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Full Length Research Paper

Phytochemical and antimicrobial studies on essential oils of some aromatic plants

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The antimicrobial activity of the volatile constituents of five different plant essential oils, that is, *Ocimum sanctum* (leaves), *Eucalyptus globulus* (leaves), *Mentha arvensis* (leaves), *Citrus lemon* (fruit epicarp) and *Citrus maxima* (fruit epicarp) was evaluated *in vitro* against seven bacteria (*Bacillus pumilus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhi*), two filamentous fungi (*Aspergillus niger* and *Aspergillus flavus*) and a yeast (*Candida albicans*). The volatile constituents of *O. sanctum*, *E. globulus* and *M. arvensis* exhibited strong antimicrobial activities against test pathogenic fungi and bacteria (both gram-positive and gram-negative). The chemical composition of essential oils determined by gas chromatograph (GC) and high performance thin layer chromatography (HPTLC) analysis consisted of eugenol (56.07%), 1, 8 cineole (17.34%) and menthol (43.45%) as the major chemical constituents in *O. sanctum*, *E. globulus* and *M. arvensis*, respectively. Limonene was the only major constituent present in the oil samples from *C. lemon* with the highest percentage (78.28%).

Key words: Essential oils, aromatic plants, phytochemical analysis, antimicrobial activity.

INTRODUCTION

Multiple drug resistance in human and plant pathogenic microorganisms have been commonly reported in recent years from all over the world, particularly in developing countries, due to indiscriminate use of commercial antibiotics in the treatment of infectious diseases (Service, 1995). Though, the resistance development by microbes cannot be stopped, appropriate action will reduce the mortality and health care costs by using antibiotic resistant inhibitors of plant origin (Ahmad and Beg, 2001). Moreover, traditional remedies utilizing plants still occupy a central place among rural communities of developing countries for curing various diseases in the absence of an efficient primary health care system (Ali et al., 2001; Pandey, 2003).

The search for antimicrobials of plant origin has been mainly stimulated by the fact that some of the major antibacterial agents have considerable drawbacks in terms of limited antimicrobial spectrum. To date, resistance in bacteria is most prevalent. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) has become a huge problem worldwide to treat nosocomial infections since 1990s (Lee et al., 2007).

Essential oils also called volatile or ethereal oils have been the active principle of many important herbal remedies since ancient times (Guenther, 1948). The antimicrobial properties of essential oils are well recognized for many years and have been used as naturally occurring antimicrobial agents in phytopathology, medical microbiology, food preservation etc. (Burt, 2004). The chemical composition of essential oils depends on a number of parameters such as environmental conditions, collection period, dehydration procedure, storage condition and isolation methods (Magiatis et al., 2002). In view of the vast potentiality of aromatic plants as sources of antimicrobial

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Abbreviations: GC, Gas chromatograph; **HPTLC,** high performance thin layer chromatography; **DMSO,** dimethyl sulfoxide.

Table 1. Conditions for GC analysis of essential oils.

Oven Temperature (°C)	Rate (C/min)	Hold (min)	Total (min)
50	0.0	1.00	1.00
100	10.00	4.00	10.00
150	10.00	4.00	19.00
200	20.00	2.00	23.50
260	20.00	3.50	30.00

components, the present investigation was undertaken to determine the main constituents and the antibacterial and antifungal activities of five different indigenous plant essential oils.

MATERIALS AND METHODS

Collection of plant material

Different parts of five medicinally important plants, that is, *Ocimum sanctum* (leaves), *Eucalyptus globulus* (leaves), *Mentha arvensis* (leaves), *Citrus lemon* (fruit epicarp) and *Citrus maxima* (fruit epicarp) were collected from various localities of Imphal and Haridwar districts, India. The plant parts were washed thoroughly with running water and then air dried.

Extraction of essential oils

Hydrodistillation method was used for the extraction of essential oil by Clevenger type apparatus (Guenther, 1949). The oils were separately collected in airtight containers, dried over anhydrous sodium sulphate and stored at 4°C for further use. Dimethyl sulfoxide (DMSO) was used for preparing different concentration of oils, that is, 700 ppm for antibacterial and 1300 ppm for antifungal testing.

Microbial strains

The reference strains used in the antimicrobial assays were: Gram-positive bacteria (*Bacillus pumilus* MTCC 1607, *Bacillus subtilis* MTCC 441, *S. aureus* MTCC 737) and gram-negative bacteria (*Escherichia coli* MTCC 1687, *Klebsiella pneumoniae* MTCC 109, *Pseudomonas aeruginosa* MTCC 1688 and *Salmonella typhi* MTCC 1251). Test fungi and yeast included in the present study were: *Aspergillus niger* MTCC 1344, *Aspergillus flavus* MTCC 871 and *Candida albicans* MTCC 227. All the ten bacterial, fungal and yeast strains were procured from the IMTECH, Chandigarh, India. The test bacteria were maintained on nutrient agar slants. Among test fungi, *A. flavus* and *A. niger* were maintained on Sabouraud-chloramphenicol agar, while, *C. albicans* was maintained on malt yeast agar medium.

Antimicrobial activity of volatile constituents of essential oils

Inverted Petri plate method as described by Dubey et al. (2005) was used to test the antimicrobial activity of volatile constituents of aromatic plant essential oils. Twenty ml of specific growth medium was aseptically poured into five replicated sterile Petri plates for each microbe and allowed to solidify. Agar discs (4 mm diameter) were cut from actively growing margins of 5 days old culture of each fungus or 48 h old culture of a bacterium and yeast and placed at the center of petri dishes. The inoculated plates were then inverted upside down and a pre-sterilized filter paper disc (15 mm diameter, Whatman Grade No. 44) was aseptically transferred into the center of the inverted lid. The available space of diffusion for volatile constituents of essential oils in petri plates remained 50 ml. Requisite amount of oil was spread over the filter paper to obtain desired concentration of the oil. In the control set, oil was replaced by sterilized double distilled water. The antibacterial assay plates were incubated at 32°C for 48 h while in the case of fungi, the dishes were incubated at 25°C for 120 h. After completion of incubation period, percent inhibition of growth was calculated according to the formula:

$$\% \text{ inhibition} = (C - T / C) \times 100$$

Where, C = growth in control plates; T = growth in test plates

Gas chromatography (GC) analysis of oils

The Varian gas chromatograph equipped with column VA-17, length 15 meter, I.D.-0.53 mm and film 1 µm was used. The other conditions for GC analysis of essential oils are as shown in Table 1. GC analysis was carried out by comparing the retention indices with those of authentic compounds. The compounds were identified and matched with the library.

High performance thin layer chromatography (HPTLC analysis) of essential oils

HPTLC analysis of essential oils was performed with the following analytical and chromatographic conditions: Plate material, HPTLC Precoated Plates Silica Gel MERCK 60F254; Solvent, Toluene (Ethyl acetate 93:07); Application mode, CAMAG Automatic TLC Sampler III; Development mode, CAMAG Twin Trough Chamber. HPTLC was performed to determine the percentage of main compound present in the oil.

RESULTS AND DISCUSSION

Twenty phytochemicals were identified by the GC analysis as constituents of different plant essential oils. Among these, six chemicals were common in *E. globulus* and *M. arvensis* oils (Table 2). *O. sanctum* included four constituents, while *C. lemon* and *C. maxima* showed the presence of two and three components, respectively

Table 2. Chemical constituents of essential oils[a].

Chemical constituents	Essential oils				
	O. sanctum	*E. globulus*	*M. arvensis*	*C. lemon*	*C. maxima*
Menthol	-	-	+	-	-
Menthone	-	-	+	-	-
(+) Limonene	-	-	+	-	-
Chavicol	+	-	-	-	-
Eugenol	+	-	-	-	-
Linalool	+	-	-	-	-
Caryophyllene	+	-	-	-	-
Transmethone	-	-	+	-	-
Menthol Acetate	-	-	+	-	-
Pinenediol	-	-	+	-	-
Limonene	-	-	-	+	+
Iseungenol	-	-	-	+	-
Carvon	-	-	-	-	+
Limonene II	-	-	-	-	+
Cineole	-	+	-	-	-
p-cymene	-	+	-	-	-
Eucalyptol	-	+	-	-	-
β-terpineol	-	+	-	-	-
Verbenone	-	+	-	-	-
α-thusenol	-	+	-	-	-

[a]As estimated by gas chromatography (GC analysis).

Table 3. Percentage of main compounds in the essential oils[a].

Essential oils	Main compound	Percentage of main compound
O. sanctum	Eugenol	56.07
E. globulus	1, 8 cineole	17.34
M. arvensis	Menthol	43.45
C. lemon	Limonene	78.28
C. maxima	Limonene	49.66

[a]As determined by high performance thin layer chromatography (HPTLC).

(Table 2). Maximum amount of eugenol was recorded in *O. sanctum* which accounted for 56.07% of the oil (Table 3). Major component of *E. globulus* was found to be 1, 8 cineole (17.34%). Limonene was the only major constituent present in the oil samples from *Citrus* spp. with highest percentage found in *C. lemon* (78.28%). The maximum percentage of menthol (43.45%) was detected in *M. arvensis*. Thus, overall chemical composition analysis indicates that *O. sanctum*, *E. globulus* and *M. arvensis* oils have higher diversity of phytochemicals than *Citrus* oils. The results of essential oils constituents are in accordance with the data published earlier (Gangrade et al., 1989; Pandey, 2003; Nickawar and Mojab, 2003; Brophy et al., 1998).

Volatile constituents of *O. sanctum*, *E. globulus* and *M. arvensis* oils exhibited high antibacterial activity, which may be attributed to the presence of diversity of phytochemicals as earlier mentioned. The essential oil of *O. sanctum* caused complete inhibition in growth of all the seven test bacteria while that of *E. globulus* and *M. arvensis* showed complete growth inhibition of six and five test bacteria, respectively (Table 4).

Essential oil of *O. sanctum* also showed the complete growth inhibition of all the test fungi and yeast employed in the present study (Table 5). The highest antimicrobial activity of essential oil of *O. sanctum* may be attributed to high percentage of eugenol. Presence of both anti-bacterial and antifungal activities in essential oil of *O. gratissimum* has been demonstrated by Matasyoh et al. (2007). Antibacterial activity of *E. globulus* oil is probably

Table 4. Antibacterial activity of volatile constituents of essential oils[a].

Test plants	Growth inhibition (%) [b]						
	B. pumilus	***B. subtilis***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***	***S. typhi***
O. sanctum	100	100	100	100	100	100	100
E. globulus	100	100	92.3	100	100	100	92.7
M. arvensis	100	100	100	100	100	100	91.6
C. lemon	39.1	49.2	45.2	54.2	54.5	54.8	33.5
C. maxima	38.6	48.1	41.5	52.7	49.1	50.0	30.9

[a]As determined by inverted Petri plate method; [b]Values represent an average of five replicates; concentration of oil was 700 ppm.

Table 5. Antifungal activity of volatile constituents of essential oils[a].

Test plants	Growth inhibition (%)[b]		
	A. niger	***A. flavus***	***C. albicans***
O. sanctum	100	100	100
E. globulus	80.4	86.2	9.7
M. arvensis	83.6	90.0	100
C. lemon	45.2	50.6	47.7
C. maxima	51.2	46.2	41.1

[a]As determined by inverted Petri plate method; [b]Values represent an average of five replicates; concentration of oil was 1300 ppm.

related to the high content of 1, 8 cineole (Magiatis et al., 2002) which has been found to reduce the growth and inhibit the spore production and germination of wide range of microbes (Batish et al., 2008). Our findings on antifungal assay revealed that the essential oil of *E. globulus* was least effective against *C. albicans*.

Volatile constituents of *C. lemon* and *C. maxima* displayed low antimicrobial activities against all the test bacteria and fungi (Tables 4 and 5). This may be due to the presence of low concentration of active constituents. Extraction method may also affect the antimicrobial property of volatile constituents. Moreover, *Citrus* oils may be highly effective against pathogenic micro-organisms that have not been employed in this study. All these interesting aspects need to be studied further.

Essentials oils of higher plants have also been evaluated against pathogenic microorganisms (bacteria, fungi and yeast) by many other workers (Orafidiya, 1993; Rasooli and Rezaei, 2002; Ozcan, 2003; Dubey et al., 2005) and the results support the findings of the present investigation. An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition in the lipids of bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable as a result of which leakage of ions and other cell contents can then occur (Sikkema et al., 1994). Although, a certain amount of leakage from bacterial cells may be tolerable without loss of viability, extensive cell contents loss or the exit of critical molecules and ions will lead to death (Denyer and Hugo, 1991).

It has also been reported that gram-negative strains are less susceptible to essential oils due to the presence of an outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Fredj et al., 2007). In this study, strong antimicrobial potential of oils against both gram-positive and gram-negative strains are not in accordance with the previous reports. It seems that volatile constituents of active essential oils (*O. sanctum*, *E. globulus* and *M. arvensis*), possess broad spectrum activity, and therefore, might be used to target multi-drug resistant pathogenic microorganisms.

A couple of decades ago, *C. albicans* was commonly regarded as little more than culture contaminant, however, because of developed antimicrobial resistance in less than two decades, this organism has become a major human pathogen. The increase in the infections of *Candida* sp. is directly related to the growing number of immuno-suppressed and immuno-compromised patients, who frequently develop opportunistic systemic and superficial mycoses such as candidiasis (Michael and Pharm, 2001). The presence of strong antifungal activity of volatile constituents of *O. sanctum* and *M. arvensis* against *C. albicans* shows that these oils may be exploited for their potential as an additional source of antibiotics with less toxicity.

Toxigenic strains of *A. flavus* produce aflatoxins on a

wide range of agricultural products under warm climatic conditions coupled with high relative humidity (85%). Our findings revealed that essential oils may constitute an ideal storage toxicant for protection of stored food stuffs from microorganisms. The oils of *O. sanctum*, *E. globulus* and *M. arvensis* might be used as an alternative to provide an indigenous, cheap and renewable source of plant protection in place of synthetic chemicals where most of them exhibit side effects.

Conclusion

The results of the present study clearly indicate that anti-bacterial and antifungal activities vary with the essential oils of different plant species and the plant parts used which possess variable phytochemicals that may be pharmacologically important for commercial exploitation. Further experiments are needed to evaluate the chemical structure of principle active compounds of essential oils against inhibitory mechanism and drug resistance in wide variety of pathogenic microorganisms.

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