Research Paper

Chemical Composition, *In Vitro* Antimicrobial and Antioxidant Activities of the Essential Oils of *Ocimum gratissimum, O. Sanctum* and their Major Constituents

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The essential oils of the flowering aerial parts of two *Ocimum* species viz., *Ocimum gratissimum* and *O. sanctum* were analyzed by gas chromatography and gas chromatography/mass spectroscopy. The principal constituent of *O. gratissimum* and *O. sanctum* was eugenol (75.1%) and methyl eugenol (92.4%), comprising 99.3 and 98.9% of the total oils, respectively. *In vitro* antimicrobial activity of the essential oils of *O. gratissimum, O. sanctum* and their major compounds eugenol and methyl eugenol were screened by using tube dilution methods. *O. gratissimum* oil was found highly active against *S. marcescens* while *O. sanctum* oil showed significant activity against *A. niger* and *S. faecalis*. Methyl eugenol exhibited significant activity against *P. aeruginosa* while eugenol was effective only against *S. aureus*. Antioxidant activity of oils, eugenol, and methyl eugenol was determined by 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) assays. Essential oil of *O. gratissimum* showed comparative antioxidant activity with IC_{50} values 23.66±0.55 and 23.91±0.49 μg/ml in 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) models, respectively. Eugenol showed slightly weaker antioxidant activity compared to oil of *O. gratissimum*, while *O. sanctum* oil demonstrated very feeble antioxidant activity and methyl eugenol did not show any activity. Eugenol and methyl eugenol would be elite source from *O. gratissimum* and *O. sanctum,* respectively, of this region could be consider as a source of natural food antioxidant, preservatives, and as an antiseptic.

Key words: Antimicrobial activity, antioxidant activity, eugenol, GC/MS, methyl eugenol, *Ocimum gratissimum, O. sanctum*

Various medicinal plants have been used from time immemorial to treat diseases all over the world. The use of traditional medicine and medicinal plants in developing countries, to meet some of their primary health care needs, has been widely documented[1]. About 80% of individuals from developing countries are using traditional medicines, derived from medicinal plants. Essential oils are secondary metabolites, produced by various medicinal and aromatic plants are known to possess a wide spectrum of antimicrobial and antioxidant activities. Essential oils have been known to possess notably antibacterial, antifungal, and antioxidant properties[2]. Plants have almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives[3]. These compounds protect the plant from microbial infection and deterioration[4].

The antioxidant property of the plant extracts has been attributed to their polyphenolic contents[5]. Plants containing a high level of polyphenols are of great importance as natural antioxidants. Oxidation process is very important in all organisms. The uncontrolled production of oxygen free radicals and inadequate antioxidant protection has been implicated with pathogenesis of cancer, diabetes, and coronary heart diseases[6,7]. The antioxidants are important ingredients in food processing, where butylated hydroxytoluene and butylated hydroxyanisole is widely used synthetic antioxidants in food. However, their use in food products have been declining due to their instability and potential to act as promoters of

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carcinogenesis[8]. Therefore, there is a need to develop natural and healthy (non toxic) additives as potential antioxidants[2].

The chemical composition, antimicrobial, and antioxidant activities of the essential oils of *O. gratissimum* and *O. sanctum* have been reported from the different regions[9-16]. No reports related to the chemical composition, *in vitro* antimicrobial and antioxidant activities of the volatile constituents of *O. gratissimum* and *O. sanctum* from Western Ghats region of North Karnataka have been found in the literature. Thus, this work was aimed to identify the volatile constituents of *O. gratissimum* and *O. sanctum*, to isolate their major compounds and to test *in vitro* antimicrobial and antioxidant activities.

MATERIALS AND METHODS

The flowering aerial parts of *O. gratissimum* and *O. sanctum* were collected from the medicinal garden of Regional Medical Research Centre (RMRC), Belgaum. The plants were identified and authenticated by a taxonomist at RMRC, Belgaum, where the voucher specimens have been deposited (RMRC-530: *O. gratissimum* and RMRC-531: *O. sanctum*).

Extraction of oils:
Fresh flowering aerial parts (1.0 kg each) of *O. gratissimum* and *O. sanctum* were steam distilled separately using copper still fitted with spiral glass condensers for 3 h. Water distillate was extracted with hexane and dichloromethane. The organic phase was dried over anhydrous sodium sulfate and the solvent was distilled off using thin film rotary vacuum evaporator at temperature range 25-30°. The oils were stored in tightly closed dark vials at 4° until analysis.

Charactrerization of oils:
The oil was analyzed by using a Varian 450 (TG-5, 30 m×0.25 mm i.d., 0.25 μm film thickness) gas chromatograph under the experimental conditions reported earlier[17]. The oven temperature was programmed from 60 to 220° at 3°/min, using nitrogen as carrier gas. The injector temperature was 230° and the detector (FID) temperature 240°. GC-MS utilized a Thermo Scientific Trace Ultra GC interfaced with a Thermo Scientific ITQ 1100 mass spectrometer fitted with a TG-5 (Thermo Scientific) fused silica capillary column (30 m×0.25 mm; 0.25 μm film thickness). The oven temperature range was 60-220° at 3°/min using helium as carrier gas at 1.0 ml/min. The injector temperature was 230°, and the injection size 0.1 μl in *n*-hexane, with a split ratio of 1:50. MS were taken at 70 eV with a mass range of *m/z* 40-450.

The essential oils of *O. gratissimum* and *O. sanctum* (5.0 g each) were chromatographed separately on a silica gel (230-400 mesh; Qualigens Fine Chemicals, Mumbai, India) column (3.5×100 cm^2) eluted with increasing polarity mixtures of n-hexane/diethyl ether to give eugenol and methyl eugenol. The purity (99.80% for eugenol and 99.65% for methyl eugenol) was determine by GC (Varian 450 gas chromatograph) using the above analytical conditions. Each oil component and their isolated constituents were identified on the basis of their retention index (RI, determined with reference to homologous series of *n*-alkanes C_8-C_{25}, under identical experimental condition), from MS library searches using the NIST and Wiley GC-MS databases and by comparison with literature mass spectral data[18]. The structure of eugenol and methyl eugenol was further confirmed by ^{1}H- and ^{13}C-NMR.

Antimicrobial activity:
The microorganisms were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune. The microorganisms includes, Gram-positive bacteria (*Staphylococcus aureus* NCIM 2079, *Staphylococcus epidermidis* NCIM 2493, *Streptococcus faecalis* NCMI 2080, *Micrococcus flavus* NCIM 2379, *Micrococcus luteus* NCIM 2103, *Bacillus subtilis* NCIM2063), Gram-negative bacteria (*Escherichia coli* NCIM 2574, *Klebsiella pneumoniae* NCIM 2957, *Serratia marcescens* NCIM 2078, *Proteus vulgaris* NCIM 2813, *Proteus mirabilis* NCIM 2241, *Pseudomonas aeruginosa* NCIM5029, *Salmonella typhimurium* NCIM 2501, *Enterobacter aerogenes* NCIM 2694) and fungal strains (*Aspergillus niger* NCIM 620, *Aspergillus fumigatus* NCIM 902 and *Penicillium chrysogenum* NCIM 733).

The essential oils, eugenol and methyl eugenol, were dissolved in 10% dimethyl sulfoxide (DMSO). DMSO is reported to be non-toxic to microorganisms at this percentage[19,20]. Erythromycin (Alembic Ltd. Solan, Himachal Pradesh, India), amikacin (Iskon Remedies, Sirmour, Himachal Pradesh, India) and

amphotericin B (Chandra Bhagat Pharma Pvt. Ltd., Ankleshwar, India) were used as the positive reference standards for Gram-positive, Gram-negative bacteria, and fungi, respectively. The inocula of microbial strains were prepared from 18 h old culture and suspensions were adjusted to 0.5 McFarland standard turbidity (~10^4 for bacteria and ~10^3 for fungi colony forming unit (CFU) per milliliter)[21].

Tube-dilution method was used to determine the minimum inhibitory concentration (MIC). Essential oils, eugenol, and methyl eugenol were dissolved in 10% DMSO separately with final concentrations of 5.0 mg/ml. Serial two-fold dilutions were prepared from the stock solution to give concentration ranging from 5.0 to 0.009 mg/ml. Erythromycin, amikacin, and amphotericin B were dissolved in sterile distilled water and two-fold dilutions were prepared (1.0-0.002 mg/ml). A total of 1 ml of each concentration was mixed with 1.0 ml of sterile nutrient broth for bacteria except for *Streptococcus faecalis* (MRS broth) while peptone water was used for fungi (at 0.5 McFarland turbidity standard). Solvent control was prepared with DMSO (10%) while blank control was prepared from virgin media. Tubes were incubated for 24 and 48 h at 37º for bacteria and fungi, respectively. Assay was performed in replicates and the mean value of six experiments was recorded (n=6) with SEM. MIC was determined as the lowest concentration that inhibits the visible microbial growth[22].

Antioxidant activity:

The antioxidant activity of the essential oils, eugenol, and methyl eugenol was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to the method of Hou *et al.*[23]. DPPH was dissolved in ethanol to give a 0.05 mM solution. Final concentration of trolox (antioxidant reference), essential oil of *O. gratissimum* and eugenol was 2.4 mg/ml, while 10 mg/ml was used for essential oil of *O. sanctum* and methyl eugenol. Aliquots containing various concentrations (5-30 µg/ml) of trolox in the final volume of 1.0 ml were mixed with 1.0 ml of ethanol DPPH solution. The oil of *O. gratissimum* and eugenol were tested at concentration range of 25-50 µg/ml while oil of *O. Sanctum* and methyl eugenol at 50-300 µg/ml using the same method. The ethanol solution of DPPH (1.0 ml) with equal amount of ethanol served as control. Reaction mixtures were incubated at 37º for 20 min and the DPPH radical scavenging activity was determined by measuring the absorbance at 517 nm using a spectrophotometer.

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) diammonium salt radical cation decolorization test was performed using spectrophotometric method of Pellegrini *et al.*[24]. The ABTS reagent stock was prepared by mixing 88 µl of 140 mM potassium persulfate ($K_2S_2O_8$) with 5 ml of 7 mM of ABTS stock solution (pH 7.4). The working ABTS reagent was prepared by diluting the stock solution with ethanol to give an absorbance of 0.7±0.05 at 734 nm. The tested concentrations of trolox, oil of *O. gratissimum*, *O. sanctum*, and eugenol and methyl eugenol were same as represented in DPPH assay. The ABTS working solution (1.0 ml) with equal amount of ethanol served as control. The reaction mixtures were incubated at room temperature (28º) for 30 min and the absorbance was measured at 734 nm. The scavenging DPPH and ABTS activities of the tested samples were calculated as the following formula: Percent (%) inhibition of DPPH or ABTS=(1–absorbance of sample/absorbance of control)×100.

RESULTS AND DISCUSSION

The yields of essential oils from *O. gratissimum* and *O. sanctum* were 1.05 and 1.20%, respectively. Table 1 shows the compounds identified in the oils of *O. gratissimum* and *O. sanctum* along with their percentage composition and retention index on a TG-5 capillary column. Volatile constituents identified in the *O. gratissimum* were 10 monoterpene hydrocarbons (15.5%), 6 oxygenated monoterpenes (1.6%), 9 sesquiterpene hydrocarbons (6.1%), 1 oxygenated sesquiterpene (0.1%), and 5 phenyl derivatives (76.0%) comprising 31 constituents (99.3%) of the total oil. Quantitatively, the most important compound was eugenol (75.1%). The other minor compounds were terpinolene (14.2%) and germacrene D (3.9%).

The major volatile constituent identified in the essential oil of *O. sanctum* was methyl eugenol (92.4%). The other minor constituents were eugenol (2.4%) and β-caryophyllene (1.3%). The class compositions were 5 monoterpene hydrocarbons (0.3%), 3 oxygenated monoterpenes (0.2%), 9 sesquiterpene hydrocarbons (2.6%), 5 oxygenated sesquiterpenes (0.9%) and 3 phenyl derivatives (94.9%), comprising 25 constituents (98.9%) of the total oil. Ten compounds

TABLE 1: ESSENTIAL OIL COMPOSITION OF *O. GRATISSIMUM* AND *O. SANCTUM*

Compounds	RI	OG	OS
α-Thujene	937	0.1	-
α-Pinene	943	0.1	t
Camphene	459	-	t
Sabinene	979	0.3	-
β-Pinene	985	0.1	-
Myrcene	994	t	-
α-Terpinene	1022	t	-
p-Cymene	1030	0.3	-
1,8-Cineole	1036	-	0.1
(Z)-*β*-Ocimene	1045	-	0.1
(E)-*β*-Ocimene	1058	0.3	0.2
γ-Terpinene	1065	0.1	-
cis-Sabinene hydrate	1073	0.1	-
Terpinolene	1092	14.2	t
trans-Sabinene hydrate	1103	1.0	-
Borneol	1173	-	t
Terpin-4-ol	1181	0.1	-
α-Terpineol	1193	0.1	0.1
Methyl salicylate	1197	t	-
Methyl chavicol	1202	-	0.1
trans-4-Caranone	1204	0.1	-
cis-4-Caranone	1209	0.2	-
Thymol	1295	0.5	-
Carvacrol	1304	0.2	-
α-Cubebene	1353	-	t
Eugenol	1361	75.1	2.4
α-Ylangene	1379	-	0.1
α-Copaene	1381	0.7	-
β-Cubebene	1393	0.2	0.1
β-Elemene	1395	-	0.4
Vanillin	1398	0.2	-
Methyl eugenol	1407	-	92.4
β-Caryophyllene	1423	0.9	1.3
β-Copaene	1438	0.1	-
α-Humulene	1457	-	t
cis-Muurola-4 (14), 5-diene	1473	0.1	-
γ-Muurolene	1485	t	0.1
Germacrene D	1493	3.9	-
trans-Muurola-4 (14), 5-diene	1502	t	-
epi-Cubebol	1506	0.1	0.4
trans-*β*-Guaiene	1513	-	0.5
Cubebol	1527	-	0.1
δ-Cadinene	1533	0.2	0.1
10-*epi*-Cubebol	1546	-	0.2
1,10-di-*epi*-Cubenol	1629	-	0.1
epi-*α*-Cadinol	1651	-	0.1
Total identified (%)		99.3	98.9
Class composition			
Monoterpene hydrocarbons		15.5	0.3
Oxygenated monoterpenes		1.6	0.2
Sesquiterpene hydrocarbons		6.1	2.6
Oxygenated sesquiterpenes		0.1	0.9
Phenyl derivatives		76.0	94.9

RI=Retention index relative to C_8-C_{25} *n*-alkanes on TG-5 column, t=trace (<0.1%), OG=*O. Gratissimum*, OS=*O. Sanctum*

were found to be common in the oils of *O. gratissimum* and *O. sanctum*, which were *α*-pinene, *E*-*β*-ocimene, terpinolene, *α*-terpineol, eugenol, *β*-cubenene, *β*-caryophyllene, *γ*-muurolene, *epi*-cubebol, and *δ*-cadinene in less quantity.

The results of antimicrobial activity of the essential oils, eugenol and methyl eugenol are presented in Table 2. The minimum inhibitory concentration (MIC) values are represented as mg/ml. The essential oil of *O. gratissimum* was found highly active against *E. coli*, *S. marcescens*, and *K. pneumoniae*. The essential oil of *O. sanctum* was found to posses significant antimicrobial activity against the microorganisms like *S. epidermis*, *P. aeruginosa*, *A. niger*, and *S. faecalis*. The comparison of eugenol and methyl eugenol was carried out against the tested microorganisms. Methyl eugenol was found to possess significant antimicrobial activity against *P. aeruginosa* and *P. mirabilis*, while eugenol was found to be effective only against *S. aureus*.

Compared to the standard antibacterial agents used in the study (erythromycin, amikacin, and amphotericin B), the essential oils of *O. gratissimum* and *O. sanctum* and their main components have a weaker antibacterial activity. According to Wan *et al.*[25], the majority of the essential oils assayed for their antibacterial properties showed a more pronounced effect against the Gram-positive bacteria. The resistance of Gram-negative bacteria to essential oil has been ascribed to their hydrophilic outer membrane which can block the penetration of hydrophobic compounds into target cell membrane[26]. Thus, the methyl eugenol is more hydrophobic than eugenol; however, hydroxyl group of eugenol may not be involved for hydrogen bonding due to the presence of –OMe group in ortho position of eugenol produced steric hindrance. This may explain the weaker antimicrobial activity of the eugenol and methyl eugenol against Gram-negative bacteria. The other phenolic components though low in the essential oils of *O. gratissimum* and *O. sanctum* could be contributing for antimicrobial activity by causing leakage of intracellular ATP and potassium ions leading to cell death[27,28].

DPPH and ABTS assay were adopted for evaluation of antioxidant properties of essential oils, eugenol, and methyl eugenol. Trolox was utilized as positive control. The antioxidant activity of

TABLE 2: MIC VALUES OF *O. GRATISSIMUM* AND *O. SANCTUM* ESSENTIAL OILS AND EUGENOL AND METHYL EUGENOL

Microbial strains	MIC mean±SEM				
	O. Gratissimum	*O. Sanctum*	Eugenol	Methyl eugenol	RA
Gram-positive					
Staphylococcus aureus	1.14±0.40	0.41±0.06	1.04±0.13*	1.97±0.33	0.002±0.001
Staphylococcus epidermidis	0.54±0.08	0.20±0.03*	1.35±0.25	1.25±0.27	0.002±0.001
Streptococcus faecalis	0.49±0.08	0.23±0.03*	1.66±0.26	1.19±0.30	0.002±0.001
Micrococcus flavus	0.35±0.03	0.25±0.04	0.33±0.06	0.36±0.05	0.002±0.001
Micrococcus luteus	0.42±0.07	0.29±0.04	0.49±0.08	0.57±0.05	0.001±0.001
Bacillus subtilis	0.36±0.08	0.22±0.03	1.45±0.20	1.87±0.39	0.001±0.001
Gram-negative					
Escherichia coli	0.49±0.13*	2.29±0.20	2.91±0.41	2.70±0.51	0.009±0.004
Klebsiella pneumoniae	0.52±0.06*	1.24±0.27	2.08±0.61	3.12±0.62	0.005±0.002
Serratia marcescens	0.29±0.10*	1.77±0.33	2.08±0.26	3.33±0.52	0.005±0.003
Proteus vulgaris	0.98±0.16	2.29±0.59	3.33±0.52	2.29±0.20	0.005±0.003
Proteus mirabilis	0.72±0.10	0.62±0.13	2.18±0.31	1.04±0.13*	0.002±0.001
Pseudomonas aeruginosa	1.04±0.13	0.39±0.07*	2.29±0.20	0.72±0.10*	0.004±0.003
Salmonella typhimurium	1.51±0.34	1.87±0.62	2.91±0.41	2.70±0.50	0.002±0.002
Enterobacter aerogenes	1.14±0.10	2.08±0.61	3.12±0.62	3.75±0.55	0.009±0.004
Fungi					
Aspergillus niger	0.61±0.10	0.18±0.02*	0.38±0.08	0.20±0.03	0.001±0.00
Aspergillus fumigatus	0.75±0.27	0.20±0.03	0.46±0.13	0.57±0.13	0.001±0.00
Penicillium chrysogenum	0.42±0.07	0.52±0.08	0.34±0.03	0.28±0.02	0.001±0.00

*P<0.05, values are mean±SEM of six experiments in replicate. RA (Reference antibiotics)=erythromycin (for Gram-positive bacteria), amikacin (for Gram-negative bacteria), amphotericin B (for fungi). MIC=minimum inhibitory concentration

oils and compounds were expressed as inhibition concentration, IC_{50}. The results of scavenging activities are listed in Table 3. Antioxidant activity in series of two-fold dilutions, the concentration of each oil and isolated compounds were used to calculate the inhibition concentration IC_{50} in µg/ml. The amount of sample required to decrease the absorption of DPPH and ABTS by 50% were calculated graphically (% of inhibition was plotted against the concentration in µg/ml). The essential oil of *O. gratissimum* was most active using both the models. The IC_{50} values were 23.66±0.55 and 23.91±0.49 for DPPH and ABTS models, respectively. The essential oil of *O. gratissimum* showed potent antioxidant activity, which could be due to high amount of eugenol (>75%). The IC_{50} values for eugenol were high viz., 27.16±0.33 and 32.83±0.40 in DPPH and ABTS models, respectively. This higher antioxidant capacity of the oil of *O. gratissimum* could be the synergistic property due to the presence of other phenolic constituents viz. *cis*-sabinene hydrate, *trans*-sabinene hydrate, terpine-4-ol, α-terpineol, thymol, carvacrol, and *epi*-cubebol present in small quantity (2.1% of the total oil). Hence, the oil of *O. gratissimum* showed better antioxidant property than pure eugenol. On the other hand, essential oil of *O. sanctum* expressed feeble antioxidant activity with DPPH and ABTS models with IC_{50} values of 219.16±1.01 and 241.50±1.08, respectively, while methyl eugenol did not show any initial antioxidant activity, which may be due to the absence of participating electron delocalization group or proton (s). The presence of the cineol-1,8, α-terpineol, eugenol, *epi*-cubebol, cubebol, 10-*epi*-cubebol, 1,10-di-*epi*-cubenol, and *epi*-α-cadinol comprising 3.50% of the total oil could show weak antioxidant activity. The essential oils, eugenol, were compared with known synthetic antioxidant trolox (Table 3). The most obvious are the nonspecific responses and synergistic effects of the compounds present in the crude extracts. For this reason a bioassay-directed fractionation of an active extract does not always lead to the isolation of active compounds. An apparent loss of activity on separation of synergistically acting components of low individual potency cannot be easily distinguished from the loss of activity resulting from chemical changes induced by a particular isolation technique. In this study, we concluded that *O. gratissimum* and *O. sanctum* of this region can be used as a source of food additives, preservatives, and as an antiseptic.

TABLE 3: ANTIOXIDANT ACTIVITY OF *O. GRATISSIMUM*, *O. SANCTUM* OILS, EUGENOL, METHYL EUGENOL AND TROLOX DETERMINED BY DPPH AND ABTS METHODS

Antioxidant	IC_{50}	
	DPPH	ABTS
O. gratissimum	23.66±0.55	23.91±0.49
O. Sanctum	219.16±1.01	241.50±1.08
Eugenol	27.16±0.33	32.83±0.40
Methyl eugenol	NA	NA
Trolox	18.92±0.30	9.83±0.30

IC_{50}=µg/ml, values are mean±SEM of three experiments in replicate. NA=Not active, DPPH=diphenyl-1-picrylhydrazyl, ABTS=2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid

ACKNOWLEDGEMENTS

The author is grateful to Officer-in-Charge, RMRC, Belgaum for providing necessary facilities and thankful to Miss Vijayalaxi, Lab Technician for carried out antimicrobial screening of oils and Mr. Manjunath Patil, Lab Attendant, Phytochemistry Department, RMRC, Belgaum for collection of plant materials and extraction of essential oils.

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Accepted 6 June 2013
Revised 28 May 2013
Received 18 January 2013
Indian J Pharm Sci 2013;75(4):457-462