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Antimicrobial and antioxidant activities of essential oils extracted from leaves of Vinh orange, Dao lime and Thanh Tra pomelo in Vietnam

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Abstract
Essential oils (EOs) are mixtures containing complex biologically active substances, and they are being used as flavoring agents, as well as preservatives for a number of commercial products. Antimicrobial and antioxidant activities are two important characteristics when determining the potential of essential oils. In this study, essential oils from leaves of three citrus varieties including Dao Lime, Vinh orange and Thanh Tra pomelo were extracted using hydro-distillation method. For antimicrobial capacity, the EOs of leaf of Vinh orange showed the strongest capacity against *S. aureus*, with an inhibition zone of 22.25 mm and the lowest capacity against *F. flavus* with the inhibition zone of 11.08. In contrast, Dao lime leaf EOs showed the lowest antimicrobial capacity, with an inhibition zone of 12.67 mm against *S.aureus* and the strongest capacity against *F. flavus* with the inhibition zone of 11.4 mm. Against *P. aeruginosa, S. typhi, B. cereus and A. flavus,* Vinh orange leaf EOs also showed the highest result, with the inhibition zone corresponding to 21.42 mm, 12.58 mm, 18.08 mm and 15 mm, respectively. Thanh Tra Pomelo leaf EOs showed the lowest result against *P. aeruginosa* with an inhibition zone of 14.67 mm and *S. typhi* with the inhibition zone of 10.17 mm. Otherwise, Dao lime leaf EOs showed the weakest capacity against *B. cereus and A. flavus,* corresponding to 11.17 mm and 10.2 mm of diameter inhibition zone. The minimal inhibition capacity (MIC) was a range of 10.5-84 mg/ml for orange leaf EOs, 84-168 mg/ml for lime leaf EOs and 21 – 168 mg/ml for pomelo leaf EOs. For antioxidant capacity, orange leaf EOs also showed the highest, similar to antimicrobial activities. The results of this study showed that essential oils of citrus leaves could be widely used as flavoring and preservatives in food, cosmetic and pharmaceutical industries.

Keywords: essential oil, citrus leaves, hydro-distillation, antimicrobial activities, antioxidant activities

1. Introduction

Citrus essential oils are mixtures of more than a hundred compounds which can be divided into three fractions: terpene hydrocarbons, oxygenated compounds and non-volatile compounds, while terpene hydrocarbons are mainly monoterpene hydrocarbons. The citrus essential oils (EOs) contain many chemical compositions which are limonene, linalool, a-terpineol, a-pinene, b-pinene and myrcene. In details, for Vietnamese orange, its essential oil consists of limonene (94.43%), linalool (0.18%), a-terpineol (0.14%), a-pinene (0.52%), b-pinene (0.02%) and myrcene (2,03%). On the other hand, Vietnamese lime and pomelo have different concentrations of those chemical compositions: limonene (50.64% and 1.08%), linalool (0.24% and less than 0.01%), a-terpineol (0.02% and 0.25%), a-pinene (1.98% and 1.08%), b-pinene (21.89% and 0.82%), and myrcene (1.15% and 1.82%) (Lan-Phi *et al*., 2010; Sharma and Tripathi, 2008) [8, 13].

Hydro-distillation involves the use of water or steam to recover volatile compounds from plant materials. There are three types of distillation: water distillation, water/steam distillation and steam distillation. In water distillation, plant materials are submerged in a large water chamber, then, the chamber is heated. Through an evaporation process, an essential oil is collected. The resulting steam also carries volatile oils and go through a condenser. After cooling, the essential oils are separated from water and returned to its former state. In the water/steam method, the grill is provided for plant materials to be placed distributively on above hot water. Steam then passes through samples. Lastly, in steam distillation, instead of placing water in the tank, steam is directed from the outside source to burst the sacs containing oils molecules. From this stage, the process of condensation and separation is standard (Chemat, 2010) [3]. The hydro-distillation method is one of the simplest methods with high yield extraction and low-cost requirement. The main advantages of this method are the less steam is used, the shorter processing time and a higher oil yield. However, controlling temperature is still a big problem during extracting, since, most volatile molecules are often loss (Chemat, 2010) [3]; therefore, quality of essential oils are deteriorated. In present time, improving the hydro-distillation method has been conducted, however, there is no public research that reports the new method replacing hydro-distillation yet. In addition, although the outcome when using hydro-distillation are lower than natural oils, their quality is still accepted in an appropriate range limit.

Citrus essential oils were known to have powerful antimicrobial activities, since they contained terpenes

compound which was the main reason for antimicrobial activities (Kalemba *et al*., 2003) [7]. Monoterpenes also had the ability of antimicrobial activities, however, hydrocarbon monoterpenes had the lowest antimicrobial activities. Oxygenated monoterpenes had higher potential, especially phenol-type compounds as thymol, carvacrol. With different regions of citrus plants, different leaf essential oils will have different antimicrobial activities. Antimicrobial activities of essential oil of lime from South Africa were determined by using the diffusion method (inhibition zone diameter). The results were 1 mm, 4 mm and 2 mm zone of inhibition, corresponding to *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*, respectively. Comparing to lime, essential oil of orange from South Africa had no antimicrobial activity according to the results of no inhibition zone against the entire microorganism above (Vimol *et al*., 2012) [16].

Another functional property of citrus essential oils is antioxidant activity. Antioxidant activity is action against linoleic acid oxidation and 2,2-diphenyl-1-picrylhydrazyl radical scavenging. Some recent publication showed antioxidant activities of these essential oils (Baik *et al*., 2008) [1]. Antioxidant of natural essential oils is the best way to extend the shelf life of food. Synthetic agents were found to have toxic and carcinogenic effects on human body and food. Therefore, safer compounds extracted from natural sources were researched and developed to prevent the deterioration of foods. Citrus leaves contained an amount of essential oils which contributed to antioxidant capacities. Three active antioxidant compounds which are monoterpenes (camphor and y-terpinene), linalool and caryophyllene were proved to be the main reason for antioxidant capacities in EOs.

Because the antimicrobial and antioxidant capacities of citrus essential oils vary depending on the source and varieties of plants. The objectives of this study are to investigate the antioxidant and antimicrobial activities of essential oils extracted from leaves of three citrus varieties including Dao lime, Vinh orange and Thanh Tra pomelo grown popularly in Vietnam.

2. Materials and Methods

2.1 Materials

Leaves from different citrus varieties (Dao lime, Vinh orange and Thanh Tra pomelo) were collected in the Spring season because citrus trees at that season were mature enough to be collected. The period of time, from collecting to researching was controlled. If it was too long, some important chemical composition inside the leaves was changed and affected the results of this project. After collecting, the samples were transferred to the laboratory by airplane in the same day. Therefore, the quality of fruits was not affected and the extraction was carried out as soon as possible. And collected leaves were stored at 4ºC for further analysis.

2.2 Extraction of essential oils using hydro-distillation method

The samples (30 g) was put into boiling flask contained 250 ml distilled water. Then the system was heated to boil the leaves. The EO molecules and steam were carried along a pipe and condensed into a cooling tank, where they returned to liquid form and were collected in a vat. The mixture includes oil and water, due to the water-insoluble capacity of oils, they can easily be separated from water by floating on the surface. (Chemat, 2010) [3].

2.3 Antimicrobial activities of essential oils

2.3.1 Microbial strain

In order to determine antimicrobial activities of citrus essential oils, four bacteria and two fungi were used, including two gram-positive bacteria (*Staphylococcus aureus* from Institute of Drug Quality Control in Ho Chi Minh city and *Bacillus cereus* from Institute of Microbiology and Biotechnology, Vietnam National University), two gram-negative bacteria (*Salmonella typhi* and *Pseudomonas aeruginosa* from Institute of Drug Quality Control in Ho Chi Minh city) and two fungi (*Aspergillus flavus* and *Fusarium solani* from Institute of Microbiology and Biotechnology, Vietnam National University). The concentration of tested bacteria was 10^6 colony forming unit (CFU/ml), while fungi were 10^5 CFU/ml.

2.3.3 Diffusion method

The antimicrobial activities of essential oils were tested on TSA discs (NCCLS, 1997), by spreading 100 µl of bacteria of tube with r = 10^6 CFU/ml. Disc had the diameter of 90 mm, contained approximately 22 ml of TSA. After spreading, 3 wells, each with 9 mm diameter were made. Two of the wells, containing 50 µl mixture of essential oils and absolute ethanol with a ratio of 1:1. The last well was the control one, containing only ethanol. Discs were then incubated at 37ºC for 24 hours. The antimicrobial activities were determined by measuring the diameters of inhibition area (including size of the well). For fungi, the test was carried out in PDA discs and incubated at 24ºC for 48 hours (Dorman and Deans, 2000) [4].

2.3.4 Dilution method (minimum inhibition concentration, MIC)

MIC stands for minimum inhibitory concentration, means that the minimum concentration of EOs can inhibit the growth of microorganisms (Shapiro *et al*., 1994) [14]. A range of concentration of EOs from 1.31 - 168 mg/ml was made in tubes. Each tube contained 500 µl samples diluted in absolute ethanol, 4 ml of sterilized TSB or PDB and 500 µl of bacteria culture with r = 10^6 CFU/ml. For bacteria, tubes were then incubated at 37ºC for 24 hours, while fungi were incubated at 24ºC for 48 hours. After incubation, these tubes were spread on TSA or PDA discs by using a cotton swab. The lowest concentration showed no growth of microorganisms were considered as the MIC.

2.4 Antioxidant activities of essential oils

2.4.1 DPPH assay

By radical scavenging ability, the antioxidant activities of EOs were measured by using 2, 2-diphenyl-1-picrylhydrazyl stable radicals (Ghasemi *et al*., 2010) [6]. Different concentrations: 0.5, 1, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 100, 120 and 140 mg/ml were prepared with methanol. A volume of each diluted EO in methanol was added to 100uM of DPPH solution with final ratio 1:1. After 15 minutes, results are then recorded, by using spectrophotometer at 517 nm. Graphing a standard curve by Microsoft Excel was then carried out. Based

on the standard curve and its formula, the concentration of EO with 50% inhibition was then calculated (Ghasemi *et al*., 2010) [6].

2.4.2 FTC assay

Ferric thiocyanate method was used in FTC assay. Different concentrations of essential oils were prepared at 1, 3, 5, 10, 20, 40, 80, 100 and 200 mg/ml with absolute ethanol (Frankel, 1984). Each sample was mixed with the solvent mixture including 0.8 ml absolute ethanol, 0.4 ml 2.51% of linoleic acid, 0.8 ml of 0.2M sodium phosphate buffer (pH=7). After shaking, tubes were then sealed and incubated in the dark at 40ºC for 48 hours. In the first 24 hours, 0.1 ml of each tube were taken out and mixed with 0.1 ml 20 mM ferrous chloride (dissolved in HCl 3.5%). Then, solutions were measured at 500 nm using a spectrophotometer. After the next 24 hours, the process was repeated. If the result was higher than the first time, other samples were further done. If not, the first time was used as the results. The control was the solution that did not contain essential oil and incubated for 48 hours. Standard curves were graphed based on the results. The concentrations inhibited 50% of linoleic acid oxidation were determined.

2.5 Data analysis

Each parameter was tested in triplicate. Microsoft excel software was used to calculate means and standard deviations. Analysis of variance (ANOVA) was applied to the data to determine differences ($p < 0.05$). Statistical data analysis was undertaken using the Statistical Package for the Social Sciences (SPSS).

3. Results and discussion

3.1 Antimicrobial activities of citrus essential oils

Antimicrobial activities of citrus leaves were determined by measuring the diameters of the diffusing essential oils in the well of the agar. The results are shown in Table 1. Overall, the citrus leaf's extracts performed a significant inhibition activity on microorganisms. Vinh orange EOs showed the highest effects with the inhibition zone diameter of 22.2 mm against *S. aureus*, 21.4 mm against *P. aeruginosa*, 12.5 mm against *S. typhi* and 18.1 mm against *B. cereus*. Other citrus leaf's EOs were found in the inhibition zone diameters range from 10.1 mm to 20.7 mm. Other researches also reported that Citrus aurantium (bitter orange) EO had the highest antimicrobial activities with the inhibition zone of 18 mm (Periyanayagam *et al*., 2014). However, Citrus hystrix (lime) EO in another research was found to be highest in antimicrobial activities with the inhibition zone of 19 mm (Vimol *et al*., 2012) [16].

Table 1: Inhibition zone (mm) of citrus leaf's EOs against bacteria

Sample	*S. aureus*	*P. aeruginosa*	*S. typhi*	*B. cereus*	*A. flavus*	*F. solani*
Vinh orange	22.2 ± 0.2^{c}	21.4 ± 0.1^{b}	12.5 ± 0.1^{b}	18.1 ± 0.1^{b}	15.0 ± 0.1^{b}	11.0 ± 0.05^{a}
Dao lime	12.6 ± 0.1^{a}	15.1 ± 0.1^{a}	10.2 ± 0.1^{a}	11.2 ± 0.1^{a}	10.1 ± 0.1^{a}	11.1 ± 0.2^{a}
Thanh Tra pomelo	20.7 ± 0.2^{b}	14.6 ± 0.1^{a}	10.1 ± 0.1^{a}	12.2 ± 0.1^{a}	10.7 ± 0.1^{a}	11.5 ± 0.3^{a}

*Values followed by the different small letters within the same column are significantly different ($p<0.05$).

For fungi, Vinh orange leaf's EOs also showed the highest affection on *A. flavus* with the inhibition zone of 15.0 mm, following by Thanh Tra pomelo leaf's EOs with 10.7 mm and Dao lime leaf's EOs with 10.1 mm of diameter. The results showed that *F. solani* was more difficult to be inhibited, since their inhibition zones were lower than *A. flavus* with 11.0 mm, 11.1 mm and 11.5 mm corresponding to Vinh orange, Dao Lime and Thanh Tra pomelo leaf's EOs, respectively.

The minimum inhibition concentration (MIC) values of different citrus oils are presented in Table 2. The inhibition of leaf's EOs against the growth of *S. aureus* was found to be the highest compared to other microorganisms. The inhibition against *S. typhi* was found to be the lowest among three leaves' EOs. The EO extracted from orange leaves showed the strongest antimicrobial activities similar to the results of zone diameter with a low concentration needed to inhibit the growth of bacteria and fungi (only 5.25 mg/ml against *S. aureus*). The EOs extracted from Pomelo leaves ranked second among three EOs, with the lowest concentration was 10.5 mg/ml against *S. aureus*, and for lime leaves extract was 21 mg/ml.

The toxic effects of leaf's essential oils against the function and structure of the cell membrane of microorganisms is the reason of antimicrobial activities, from low to high concentration of essential oils. Microorganism cell membrane was damaged and then destroyed based on the loss of homeostasis (Carson *et al*., 2002) [2]. Moreover, the enzymes and proteins of cell membrane were interacted with the chemical compositions of essential oils, producing protons to the cell exterior, causing death (Omidbeygi *et al*., 2007) [11].

Table 2: Minimum inhibitory concentration (mg/ml) of citrus leaf's EOs against bacteria

Sample	*S. aureus*	*P. aeruginosa*	*S. typhi*	*B. cereus*	*A. flavus*	*F. solani*
Vinh orange	10.5	42	84	42	42	84
Dao lime	84	84	168	84	168	84
Thanh Tra pomelo	21	84	168	84	84	42

3.2 Antioxidant activities of citrus essential oils

3.2.1 DPPH assay

Antioxidant activity of the essential oils extracted by citrus fruits leaves, as assessed by the DPPH radical scavenging assay as well as expressed in terms of 50% inhibition concentration (IC50) is given in Figure 1. DPPH is a stable free radical having maximum absorption at 517 nm that accepts an electron or hydrogen atom to become a stable

diamagnetic molecule.IC_{50} values denote the concentration of a sample, which is required to scavenge 50% of DPPH free radicals. The lower IC_{50} value is, the higher antioxidant activity is.

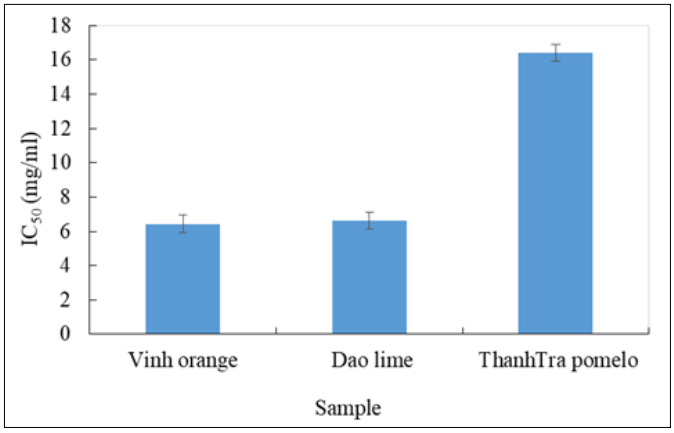

Fig 1: IC_{50} values of citrus leaf essential oils by DPPH method.

The highest IC_{50} value was obtained from the essential oil extracted from leaves of Thanh Tra pomelo (16.4 ± 0.24 mg/ml), followed by the essential oil extracted from Dao lime leaves with 6.64 ± 0.01[b] mg/ml, and the lowest was 6.44 ± 0.03[a] mg/ml for Vinh orange leaves. These results indicated that essential oil extracted from Vinh orange leaves had the highest antioxidant activities.

3.2.2 FTC assay

The antioxidant activity of citrus EOs assessed by the ferric thiocyanate method involving oxidation of linoleic acid is shown in Figure 2. The ferric thiocyanate method was originally designed for measuring lipid peroxide content in an emulsion system, whereby the end-point measure is the amount of Fe^{2+} that is oxidized to Fe^{3+} by lipid peroxides. The Fe^{3+} - thiocyanate complex produces a deep red color, which is detectable at 500 nm. The AA_{50} value is the concentration required to achieve a 50% inhibition of linoleic acid oxidation. The lower AA_{50} value, the higher the antioxidant activity is.

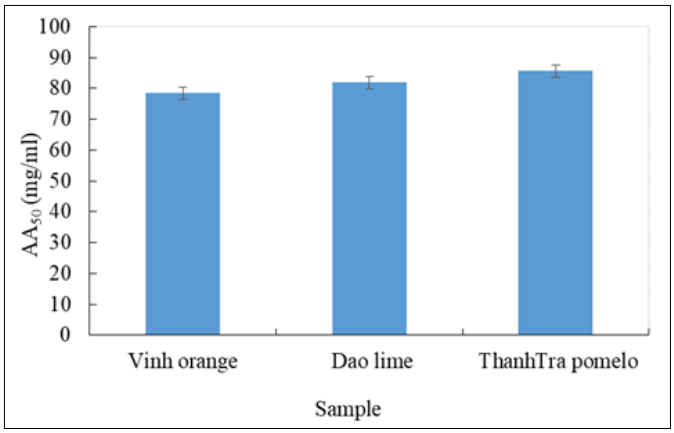

Fig 2: AA_{50} values of citrus leaf essential oils by FTC method.

All citrus EOs used in this study had significant antioxidant activities against linoleic acid peroxidation. Among the citrus EOs extracted by the hydro-distillation method, Vinh orange displayed the lowest inhibitions of peroxidation (78.42 ± 3.01 mg/ml), and thus the best antioxidant activities. The essential oil of Thanh Tra pomelo showed the least antioxidant activity in this assay because of AA50 value was 85.64 ± 0.26 mg/ml. Dao Lime EOs ranked second place with AA50 value was 81.83 ± 0.06 mg/ml. The different antioxidant activity of essential oils may be due to the different compositions of EOs. The higher antioxidant activities are related to the higher terpene compositions, especially concentration of terpinolene, geraniol, b-pinene and myrcene in the extracted essential oil (Song *et al*., 2001) [15].

4. Conclusion

In the present study, antimicrobial and antioxidant activities of essential oils from three different kinds of citrus leaves (Vinh orange, Dao lime and Thanh Tra pomelo) grown in Vietnam were investigated. The antimicrobial and antioxidant activities of the orange essential oil were found to be the highest, whereas antimicrobial and antioxidant activities of the pomelo EOs were the lowest. The results of this study gave useful information for using citrus leaf essential oils in food and pharmaceutical industries.

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The authors have declared no conflict of interest.

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