Chemical Composition and Antibacterial Activity of Orange (Citrus sinensis) Essential Oils Obtained by Hydrodistillation and Solvent free Microwave Extraction

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Abstract: Essential oils (EOs) have gained much media attention in recent years because of their advantages in various fields, including food preservation, pharmaceuticals, herbal medicine, and natural therapies. Phenolic components which exhibit antimicrobial activity, along with some healthy substances, are present abundantly in essential oils, hence their use to prevent bacterial growth. Oranges are amongst the world's most popular fruits. Orange (Citrus sinensis) is widely recognized for its nutritious and medicinal properties. Since time immemorial, most parts of the orange plant from fruits, peels, flowers, leaves and juice are used as traditional medicine. Hydrodistillation (HD) and solvent free microwave extraction (SFME) were used to extract essential oils from orange fruit peels. The total volatile compound yield is obtained from 98-100% by HD and SFME. Gas chromatography-mass spectrometry (GC-MS) analysis revealed that the oils obtained from both method contained limonene (98.238% and 98.415), β-myrcene (1.169% and 1.172%) and α-pinene (0.548% and 0.413%). A small amount of sabinene (0.071%) and β-pinene (0.0032%) were only found in SFME-produced orange essential oil. The essential oils obtained from the two extraction methods were able to inhibit against Bacillus cereus.

1. Introduction
In recent years, traditional medicines have recognized the importance of plant-based products [1-5]. Plant essential oils are commonly used in daily life, especially in the cosmetics industry, disinfectant and insecticide components [6-8]. Applications of plant essential oils are diverse, ranging from manufacture of pharmaceuticals, ingredient in alternative medicine to food preservatives [9-11].

Orange oil is commonly used in dairy, medicinal and cosmetic products owing to its taste and fragrances. It is a complex natural mixture which consists of over 100 compounds. Previous study had reported various method used to extract EOs from orange peel (Citrus sinensis L.) by using three different methods, i.e. traditional hydrodistillation (HD), cold-pressing (CP) and solvent-free microwave-assisted extraction (SFME) [12]. However, different methods could change the properties of EOs. In addition, the extracted orange oils were shown to inhibit Aspergillus niger, Aspergillus flavus, Penicillium verrucosum and Penicillium chrysogenum at < 1% of concentration. The research of R. Dabbah et al. (1970) on various citrus oils and their derivatives such as orange and lime terpenessless oil
found that d-limonene, terpineol, and geraniol present in the oil can inhibit S. senftenberg, E. coli, Pseudomonas spp. and especially Staphylococcus aureus [13]. Orange oil and terpineol helped increasing pasteurized milk shelf-life to more than 56 days when processed at 4°C. In Vietnam, although Citrus sinensis is a common crop, it still has no evidence of biological activities.

To date, an alternate approach using microwave energy termed as SFME has been established for natural product extraction [14-15]. SFME appears especially appealing in isolating essential oil from rosemary [16]. Some advantages of this method over HD include the extraction speed at 100 °C for the first oil droplet, high oil yield, low energy requirements, and high purity [17]. Therefore, the present study aimed to compare the antibacterial activity of C. sinensis essential oils extracted from HD and SFME.

2. Materials and methods

2.1. Plant material

500 g of C. sinensis fruits were collected between January to February, 2019 in Tien Giang province (10 ° 25 13.04 N, 106 ° 17 48.64 E), Vietnam. The chosen oranges were freshly ripe and had smooth skin. After washing thoroughly with water, the peel was removed and stored in a cooler. The total amount of peels was divided by half to use for two extraction methods, which were HD and SFME.

2.2. Solvent free microwave extraction (SFME)

SFME was performed with a Milestone DryDIST (2004) method (Figure 1). The reactor is composed of a twin magnetron (800 W, 2450 MHz) coupled with a maximum power delivered in 5W increments of 500W. A revolving microwave diffuser enables consistent propagation of microwaves in the plasma-coated PTFE cavity. An external IR-sensor monitored the temperature. Constant temperature and water temperatures were maintained by the reflux of concentrated water, which was accomplished at 5°C using a rotating cooling system. By including water or any chemical, 250 g of orange peels were placed into the reactor. The orange essential oil was collected after 40 min of SFME extraction, dehydrated by \( \text{Na}_2\text{SO}_4 \) and stored in a jar.

2.3. Hydrodistillation (HD)

Hydrodistillation of orange peels (250 g) was performed in a Clevenger all-glass apparatus, as defined in the British Pharmacopeia (1980) (Figure 1). Heating mantle was heated to 50 °C with peel:water ratio of 4:1 in 3 h. After the process completed, the orange peel essential oil was collected, dehydrated by \( \text{Na}_2\text{SO}_4 \) and stored in a jar. The critical oil was subsequently extracted and analyzed.

![Figure 1. Orange essential oil extraction process by HD and SFME](image-url)
2.4. Extraction procedures
The collected yields of essential oils were determined as follows:

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\text{Yield (\%) = \frac{\text{Weight of extract recovered}}{\text{Weight of fresh citrus peel}} \times 100}
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2.5. GC–MS analyses
The structure of essential oils in both production methods was studied using GC-MS analysis. 25 μL of n-hexane basic oil in 1.0 mL. Equipment Name: GC Agilent 6890N, MS 5973 inert with column HP5-MS, pressure of column head 9.3 psi. The conditions to maintain GC-MS device included: carrier gas He; flow rate 1.0 mL/min; break 1:100; injection volume 1.0 μL; injection temperature 250 °C; oven temperature development involved an initial keep at 50 °C for 2 min and an improvement to 80 °C at 2 °C/min and a continuous rise to 150 °C at 5 °C/min to 200 °C at 10 °C/min and an improvement to 300 °C at 20 °C/min.

2.6. Antimicrobial activity
Six bacterial strains used in this study consisted of Staphylococcus aureus NRRL B-313, Bacillus subtilis NRRL B-354, Pseudomonas aeruginosa NRRL B-14781, E. coli NRRL B-409, Clostridium perfringens and Salmonella typhi YS1646. Antibacterial activity of essential oil samples was assayed by the agar-well diffusion method. Three milliliters of liquid cultures with aeration (150 rpm) were developed overnight at 37 °C on LB agar plates. Wells (5 mm) were made in the agar surface using a cork borer and loaded with 20 μl of orange peel essential oil. Plates were incubated overnight at 37 °C. Amoxicillin (100 μg/mL) was used as positive indicators, with distilled water being used as negative controls. The diameter of inhibition zones around the wells which indicate antibacterial activity were measured. Both experiments were performed in triplicate.

3. Results and discussion
3.1. Yields and chemical composition of the essential oil
The composition, yield and retention indices of the volatile compounds of C. sinensis essential oil obtained from HD and SFME were listed in Table 1. The yield from SFME and HD approaches was insignificant (0.39 and 0.31%, respectively), although the variation is linked to the time of extraction. The yield obtained from 40 min of SFME was insignificantly different from that obtained from 3 h of HD. To obtain the temperature for extraction and the first drop of essential oil, the heating process is very important, requiring only 2-3 min of heating with SFME compared to 30-40 min in HD. Factors that have caused such difference have been reported by previous studies [18] [19].

In the hydrodistilled oil, three compounds were identified amounting to 100% of total content. Monoterpenoids such as limonene, β-pinene, β-myrcene, sabinene, and α-pinene were abundant in this oil. In the oil obtained from SFME, five compounds of the oil were detected, comprising 99.9% of the total oil composition. It is possibly due to the dimmed thermal and hydrolytic effects of SFME compared to hydrodistillation which requires a significant amount of water and consumes time and energy [20]. Water involves in several reactions that progress by intermediate carbocation. Microwave irradiation has significantly improved the extraction cycle even without major improvements in the volatile oil composition, as reported by Figueredo (2012) [21]. Moreover, the critical oil is derived using HD and SFME approaches comprising hydrocarbon monoterpenic components such as limonene (98.238%; 98.415%), sabinene (0.071%), β-myrcene (1.169%; 1.172%), β-pinene (0.032%) and α-pinene (0.548%; 0.413%), respectively.

The quality and aroma of pure essential oils cannot be the same despite being produced in the same place, which is caused by many factors [22]. The evaporation component in essential oils can be affected by weather, environmental factors, the extraction process, and raw materials can be parts of plants (flowers, leaves and shells) [23]. Of course, the effect is that the scent of these 3 types of extracted ingredients is different, it is separated into 3 completely different essential oils. The esters in essential
oils are often susceptible to hydration, which gives acid and alcohols when heated for a long time with water [24]. Therefore, to limit this phenomenon, steam distillation must be done for a short time as possible. In this study, essential oils contained almost no sesquiterpene, alcohol, phenol and ethylphenol, and aldehydes. Limonene is the main ingredient that makes up the aroma of orange peel oil [25]. We can see the presence of limonene in the composition of many aromas, fragrances, fragrant soaps, with the main scent of orange [26]. Besides the aroma value, limonene has long been found to have remarkable biological activities [26]. Many studies indicate that limonene is the most significant ingredient that plays an essential role in orange peel oil consistency, and demonstrates its antimicrobial, antioxidant and toxic agents. [27].

According to Gachkar et al .(2007), variations in essential oil content of orange may be due to climatic influences on plants developing in various environments [28]. Rodríguez, Ysambert, & Ferrer, 2003 showed in the report that the quantitative concentration of overall hydrocarbon monoterpenes in the volatile component of Venezuelan essential oil is 96.83%, primarily attributed to limonene (94.55%), myrcene (1.22%) and α-pinene (0.51 %) [29]. Hosni K, Zahed N, Chrif R, et al stated earlier on the content of Limonene (96.0-97.3%) as the key component of Tunisian orange essential oil. [30]. Similarly, in another study conducted on essential oil extracted from oranges collected from Italy, limonene and myrcene were found as major ingredients [31].

Table 1. Constituents detected in orange essential oils obtained by hydrodistillation and by solvent free microwave extraction.

| No | R.T  | Compounds | HD (%) | SFME (%) |
|----|------|-----------|--------|----------|
| 1  | 7.272| α-pinene  | 0.548  | 0.413    |
| 2  | 8.997| Sabinene  | -      | 0.071    |
| 3  | 9.949| β-myrcene | 1.169  | 1.172    |
| 4  | 11.925| Limonene | 98.283 | 98.415   |
| 5  | 9.081| β-pinene  | -      | 0.032    |

3.2. Antibacterial activity

Generally, orange essential oils have only been shown to inhibit against Bacillus cereus, with 6 mm of inhibition zone diameter when using essential oil extracted from HD and 4 mm when applying the essential oil extracted from SFME (Figure 2).

The antibacterial activity against a bacterial strain is expressed by the diameter surrounding the well in the agar plate containing that bacteria. The highest antibacterial activity of orange essential oil is most evident in Bacillus cereus strains of essential oils from HD and SFWE methods. However, such antibacterial effect of orange essential oil was not observed in the remaining Gram-positive and Gram-negative bacteria. Previous studies have indicated that many bioactive compounds commonly found in different plant species including thymol, citral, eugenol, limonene, pinene and linalool exhibited strong inhibitory activity against these microorganisms by interrupting bacterial integrity [32]. In addition, another mechanism through which essential oils puncture cell membrane is their hydrophobicity, which causes strong interaction with lipid components. This in turn ruptures cell structure and in turn cause leakage of component within bacteria.
Figure 2. Inhibition of orange essential oil by the agar well diffusion assay. Wells contained 20µl essential oil obtained by microwave extraction (well C1), 20µl essential oil obtained by hydrodistillation (well C2), add 20µl Amoxicillin (100 µg/ml) (well +) and 20µl sterile water (well H2O).

4. Conclusion
Since the increasing use of natural products as food additives, their bacterial inhibition capability has been of considerable concern in the food, pharmaceutical and cosmetics industries. This research showed that the oil collected from SFEM was more effective against tested microorganisms than HD. The oils contained a significant amount of limonene (98.238%, 98.415%), sabinene (0.071%), β-myrcene (1.169%), β-pinene (0.0032%), and α-pinene (0.548%, 0.413%). Inhibitory properties of
essential oils against gram-negative bacteria is generally weaker than against gram-positive bacteria, which may be attributed to variations in certain bacterial groups’ cell membranes. Overall, our findings showed that the orange peel oil obtained from SFME has a higher antibacterial effect than HD.

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