Gram-negative bacteria are different in their structures, there was no obvious difference between them in the susceptibility test. This result was in agreement with the study by Ouattara [11]. One study found that Gram-positive bacteria to be more sensitive to essential oils than Gram-negative because of the relatively impermeable outer membrane surrounding Gram-negative bacteria [12]. However, another study suggested that there was a time delay in the Gram-negative growth. Therefore, over time, these essential oils would have the same effect on both Gram-positive and Gram-negative bacteria [13].

3.3. MIC and MBC values

After screening for the presence of antibacterial activity, the MICs of selected essential oils were determined by the broth macro-dilution method. Not only presenting qualitative data like the disk diffusion technique, this method also shows quantitative values of antibacterial activity. These MIC values can be serves as a guideline for adjusting an optimal concentration of drugs to treat patients, which reduces the spread of antibiotic resistance in the community.

In order to have accurate results, surfactants were introduced to lower the surface tension between essential oils and broth; thus, allow better solubility of apolar particles in broth (polar solution). Despite the fact that DMSO is more preferable to other solvents in scientific literature, Tween 80 is applied in this experiment due to its low toxicity and safety regarding food and pharmaceutical applications [14]. Furthermore, DMSO at a concentration of 4% and beyond was reported to decrease the growth of bacteria, which unfortunately causes errors in experiments [15].
Figure 2. MIC values of essential oils against B. subtilis, E. coli, S. aureus

As can be seen from figure 2, the MIC range of those essential oils varied from 62.5 μL/L to 500 μL/L. Generally, Mexican mint and eugenol showed low MICs (62.5-125 μL/L). The MIC of Mexican mint (125 μL/L) against E. coli was supported by Aguiar and partners as their result shared almost the same value, which was 128 μL/L [9].

Similarly, the MIC values of eugenol against all bacterial strains were in line with Atanasova-Pancevska’s research (195μL/L) [16]. Followed by these two strong antibacterial properties, clove, clove basil, and terpinen-4-ol had higher MIC values from 125 to 500 μL/L. Both clove and clove basil had eugenol as a major constituent with 70.7% and 81.1% content, respectively. Lots of studies from Murbach Teles Andrade [17], Fu [18], and Adebolu [19] had MICs higher than these three examined essential oils. This difference can be explained by the compositions of essential oils that depend heavily on the geographical region of plant cultivation, the phenological stage of the plant and its chemotype, and the extraction of the oils.

Figure 3. MBC values of essential oils against B. subtilis, E. coli, S. aureus.
Based on the disk diffusion method results, it was clear that the bigger the zones of inhibition were, the lower the MICs would be and the higher the antibacterial properties of EOs would exhibit. Besides, MIC values were approximately equal to or as twice as MBCs (figure 3). Therefore, Mexican mint, eugenol, as well as terpinen-4-ol could be used as active ingredients in mouthwash due to their effective antibacterial activities.

4. Conclusions
Of all the essential oils tested, Mexican mint oil, eugenol, and terpinen-4-ol strongly inhibited the growth of B. subtilis, E. coli, and S. aureus. Their application into the mouthwash product will be full of promise as it showed great antibacterial properties with a low concentration of essential oils and good sensory quality.

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