

Chemical Compositions and Antimicrobial Activities of *Ocimum sanctum* L. Essential Oils at Different Harvest Stages

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Background: Essential Oils (EOs) possess antibacterial properties and represent a natural source to treat infections and prevent food spoilage. Their chemical composition might be affected by the environmental condition and the developmental growth stages of the plant.

Objectives: The current study aimed to determine the variations in chemical compositions and antimicrobial activities of the EOs of *Ocimum sanctum* L. at different stages of harvesting.

Materials and Methods: The oils constituents were analyzed by gas chromatography/mass spectrometry (GC/MS). The effects of three different harvest stages of *O. sanctum* EOs against most common causes of food-borne were evaluated by broth micro-dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI).

Results: The analysis of the EOs indicated that eugenol was the major compound of the EOs at all developmental stages which reached its maximum level at the second stage. The results showed that the tested EOs exhibited antimicrobial activities against all of the examined pathogens at concentrations of 0.125-32 µL/mL, except *Pseudomonas aeruginosa* which was only inhibited by high concentrations of the floral budding and full flowering EOs. EO distilled from the second developmental growth stage (floral budding) of *O. sanctum* exhibited the strongest antibacterial activities against the food borne bacteria.

Conclusions: Considering the wide range of antimicrobial activities of the examined EOs, they might have the potential to be used to manage infectious diseases or extend the shelf life of food products.

Keywords: Essential Oil; Eugenol; Anti-Infective Agents Fungi; *Ocimum Sanctum* Linn; Foodborne Diseases Food-Borne Infection

1. Background

Foods provide a suitable media for many microorganisms to grow and produce byproducts and metabolites. Consumption of the contaminated food with a toxin or secondary metabolite due to bacterial growth in food, such as *Staphylococcus aureus* or *Aspergillus* species, is called food poisoning. On the other hand, certain types of live bacteria such as *Enterobacteriaceae* family might be transmitted through eating contaminated foods and cause food-borne infection. Contaminated foods are among the main concerns of the public health policy makers and food industries. Traditionally, in tropical areas where the food-borne infections are more frequent, the aromatic plants and spices are used in foodstuff for their flavoring and preservative properties (1-4). It is observed that many of these plants and their aromatic products have potential antimicrobial activities (5-8). In the recent decades, there is a global tendency to use organic and chemical-free foodstuffs. The use of aro-

matic plants with known medicinal and antimicrobial properties might be a solution to increase the shelf life of products or overcome antibiotic resistance (8).

Parallel to the invention of new generations of antibiotics, clinicians encounter antibiotic resistance which has dramatically increased during the recent years. This problem is increasing worldwide, imposing a huge burden of costs, morbidity, and mortality. Therefore, it is necessary to find new antimicrobial agents to apply in the cases of antibiotic resistance. Methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) species, third-generation cephalosporin-resistant (TGCsR) *Escherichia coli*, imipenem and quinolone-resistant *Pseudomonas aeruginosa*, antibacterial-resistant *Salmonella* and *Shigella* species are the top resistant pathogens. Plants and their EOs are among the resources of new anti-microbial compounds. The number of studies on antimicrobial activities of plant compounds

against many different types of microbes, including food-borne pathogens has increased in the recent decades.

Ocimum sanctum L. (Tulsi in Sanskrit or Reyhan-e-Moghaddas in Farsi) belonging to Lamiaceae family, is native to Asia, and Central and western parts of Africa. Tulsi is traditionally used as a medicinal plant (9). Pharmacological studies and clinical practices have demonstrated that this species possesses anti-oxidative (10, 11) and antimicrobial functions including antibacterial (9, 12), antifungal (13-15), antimalarial (9) and anti-helminthic (16). It has been also recommended to treat diabetes, bronchitis, diarrhea, dysentery, dyslipidemia, hypertension and skin diseases (9, 17, 18).

Chemical and biological diversity of the aromatic and medicinal plants differ significantly depending on the factors such as cultivation area, climatic conditions, genetic modification, different plant parts, developmental stages and collection time (19-21). In the recent years, numerous publications have reported the chemical compositions of the EOs of medicinal and aromatic plants demonstrating that the growth stage and harvesting time have a major impact on the EO content and compositions (21-23). Therefore, it is necessary to determine the proper time and plant growth phase to harvest by analyzing the EO and its compositions at various growth and developmental stages. To the best of authors' knowledge, literature pertaining to the EO content and composition of Tulsi from Iran is not available. Moreover, there is no report on EO compositions of Tulsi at different collection time such as vegetative, floral budding and flowering stages. It is, therefore, imperative to determine the appropriate harvesting time by analyzing the oil yield and composition of the plant.

2. Objectives

The current study, for the first time, aimed to assess the EO content and chemical constituents of three different growth and developmental stages of *O. sanctum*. In addition, the antimicrobial effects of these EOs were evaluated against standard strains and clinical isolates responsible for food borne infections.

3. Materials and Methods

3.1. Plant Material

This experiment was carried out in Agricultural Research Center of Bushehr province, located in nine miles South-east of Borazjan, Iran, with an altitude of 110 meters, 51°17' longitude and 21° 2' latitude in a 1350 square meters land, which was a fallow land last year. The average annual rainfall in this region is 300 to 350 mm, the maximum temperature is 51°C in August, and minimum temperature is -1°C in February. Some chemical characteristics of the experimental soils are shown in Table 1. *Ocimum sanctum* aerial parts were collected

from the cultivated plants at three stages of growth and development during June and July 2012. The samples were harvested at vegetative, floral budding (emergence of flower buds) and full flowering stages. A voucher specimen (HSU 24989) is deposited in the herbarium of Shiraz University, Shiraz, Iran.

Table 1. Some Chemical Characteristics of the Experimental Soil ^a

Variable	Value
EC, dS m ⁻¹	1.8
pH	7.5
O.M, %	0.97
T.N, %	0.53
P, mg kg ⁻¹	13.5
K, mg kg ⁻¹	540
Cu, mg kg ⁻¹	1.1
Fe, mg kg ⁻¹	4.9
Zn, mg kg ⁻¹	0.234
Mn, mg kg ⁻¹	0.394

^a Abbreviations: EC, electrical conductivity; O.M, organic matter; T.N, total nitrogen.

3.2. Essential Oils Preparation

At the above-mentioned developmental stages, the essential oil of the aerial parts of the *O. sanctum* (50 g for each stage) was obtained by hydrodistillation for 2.5 hours, using an all-glass Clevenger-type apparatus, according to the protocol outlined by the British Pharmacopoeia (24). The sample oils were dried over anhydrous sodium sulphate and stored in sealed vials at 4°C before gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS) analysis and antimicrobial assessments.

3.3. Essential Oils Analysis by Gas Chromatography/Mass Spectrometry (GC/MS)

The EOs were analyzed by GC-MS (Agilent, USA). The analysis was carried out on a Thermoquest-Finnigan Trace GC/MS instrument equipped with a DB-5 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 mm). The oven temperature was programmed to increase from 60 to 250°C at a rate of 4°C/minute and finally held for 10 minutes; transfer line temperature was 250°C. Helium was used as the carrier gas at a flow rate of 1.1 mL/minute, with a split ratio equal to 1:50. The quadrupole mass spectrometer was scanned over the 35-465 amu with an ionizing voltage of 70 eV and an

ionization current of 150 mA.

GC-FID analysis of the oil was conducted using a Thermoquest-Finnigan instrument equipped with a DB-5 fused silica column (60 m x 0.25 mm i.d., film thickness 0.25 mm). Nitrogen was used as the carrier gas at the constant flow of 1.1 mL/minute; the split ratio was the same as that of GC/MS. The oven temperature was raised from 60 to 250°C at a rate of 4°C/minute and held for 10 minutes. The injector and detector (FID) temperatures were kept at 250 and 280°C, respectively. Semi-quantitative data were obtained from FID area percentages without the use of correction factors.

3.4. Identification of EO Components

Retention indices (RI) were calculated by using retention times of n-alkanes (C6-C24) that were injected after the oil at the same temperature and conditions. The compounds were identified by comparison of their RI with those reported in the literature (25) and their mass spectrum was compared with the Wiley Library.

3.5. Determination of Antimicrobial Activities

3.5.1. Microorganisms

The antifungal activities of the EOs were determined against five standard strains of fungi including *Aspergillus flavus* (ATCC 64025), *A. fumigatus* (ATCC 14110 and CBS 144.89), *A. clavatus* (CBS 514.65) and *A. orizae* (CBS 818.72). The antibacterial activities of the EOs against standard species of *Staphylococcus aureus* (ATCC 25923 and ATCC 29213), *Enterococcus faecalis* (ATCC11700), *Escherichia coli* (ATCC 25922), enterohemorrhagic *E. coli* (ATCC 43894), *Pseudomonas aeruginosa* (ATCC 27853) and a clinical isolate of *Shigella flexneri* collected from the Dr. Faghihi Hospital (Shiraz, Iran) were also determined in this study.

3.5.2. Determination of Minimum Inhibitory Concentration (MIC)

MICs were determined using the broth microdilution method recommended by the CLSI (26, 27) with some modifications. Briefly, to determine antifungal activities, serial dilutions of the EOs (0.06-64.0 µL/mL) were prepared in 96-well microtitre plates using RPMI-1640 media (Sigma, St. Louis, USA) buffered with MOPS (Sigma, St. Louis, USA). To determine the antibacterial activities, serial dilutions of the EOs (0.06-64.0 µL/mL) were prepared in Muller-Hinton media (Merck, Darmstadt, Germany). Test fungi or bacteria strains were suspended in the media and the cell densities were adjusted to 0.5 McFarland standards at 530 nm wavelength using the spectrophotometric method (this yields stock suspension of 1.5×10^6 cells/mL for fungi and 1.5×10^8 cells/mL for bacteria). To each well of the microtiter plates, 0.1 mL of the working inoculums was added and

the plates were incubated in a humid atmosphere at 30°C for 24-48 hours for yeasts or at 37°C for 24 hours for bacteria. To provide control (blank) samples, 200 µL of un-inoculated medium was included. In addition, growth controls (medium with inoculums, without EO) were also included.

The growth in each well was compared with that of the growth control well. MICs were visually determined and defined as the lowest concentration of the EO produced $\geq 50\%$ growth inhibition for fungi and $\geq 95\%$ growth reduction for bacteria compared with the growth control wells. Each experiment was performed in triplicate. In addition, media from wells with fungi showing no visible growth were further cultured on Sabouraud dextrose agar (Merck, Darmstadt, Germany) and from wells with bacteria showing no visible growth on Muller-Hinton agar (Merck, Darmstadt, Germany) to determine the minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC), respectively.

4. Results

The hydro-distillation of the aerial parts of *O. sanctum* at the vegetative, floral budding, and full flowering stages yielded 0.98%, 0.92% and 1.1% (w/w) EO, respectively. The composition of EOs at different growth stages is shown in Table 2, in the order of their elution from a DB-5 column. A total of 13, 15 and 15 compounds representing 88.44%, 95.09% and 92.21% of the total were detected at vegetative, floral budding and full flowering stages, respectively. GC/MS analyses showed that the main constituents of the EO from the vegetative stage were 1, 8-Cineole and β -bisabolene, while eugenol was found as the main compound of floral budding and full flowering developmental stages.

The antibacterial activities of *O. sanctum* EOs against the tested bacteria are presented in Table 3. The EOs inhibited the growth of Gram-positive cocci at the concentrations of 0.5-32 µL/mL and removed them at the concentrations ranging from 1 to 32 µL/mL. Furthermore, the EOs exhibited anti-bactericidal activity (MBC) against all of the above-mentioned Gram-positive cocci at the concentrations ranging from 8 to 32 µL/mL, except the vegetative growth stage against *E. faecalis*. The *O. sanctum* EOs inhibited the growth of all Gram-negative bacteria at the concentrations of 0.25-4 µL/mL, except *P. aeruginosa*. The growth of *P. aeruginosa* was inhibited only in concentrations ≥ 64 µL/mL. The EOs exhibited anti-bactericidal activity (MBC) against all the above-mentioned Gram-negative bacteria at concentrations ranging from 0.5 to ≥ 64 µL/mL. The antimicrobial activities of *O. sanctum* EOs against the tested fungi are shown in Table 3. The essence of *O. sanctum* at different growth stages exhibited fungistatic and fungicidal activities against the *Aspergillus* species at the concentrations of 0.125-2 µL/mL and 0.125-4 µL/mL,

respectively.

Table 2. Chemical Components of the Essential Oils Distilled From Three Developmental Stages of *Ocimum sanctum*^a

Compound	Developmental Stages, %			
	RI	Vegetative	Floral Budding	Full Flowering
Ethyl Isovalerate	854	-	-	0.83
α -Pinene	954	0.76	0.41	0.66
Sabinen	985	0.55	0.44	0.49
β -Pinene	1010	1.08	1.53	1.73
Myrcene	1059	0.53	1.128	0.93
1,8-Cineole	1068	20.78	19.41	20.45
Linalool	1109	0.22	0.19	0.15
Terpinen-4-ol	1184	-	0.28	-
α -Terpineol	1211	-	0.37	0.42
Estragol	1229	11.49	10.61	11.40
Eugenol	1382	15.70	37.15	24.63
α -Cis-Bergamotene	1469	3.13	1.30	2.69
α -Humulene	1482	2.34	1.29	1.27
β -Bisabolen	1542	20.99	13.29	18.76
γ -Elemene	1565	10.47	7.7	7.8

^a abbreviation: RI, retention index.

Table 3. Antimicrobial Activity of *OcimumSanctum* Essential Oil Against Standard and Clinical Isolates of Bacteria and *Aspergillus* spp.^a

Bacteria	Vegetative Stage		Floral Budding Stage		Full Flowering Stage	
	MIC	MMC	MIC	MMC	MIC	MMC
Gram positive						
<i>S. aureus</i>	1	16	0.5	8	1	8
<i>S. aureus</i>	8	32	2	16	1	8
<i>E. faecalis</i>	32	> 64	4	8	16	32
<i>B. cereus</i>	2	8	0.5	4	0.5	2
Gram negative						
<i>E. coli</i>	4	8	1	2	2	4
<i>E. coli</i> O157	4	8	1	2	1	2
<i>P. aeruginosa</i>	> 64	> 64	64	64	64	> 64
<i>Shigella flexneri</i>	2	2	0.25	0.5	0.25	0.5
Fungi						
<i>A. flavus</i>	2	4	0.5	1	0.25	0.5
<i>A. fumigatus</i> 1	1	4	0.25	1	0.125	0.5
<i>A. fumigatus</i> 2	1	4	0.25	1	0.125	0.5
<i>A. clavatus</i>	1	2	0.25	0.5	0.125	0.125
<i>A. orizae</i>	1	4	0.5	1	0.25	0.5

^a Abbreviations: MIC, minimum inhibitory concentration; MMC, minimum antimicrobial concentration.

5. Discussion

EOs and their components are generally recognized as safe (GRAS) for human and animal consumption under US Federal Regulations and have antimicrobial activity

against a wide variety of foodborne pathogens. The current study showed anti-bacterial and anti-fungal characteristics of a traditional plant called *O. sanctum* on

food-borne Gram-positive, Gram-negative bacteria and *Aspergillus* species. The compositions of EOs might be affected by the developmental stage of the plant (6, 28). Although some authors reported Methyl Chavicol (14, 29) as the major compounds of the EO, others reported eugenol as the main constituent of the EO (13, 16). In the present study, β -bisabolene was the dominant compound of the oil at the vegetative stage which declined gradually from 20.99% to 13.29% at the floral budding stage. Eugenol which reached its maximum level at the floral budding stage was identified as the main compounds of both floral budding and full flowering stages. The lower concentration of eugenol in this study, compared with that of some previous reports (13, 16), may reflect variations due to geographical location. During the various developmental stages of *O. sanctum*, the concentration of 1, 8-Cineole gradually declined from 20.78% to 20.45%.

The antimicrobial activity of *O. sanctum* against some microorganisms has been investigated in some previous studies (12). Agarwal et al. in an experimental study demonstrated that *O. sanctum* extract was effective against *Streptococcus mutans* (12). Others showed antimicrobial properties of the extract of this plant against *S. aureus* and enteric bacteria (30). In another study, its efficacy against multi-resistant strains of *Neisseria gonorrhoea* was demonstrated (31). In the present study, the EOs inhibited the growth of all Gram-positive bacteria and removed them at the concentrations up to 32 μ L/mL, except *E. faecalis*. Similar to the previous studies (30), nearly all the studied Gram-negative bacteria were susceptible to EOs of this plant at the concentrations of 0.25-4 μ L/mL, except *P. aeruginosa* which was only inhibited by high concentrations of the floral budding and full flowering EOs. This bacterium is a well-known nosocomial and burn wound pathogen. It has a low susceptibility to many antibiotics which is caused by the action of multidrug efflux pumps and the low permeability of its outer membrane (32). The higher MICs of the EOs against *P. aeruginosa* in comparison with the other bacteria might be attributable to the presence of some constructive proteins (such as Protein F) in its outer membrane (32).

The antimicrobial properties of *O. sanctum* are not confined to bacteria. It possesses anti-fungal and anti-helminthic activities. Of *Aspergillus* species, *A. flavus* and *A. parasiticus* are responsible for producing a number of secondary metabolites including aflatoxins. These secondary by-products are well-known for carcinogenic, mutagenic and teratogenic effects on humans and animals (33). In addition, *Aspergillus* species, in particular *A. fumigatus* and *A. flavus*, are responsible for severe invasive infections especially in immunocompromised hosts (34). Antifungal activities of the *O. sanctum* against *Candida* spp. and dermatophytes were previously indicated (6, 14, 15). In the current study, the developmental growth stages of the EOs, in particular full flowering stage, exhibited significant fungistatic and fungicidal activities against *Aspergillus* species. These results support the previously

reported data (13), and suggest that the EO of *O. sanctum* has the potential to be used in the food industries as preservative to prevent the growth of aflatoxicogenic fungi.

Since the EOs exhibited similar antimicrobial effect. One of the main characteristics of EOs is their hydrophobicity, which enables their incorporation into the cell membrane (35). Of the developmental stages, the second stage was more potent against most of the examined bacteria. Its mechanism may yield behind the active ingredients of *O. sanctum*, since biological activity of the EOs is determined by its various chemical constituents. The most potent antibacterial component of *O. sanctum* in the second stage was eugenol and the noticeable antibacterial properties of this stage might be related to this compound. Eugenol is a phenylpropanoid compound used in perfumes and flavorings as well as medicines as an antiseptic and anesthetic.

Antimicrobial activities of eugenol were shown previously (36-42). Eugenol inhibited the growth of *Helicobacter pylori* strains at a concentration of 2 μ g/mL (36). Eugenol was found to be effective against *Listeria monocytogenes*, *Aeromonas hydrophila* and autochthonous spoilage flora in microbial media (37). In other studies, eugenol exhibited strong antimicrobial activities against *E. coli*, *S. aureus*, *Bacillus cereus*, *L. monocytogenes*, *P. aeruginosa*, *Salmonella typhi*, and *Proteus mirabilis* (40-42). It has also been used in mucoadhesive tablets to treat periodontal diseases (38). An important characteristic of eugenol is its hydrophobicity which enhances its incorporation into the cell membrane.

It is proposed that the effective microbial damage of this molecule might be due to disruption of bacterial cell membrane (41, 42), which leads to increased cell membrane permeability and protein leakage (39). Other possible mechanisms of antimicrobial action of eugenol are conversion of cytochrome P-450 mediated into cytotoxic quinone methide (40, 43, 44) and inhibition of energy generation (45). Other ingredients of the EOs with significant antibacterial potency were cineol and β -bisabolene. These two latter ingredients had higher concentrations at the vegetative stage than the other stages. Synergistic activity of the combination of ampicillin with β -bisabolene against *S. aureus* was confirmed by previous studies (46-48). Moreover, it was shown that 1, 8-cineol has significant antimicrobial activities alone or in combination with other monoterpenes or drugs (49). All together, these facts and results support the broad spectrum antimicrobial activities of *O. sanctum* EO by the mechanisms different from those of the antibiotics, and its possible application in the cosmetic, medicinal and food products.

Chemical composition and subsequent biological activities of EOs are not only determined by the origin and environmental conditions, but also by developmental stage in which the plant is collected. The EO distilled from the second developmental growth stage of *O. sanctum* (floral budding) exhibited strong antibacterial activities against several food-borne bacteria as well as *Aspergillus*

species. Therefore, any attempts for clinical or industrial application of this EO should focus on the second developmental stage and its major ingredient (i.e. eugenol). Regarding the considerable antibacterial activities of the tested EOs, they might be the candidates to develop new antibiotics and disinfectants to control infective agents. The desirable flavor and odor of this EO at the examined concentrations is an additional benefit to its antimicrobial activities which makes it a suitable candidate to be used as a food preservative.

Authors' Contributions

Study concept and design: Kamiar Zomorodian; sample collection and laboratory examinations: Amir Alam Kamyab, Mohammad Javad Rahimi, Keyvan Pakshir, Narges Khatoon Kazerani; data interpretation: Keyvan Pakshir, Kamiar Zomorodian, Mohammad Jamal Saharkhiz; manuscript: Kamiar Zomorodian, Amir Alam Kamyab and Mohammad Jamal Saharkhiz; Critical revision of the manuscript for important intellectual content: All the Authors.

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