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ANTIMICROBIAL ACTIVITIES OF VIETNAMESE HOLY BASIL (*OCIMUM SANCTUM*) ESSENTIAL OIL AGAINST FOOD-BORNE BACTERIA AND FUNGI

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ABSTRACT

The essential oil (EO) obtained from the leaves of the holy basil (*Ocimum sanctum* L.) growing in Vietnam was found to contain eugenol (53.61 %), (*E*)-caryophyllene (25.37 %), *β*-elemene (15.86 %), and germacrene A (2.03 %) as the main volatile components. The antimicrobial activities of this oil were screened against several food-borne bacteria and fungi species. Significant growth inhibition effects against food-borne bacteria *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis,* and *Bacillus cereus* were observed using the standard disc diffusion method. The diameters of the inhibition zones against tested strains ranged from 15.4 to 16.4 mm; 16.7 to 18.4 mm; and 17.7 to 20.4 mm for 5 µl, 10 µl and 15 µl of EO, respectively. The minimum inhibitory concentration (MIC) determined by micro-dilution method in MHB liquid medium was 0.78 µl/ml. The bactericidal concentrations (MBC) was 1.56 µl/ml for all isolates tested. The antifungal properties of the holy basil EO were also determined in this study against three important pathogenic fungi such as *Candida albicans*, *Rhizoctonia solani* and *Fusarium oxysporum* with the inhibition zones ranging approximately from 20 to 30 mm. The results suggested that holy basil EO can be applied in food industries as natural flavoring preservatives/additives to control food spoilage and food born bacteria and fungi.

Keywords: Ocimum sanctum L., holy basil essential oil, antibacterial activity, antifungal activity.

1. INTRODUCTION

Food safety is critically related to public health and this field has received an increasing attention in recent years. The emergence of new outbreaks caused by food-borne spoilage and pathogenic bacteria is one of the major food safety challenges [1]. Synthetic preservative has been used in food industry over the last few decades. However, the use of these artificial compounds in controlling food spoilage and pathogen bacteria has been a controversial topic.

Thus the natural preservatives such as herb essential oil (EO) have been applied to improve the flavor of food and to extent of shelf - life and reduce pathogenic bacteria in food. Medicinal and aromatic plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value [2].

Holy basil (*Ocimum sanctum* L.), a species native to India, is widely used for spice and medicine. The essential oil obtained from these leaves has a sweet, pungent, herbaceous aroma. Many pharmacological studies demonstrated that *Ocimum sanctum* L. possesses the antioxidative and antimicrobial functions including antibacterial, antifungal, antimalarial and antihelminthic activities [3-5]. It has been also recommended to treat diabetes, bronchitis, diarrhea, dysentery, dyslipidemia, hypertension and skin diseases [3, 4].

The number of researches on the essential oil composition of the holy basil leaves has been reported. Generally, the essential oil from these leaves is quite rich in eugenol, however the considerable confusion that exists over the composition ratio depending on different parts, development growth stages of the plant to be used, and harvesting conditions. Mondal *et al.* [5] studied the holy basil from India and found that the chemical constituents of essential oil obtained from fresh and dried leaves differ greatly. Eugenol (57.94 %), (β)-caryophyllene (15.32 %), (β)-elemene (7.57 %) and germacrene D (9.10 %) were reported as dominant components in the essential oil of fresh leaves, while caryophyllene oxide (29.36 %), (β)-caryophyllene (18.20 %), (β)-elemene (11.38 %) and eugenol (6.34 %) were the highest abundance in the oil composition of dried leaves. The obtained essential oils showed antimicrobial and fungal activities against *Escherichia coli*, *Shigella* spp., *Salmonella typhimurium* and *Candida albicans*. Similar results were reported by Saharkhiz MJ *et al.* [6] and showed that eugenol was the major compound of the EOs obtained from all stages of growth (vegetative, floral budding and full flowering) in Iran, 2012. In addition, EO distilled from the second development growth stage (floral budding) of *O. sanctum* L. exhibited the strongest antibacterial activities against several food-borne pathogens as well as *Aspergillus* species.

In Viet Nam, holy basil has been used for many years as a food additive and traditional folk medicine. However, the essential oil composition and the antimicrobial activities of the EO of Vietnamese *Ocimum sanctum* L. have not been fully understood. The purpose of this study was to determine the EO chemical composition and also to investigate the antimicrobial activities of *Ocimum sanctum* L. essential oil against several food-borne and pathogenic bacteria and fungi.

2. MATERIALS AND METHODS

2.1. Material, reagents, bacterial strains and culture conditions

Fresh leaves of Vietnamese holy basil were collected from the cultivated plants during April and May 2018 in Dai Yen village, Doi Can, Hanoi. The samples were preserved according to the method of Trang *et al.* [7]. Plants were confirmed as *Ocimum sanctum* L. at Department of Plant, Faculty of Agronomy, Vietnam National University of Agriculture. Mueller-Hinton broth (MHB), commercial standard discs (φ = 6 mm) and other discs were purchased from Becton, Dickinson and Company (New Jersey, USA). All other reagents were of the highest commercial grade available. Milli-Q water or sterilized water was used in all procedures.

Seven food-related species: *Escherichia coli ATCC*25922™ (*E. coli*), *Bacillus subtilis* ATCC 11774™ (*B. subtilis*), *Staphylococcus aureus* sub sp. ATCC 25923™ (*S. aureus*), *Pseudomonas aeruginosa* ATCC® 10145 (*P. aeruginosa*), *Candida albicans* ATCC 10231 (*C. albicans*), *Rhizoctonia solani*, *Fusarium oxysporum* were obtained from the American Type

Culture Collection were used in this study. All strains were grown on the MHB agar plates supplemented with 1.4 % agar and incubated at 37 ^oC under aerobic conditions for 24 hrs.

2.2. Essential oil preparation

Holy basil essential oil was prepared according to previous method described by Trang *et al.* [7]. 100 g of fresh holy basil leaves were cut into 1 cm and then transferred immediately into Clevenger-type apparatus to which 400 ml of deionized water was added. The essential oil was obtained by distillation for 6 hrs according to the Vietnamese standard TCVN 7039:2002, its physical properties were determined according to TCVN 8450:2010. The oils were preserved in a sealed vial at 4 ^oC.

2.3. Assay for antimicrobial activity

Using the filter paper disc diffusion method on the MHB agar plate, the bacterial growth inhibition by EO was assessed. Sterile standard discs (φ = 6 mm) containing essential oils were placed on the MHB agar plates previously spread with 0.1 ml of bacterial suspension (cell density = 10^6 CFU/ml) in MHB liquid medium. The plates were kept in the refrigerator for 4 hrs for the diffusion of EO in the plate and incubated at 37 ^oC for 24 hrs under aerobic conditions. The diameters of the zones of inhibition were measured and recorded in millimeters and the values were given as the average of three replicates at least. The inhibition zones were measured and recorded in millimeters. The inhibition zone representing more than 6 mm is defined as antibacterial active [7].

The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined by microbroth dilution assay in 96-well microtitre plates, according to the previous report [8]. EO is dissolved in sterile water containing 0.5 % Tween 80 to achieve the final concentration ranging from 0.39 µl/ml to 50 µl/ml. Each well (200 µl) contained 20 µL of sample; and 20 µl of bacterial suspension contained 10^6 CFU/ml and 160 µl of MHB medium. After incubation at 37 ^oC for 24 hrs, the optical density was measured at 600 nm using Elisa reader (Bio-rad Model 680, Japan). The MIC was determined as the lowest concentrations showing no growth. The MBCs were determined by spreading 10 µl of the culture on MHB agar plate then inoculated at 37 ^oC for 24 hrs. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5 % killing of the original inoculums. All tests were performed in triplicates.

2.4. GC-MS analysis

Volatile compounds of the EO were analyzed by GC-MS QP 2010 (Shimadzu, Japan) instrument equipped with a flame-ionization detector (FID) and a fused silica capillary column (DB-5 column, 30 m × 0.25 mm i.d., and 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.5 ml/min. The oven temperature was held at 60 ^oC for 4 min and increased to 230 ^oC at the rate of 3 ^oC/min and then held for 15 min. The injector and detector temperatures were set at 200 ^oC and 230 ^oC, respectively. Each compound was identified by the agreement of mass spectrum with those of the authentic compound in the GC-MS library (Willey, Chemstation).

3. RESULTS AND DISCUSSION

3.1. Volatile components in the essential oil from holy basil

After 6 hrs of distillation, the EO of holy basil leaves with light yellow color, typical flavor was obtained. Its physical properties were determined according to the respective Vietnamese standards. The result is shown in Table 1.

Table 1. Physical properties of EO from holy basil leaves.

Properties	*Values*
Density at 20 °C (g/ml)	0.86
Acid index (mg KOH/g)	2.35
Ester index (mg KOH/g)	33.21

Agreement of the mass spectra in the GS-MS library enabled 18 compounds to be identified as the volatiles in the essential oil of holy basil leaves. The relative peak area of each compound to the total peak area (%) is expressed in Table 2. The results showed that eugenol (53.62 %) was the principal component; *β*–caryophyllene and *β*-elemene were the second and third major component, accounting for 25.37 % and 15.87 % of total peak area, respectively, in this essential oil. These findings are quite in agreement with those of the previous study [3-6] in which eugenol and *β*-caryophyllene accounted for the major proportions of EO of the holy basil leaves. On the other hand, some authors reported methyl chavicol as the major compound of the EO [9, 10]; others reported eugenol as the principal component in this EO. However, in our study methyl chavicol was not detected by GC-MS analysis. The different contents of eugenol and *β*-caryophyllene were found comparing with those of some previous reports or the absence of methyl chavicol may reflect variations due to the origin, geographical location, etc. This result is in line with those reported by Saharkhiz *et al.* [6] and Joshi R.K [3].

The chemical composition also affects antimicrobial activities of the essential oil. It might have been due to the presence of eugenol (antibacterial component) and (*β*)-caryophyllene and caryophyllene oxide (antifungal components) [5, 6]. Thus, the next experiments on antibacterial activities were conducted on several food-borne bacteria and fungi.

3.2. Antimicrobial activity of Vietnamese holy basil EO against food-borne bacteria and fungi

The Vietnamese holy basil EO contains a wide variety of secondary metabolites that are capable of inhibiting or slowing the growth of bacteria and fungi. The results were shown in Table 3 and 4. Holy basil EO showed the antibacterial effect against all the test pathogenic strains with the inhibition zones ranging from 16.4 mm to 19.7 mm in diameter against *S. aureus*, from 16.0 mm to 18.4 mm against *B. subtilis*, and from 15.4 mm to 17.7 mm against *P. aeruginosa*. The strongest antimicrobial activity was found against *E. coli* with the inhibition zones ranging from 16.4 mm to 20.4 mm in diameters. The results indicated that the holy basil EO showed the potential activities against both Gram positive and Gram negative tested bacteria. Several authors have been investigating the mechanism of antimicrobial activities of EO against pathogenic bacteria [11]. EO and their components showed activities against a variety of targets, particularly the membrane and cytoplasm and they even changed the morphology of the cells. Vietnamese holy basil EO was found in this study (Table 2) as a variable mixture of active compounds including eugenol (53 %) which was shown very strong inhibition growth against Gram negative bacteria because of its hydrophobicity which enhances its incorporation into the cell membrane and breaking down the cell [6].

Table 2. Volatile compounds identified in the essential oil obtained from holy basil leaves.

No.	Retention time	Compounds	Relative area* (%)
1	6.027	pyruvic acid, butyl ester	0.04
2	9.921	2-methyl-nonane	0.10
3	***26.857***	***eugenol***	***53.61***
4	***28.369***	***β-elemene***	***15.86***
5	***29.680***	***β-caryophyllene***	***25.37***
6	*31.251*	*α*-humulene	1.36
7	32.034	3-tetradecen-5-yne	0.04
8	32.706	selinene	0.28
9	32.919	2-propylfuran	0.01
10	33.019	aromadendrene	0.29
11	*33.134*	*α*-thujone	0.01
12	33.288	casbene	0.01
13	33.495	germacrene A	2.03
14	*35.359*	*α*-terpineol	0.32
15	35.479	unknown	0.02
16	35.811	nerolidol	0.05
17	36.623	caryophyllene oxide	0.51
18	39.783	selinen-11-en-4-ol	0.09

* Peak area relative to the total peak area (%) on GC-MS with DB-5 column

Table 3. Antimicrobial activity of Vietnamese holy basil essential oil against food-borne bacteria.

Bacteria strains	Gram	Diameter of inhibition zone (mm)* used 5 µl of EO	Diameter of inhibition zone (mm)* used 10 µl of EO	Diameter of inhibition zone (mm)* used 15 µl of EO
E. coli	-	16.4	18.4	*20.4*
S. aureus	+	16.4	18.4	*19.7*
B. subtilis	+	16.0	17.0	*18.4*
P.aeruginosa	-	15.4	16.7	*17.7*

* Diameter of each disc was 6 mm and the inhibition circle representing more than 6 mm is defined as anti-bacterial active; Values are the mean of triplicates at least.

The essential oil also showed antifungal activities against three important pathogenic fungi such as *Candida albicans, Rhizoctonia solani* and *Fusarium oxysporum* (Table 4) with the inhibition zones ranging from 20 to 30 mm. This EO showed strongest antimicrobial activity against *Candida albicans* and *Fusarium oxysporum* with inhibition zones was about 30 mm in diameter, followed by *Rhizoctonia solani (*20.5 ± 0.12 mm) although using only 5 µl of this EO.

Besides, *Candida albicans* is the most common human fungal pathogen, ranking as the fourth-greatest cause of nosocomial bloodstream infections [12]; while *Fusarium oxysporum* responsible for skin invasion. Although the Vietnamese EO components differ comparing with Iran holy basil, similar antimicrobial activities against pathogenic fungi were also obtained [6]. Comparing with Indian holy basil with 18.52 % of β-caryophyllene and caryophyllene oxide of total peak area, the Vietnamese holy basil showed stronger antifungal effects against *Candida albicans* with 18 mm of inhibition zones. Exact mechanism of the antifungal activities has not been understood yet, that could be because of the amount of β-caryophyllene and caryophyllene oxide (known for antifungal activities in previous study by Dey *et al.* [13]) in the essential oil composition.

Table 4. Antifungal activity of Vietnamese holy basil EO against pathogen fungi.

EO sample (5 µl)	Diameter of inhibition zone (mm)*		
	Candida albicans	*Rhizoctonia solani*	*Fusarium oxysporum*
Holy basil	30.2 ± 0.19	20.5 ± 0.12	30.1 ± 0.34

The results obtained in this study strongly indicated that Vietnamese holy basil might be the candidate to develop natural antibiotics and disinfectants to control infective agents in food. The above studied bacteria are considered to be the most common pathogen causing outbreaks of food poisoning and food-spoilage [2, 3]. Thus, the holy basil EO could be used as a natural source as food preservative to reduce and substitute or avoid chemical preservatives.

3.3. Killing effects of EO against pathogenic bacteria

Table 5. Killing ability of EO of holy basil against food-borne bacteria.

Bacteria strains	Gram	MIC (µl/ml)	MBC (µl/ml)
E. coli	-	0.78	1.56
S. aureus	+	0.78	1.56
B. subtilis	+	0.78	1.56
P. aeruginosa	-	0.78	1.56

Vietnamese holy basil EO exhibited inhibition activities and bactericidal activities against all test pathogenic strains (Table 5). To determine the bactericidal activity of this EO against pathogenic bacteria, killing experiments were performed in the presence of 2 x MIC to 64 × MIC of holy basil EO. The bactericidal effects of holy basil EO, a reduction of more than 10^{4-5} CFU ml^{-1} of culturable cells, were mainly observed after 24 hrs of incubation for all isolates at the concentration of 100 µl/ml. The bactericidal behavior of this EO did not differ significantly in all isolates. The MIC and MBC values of holy basil EO against all tested strains were 0.78 µl/ml and 1.56 µl/ml, respectively. In most cases, considering the ratio MBC to MIC was < 4, the majority of EO were bactericidal against the tested strains. The results indicated that Vietnamese holy basil EO showed strong inhibition and bactericidal effect against food borne bacteria. Besides it, eugenol is the predominant chemical constituent in the holy basil, so it could be suggested that eugenol plays an important role in the antibacterial activity of these leaves.

In another study, Deans *et al.* [14] found that the susceptibility of Gram-positive and Gram-negative bacteria to plant volatile oils had little influence on growth inhibition. The structure of the Gram-positive bacteria cell wall allows hydrophobic molecules to easily penetrate the cells and act on both the cell wall and within the cytoplasm. It is often reported that Gram-negative bacteria are more resistant to the plant-based essential oils because of the differing structures of the cell walls of Gram-negative and Gram positive bacteria [1]. However, Vietnamese holy basil EO did not show much difference in antimicrobial activity between Gram-positive and Gram-negative food-borne bacteria. The difference in the antimicrobial activities of EO depended on the composition of EO. Similar result was also obtained for Iran holy basil, the EO of which showed stronger antimicrobial activities against Gram-negative bacteria [6].

4. CONCLUSION

The growing tendency for replacing synthetic additives with natural ones has brought about great interest in the evaluation of antimicrobial properties of the plants products because of their relatively safe status, wide acceptance by consumers, and their exploitation for potential multipurpose functional use. To the best of our knowledge, this is the first report on the chemical composition and antimicrobial activities of Vietnamese holy basil (*Ocimum sanctum* L.). The results strongly indicated that Vietnamese holy basil showed strong antimicrobial affects against Gram-negative, Gram-positive pathogenic bacteria and pathogenic fungi.

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