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EVALUATION OF ANTIFUNGAL ACTIVITY OF CITRUS GRANDIS ESSENTIAL OIL AND CHEMICAL COMPOSITION

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ABSTRACT

The fungi have an important role in the environment. They cause the infections when grow on human. There is urgent need to get a new therapeutics or remedies from nature to inhibit the growth of pathogenic fungi. In the present study, the chemical composition of *Citrus grandis* volatile oil and anti-dermatophytic activity was evaluated against isolated dermatophytic species. The Clear pale yellowish color with fresh and sweet aroma oil was extracted from the leaves of *Citrus grandis* that revealed the presence of 39 volatile components. The oil has revealed excellent inhibition activity against test organisms with presence of Maximum Inhibition Zone of 14.33 mm for *Microsporum gypsium* as well as 14 mm against *Trichophyton mentagrophytes* (KU578106) as compared to standard. The present study detailed to chemical composition of *Citrus grandis* volatile oil with the confirmative presence of phenol components. The data also prove the anti-dermatophytic potential against *Microsporum* and *Trichophyton* species. The data also represent the further requirements for to investigate the various pharmacological behaviors to its commercialization for the advantage of human beings.

KEYWORDS: *Dermatophytes; Citrus grandis; Volatile; Hydro Distillation; MIC*

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INTRODUCTION

Fungus also has individual kingdom that plays an important role in environment since evolution[1]. They are the second biggest group of microorganisms after the insects and capable to colonize an extensive variety of different substrata[2]. In major classification system of fungi, the *Deuteromycota* division has a broad account of pathogenic fungi that cause infections to living organisms. Some of them also have ability to penetrate the natural keratin on living organisms and functions as keratinolytic agent[3-4]. These keratinophilic fungi also cause superficial infections i.e. dermatophytosis or Tinea[4]. During the last decades, mycotic infections are raised up to 20–25% in world's population[5-6].The leading phenomena of mycotic infections are prompted an extensive search for new drugs to treat mycoses from nature[7-8]. The plant *Citrus grandis* is also a natural citrus fruit belonging to Rutaceae family with the appearance of a big grapefruit. The fruit part is usually pale green to yellow when ripe, with sweet white flesh and very thick albedo (rind pith). It is a large citrus fruit about 15–25 centimeters in diameter. The *Citrus* oil has therapeutic, perfumery, flavoring, and antimicrobial properties[9]. So Basis on the remedial aptitudes of *Citrus,* in present study the detailed chemical composition of its volatile oil and anti-dermatophytic activity was evaluated against *Microsporum gypseum*, and *Trichophyton mentagrophytes* (KU578106) species.

MATERIAL& METHODOLOGY

Collection of plant material & extraction of essential oil

The Leaves part of Chakotra (*Citrus grandis*) was collected from fresh plants from Rishikesh and Forest Research Institute, Dehradun Region of Uttarakhand. About 500 gm of powered materials was subjected in Clevenger apparatus hydro distillation unit on 70ºC–80ºC for 3-5hrs with approximately four times water (w/v). The extracted fractions of oily and aqueous layers were separated with saturation in petroleum ether and dehydrated by anhydrous sodium sulphate[10-11].

Characterization of Essential Oil

Identification of pharmacologically active constituents from crude essential oil was characterized by gas chromatography (GC) and gas chromatography mass spectrometric (GC/MS) methods with using RTX5MS column. The detected compounds were identified by processing the raw GC-MS data of Wiley and comparing with National Institute of Standard and Technology (NIST, USA) mass spectral database and from retention times and mass spectra of standard compounds[12-13].

Fungal Isolation

To isolate the fungal isolates of *M. gypseum* and *T. mentagrophytes* (KU578106) were isolated from the skin samples collected from outdoor patients at the SMS Medical College and Hospital, Jaipur. The cultures were maintained on SDA agar slants supplemented with antibiotics and isolates of were identified from to via microscopic identification[5] and 18S rRNA sequencing. Sequences are submitted to NCBI Gene bank[14].

Inoculums Preparation

For estimation of antifungal activity, the inoculums were prepared using sterile autoclaved distilled water and mixing it with surface growth, spores and hyphae by sterile wire loop. The concentration of suspension up to 90% transmittance with approximates 1×10^6 CFU/ml spores fixed by spectrophotometer at 530 nm[5].

Determination of Antifungal activity & MIC

The antifungal activity was carried out by the agar disc diffusion method of the *Citrus* oil against isolated fungal species of dermatophytes[15-16]. In methodology, 6.0 mm discs of Whatman No.1 paper sterilized in autoclave, were soaked in pure oil was placed on an agar plates containing fungal spore suspension. Respectively Ketoconazole (10mcg/disc) and Fluconazole (10mcg/disc) were used as a positive control. The plates were incubated at 30°C for 48 to 72 hrs. Three replicates were kept in each case and average values were calculated. The diameter of the inhibition zones (including diameter of the disk) was measured in mm and the activity index was calculated on the basis of the size of the inhibition zone.

Activity index = Inhibition zone of sample / Inhibition zone of standard

In the subsequent method, the different concentrations series as Pure: 1/2: 1/4: 1/5: 1/7 (Pure: 50: 25: 20: 14%) of essential oil were also diluted in DMSO solution. In present study, minimum inhibitory concentration was evaluated using micro dilution method in essential oils using SDB Medium poured into sterile capped 2ml eppendorfs [17-20]. In tubes, different concentrations of *Citrus grandis* oil diluted with DMSO ranging in 5: 10: 15: 20: 25: 30: 50: 75: 100µl were added using micropipettes. Then 0.1 µl inoculums suspensions was inserted deep into each eppendorfs containing broth with different concentration of oil as well as a oil-free control. The tubes were then incubated at 30ºC for 48-72 hours to determine the MIC. MIC was read to be the lowest concentration at which there was no visible growth of the organism by visual inspection. Experiments were performed in triplicate and data analyzed are mean± SE subjected to one way ANOVA with significant ($P<0.05$).

RESULT & DISCUSSION

The clear pale yellowish color with fresh and sweet aroma oil was extracted from the leaves of *Citrus grandis*. The GC and GC-MS analysis revealed the presence of 37 volatile components. The percentage composition and names of the essential oil components are listed in Table-1 and Figure-1. The major components of essential oil were Spathulenol (31.66%), β-Caryophyllene (24.49%), ε-Longipianol (8.74%), Bicyclogermacrene (6.18%), P-Allylguaiacol (4.55%), α-Humulen (3.45%), δ-Cadinene (2.61%), Intermedeol (2.21%), ζ-Nerolidole (2.02%), Longifolenaldehyde (2.07%), α-Muurolene (1.44%), β-Copaene (1.00%) and other components were present in trace amounts as presented in Table1.

Table 1
Chemical composition of leaf essential oil of Citrus grandis

Peak Number	Reaction Time	Area%	Name of Compound	RI	Molecular Weight	Molecular Formula
1.	13.666	0.23	Cyclohexanol	1028	154	$C_{10}H_{18}O$
2.	15.619	0.12	Trans-Linalool Oxide	1070	170	$C_{10}H_{18}O_2$
3.	16.918	0.22	β-Linalool	1098	154	$C^{10}H^{18}O$
4.	18.750	0.16	Trans -Pinocarveol	1137	152	$C^{10}H^{16}O$
5.	20.580	0.21	4-Terpineol	1176	154	$C^{10}H^{18}O$
6.	21.221	0.14	α-Terpineol	1189	154	$C^{10}H^{18}O$
7.	21.504	0.22	Myreteneol	1195	152	$C^{10}H^{16}O$
8.	22.983	0.51	β-Citronellol	1227	156	$C^{10}H^{20}O$
9.	24.189	0.19	Geraniol	1253	154	$C^{10}H^{18}O$
10.	27.929	0.22	δ-Elemene	1337	204	$C_{15}H_{24}$
11.	28.837	4.55	P-Allylguaiacol	1357	164	$C_{10}H_{12}O_2$
12.	29.637	0.34	α-Copaene	1375	204	$C_{15}H_{24}$
13.	30.336	0.59	Germacrene A	1391	204	$C_{15}H_{24}$
14.	31.601	24.49	β-Caryophyllene	1421	204	$C_{15}H_{24}$
15.	31.926	0.99	β-Copaene	1429	204	$C_{15}H_{24}$
16.	32.340	0.53	Alloaromadendren	1439	204	$C_{15}H_{24}$
17.	32.961	3.45	α-Humulen	1454	204	$C_{15}H_{24}$
18.	33.253	0.28	Aromadendrene	1461	204	$C_{15}H_{24}$
19.	33.900	0.46	ε -Amorphene	1476	204	$C_{15}H_{24}$
20.	34.092	0.37	Germacrene D	1481	204	$C_{15}H_{24}$
21.	34.277	0.84	β-Lonene	1485	192	$C_{13}H_{20}O$
22.	34.749	6.18	Bicyclogermacrene	1497	204	$C_{15}H_{24}$
23.	34.878	1.44	α-Muurolene	1500	204	$C_{15}H_{24}$
24.	35.430	0.53	γ-Cadinene	1514	204	$C_{15}H_{24}$
25.	35.807	2.61	δ-Cadinene	1523	204	$C_{15}H_{24}$
26.	36.974	0.45	Caryophyllene Oxide	1553	220	$C_{15}H_{24}O$
27.	37.426	2.02	ζ -Nerolidole	1565	222	$C_{15}H_{26}O$
28.	38.141	31.66	Spathulenol	1583	220	$C_{15}H_{24}O$
29.	38.283	8.74	ε-Longipianol	1586	220	$C_{15}H_{24}O$
30.	38.575	0.55	Viridiflorol	1594	222	$C_{15}H_{26}O$
31.	38.994	0.28	Epiglobulol	1605	222	$C_{15}H_{26}O$
32.	39.232	0.83	Cedroxyde	1611	220	$C_{15}H_{24}O$
33.	40.265	0.67	Bicyclo[7.2.0]Undecan-5-Ol	1639	220	$C_{15}H_{24}O$
34.	40.774	0.33	β- Eudesmol	1652	222	$C_{15}H_{26}O$
35.	40.937	2.21	Intermedeol	1657	222	$C_{15}H_{26}O$
36.	41.243	0.30	Nootkatol	1665	220	$C_{15}H_{24}O$
37.	41.530	2.07	Longifolenaldehyde	1673	222	$C_{15}H_{26}O$

In Disc diffusion method (Table 2), C. grandis confirmed good antidermatophytic activity against selected test fungi. Maximum zone of inhibition was found to be 14.33mm against M. gypsium (Inhibition Zone (IZ): 14.33mm, Activity Index (AI): 0.72) as compared to standard drug. Likewise, the plant oil also revealed good T. mentagrophytes (KU578106) activity against (IZ: 14.00 mm, AI: 0.46) as compared to standard (Table-2).

Table 2
Antifungal activity of Citrus grandis essential oil against pathogenic organisms

Test Strain	Antifungal activity of *Citrus grandis* (in mm)				
	IZ of Oil Sample	IZ of standard Ketoconazole	AI	IZ of Standard Fluconazole	AI
M. gypseum	14.33±0.23	20±2.82	0.72	-	-
T. mentagrophytes (KU578106)	14±0.57	30±3.88	0.46	14±0.70	1.00

Concentration of oil used 100%. IZ = inhibition zone (in mm) including the diameter of disc (6 mm), AI = activity index.

Ketaconazole is a best antifungal standard against dermatophytes. Fluconazole was observed in resistant as compare to Ketoconazole. It has resulted only for the *T. mentagrophytes* (KU578106) for growth inhibition.

Table 3
Minimum Inhibition activity of C. grandis essential oil against pathogenic organisms

Minimum Inhibition activity for *C. grandis* Essential oil (in mm)				
Test Fungi	Oil Concentrations			
	1/2 (50%)	1/4 (25%)	1/5 (20%)	1/7 (14%)
Microsporum gypseum	11.00±0.70	10.50±0.28	9.33±0.47	8.33±0.24
Trichophyton mentagrophytes()	10.25±1.42	09.00±1.73	8.50±0.28	≥6

IZ = inhibition zone (in mm) including the diameter of disc (6 mm), AI = activity index.

The Minimum inhibition activity of *C. grandis* essential oil was evaluated by modified dilution in oil concentration method against different test fungi. The results showed (Table 2 & 3) that all selected test fungi have good records of resistance activity against neat essential oil (100% concentration) with formation of inhibition zone. In selected fungi, *T. mentagrophytes* (KU578106) shown the inhibitory action at 20% as lowest range of oil concentration mixed in DMSO Whereas the *M. gypseum* showed their highest inhibitory action at 14% oil concentration (Table-33). In The MIC Value against all selected strains was observed to start from 15µl/ml concentration as minimum of essential oil mixed in DMSO.

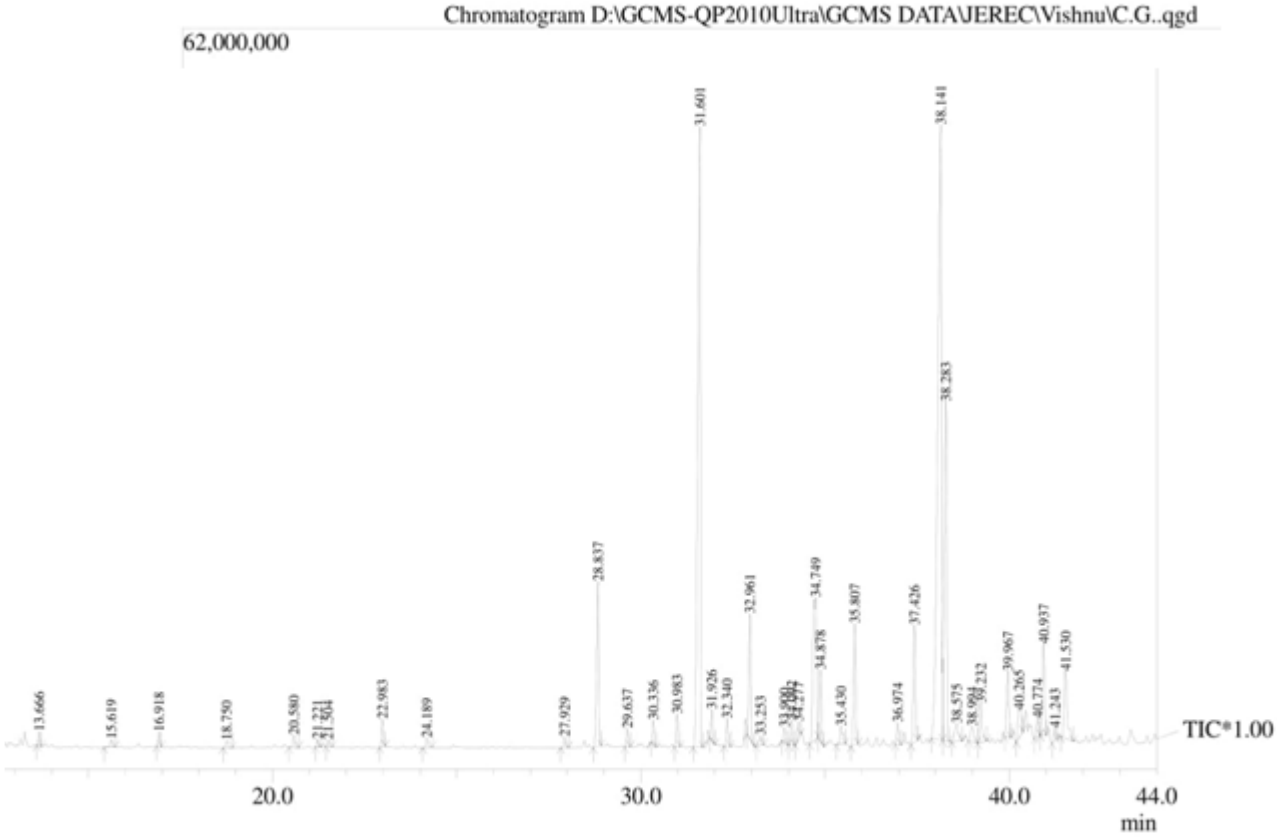

Figure 1
Chromatogram of essential oil of Citrus grandis by GC-MS.

In case of *Citrus grandis*'s Essential oil that was recorded with 37 volatile components in clear pale yellowish colored oil. In present study, the similar study was also showed by Njoroge et al. Who compared the volatile constituents of cold-pressed peel essential oils of *Citrus paradisi* and *Citrus grandis* from Kenya and reported the presence of limonene (91.1 and 94.8%), alpha-terpinene(1.3 and 1.8%), and alpha-pinene (0.5%) as the main compounds[21]. The Heptyl acetate, octanal, decanal, citronellal, and (Z)-carvone were the main constituents (0.1-0.5%). Perillene, (E)-carveol, and perillyl acetate occurred in the redblush grapefruit but were absent from the pummelo oil. From Korea, in study of Baik et al., 2008 also reported limonene (68.08%), β-myrcene (22.65%), β-pinene (14.74%), linalool (6.23%) and γ-terpinene (1.63%) as main ingredients of *C. grandis* oil essential oils[22]. Subsequently, Kamal et al. investigated the variation in the yield and chemical composition of the essential oils isolated from fresh, ambient-, and oven-dried peels of three Citrus species namely *Citrus reticulata, Citrus sinensis* and *Citrus paradisii* and documented The limonene, the most prevalent chemical constituent ranged from 64.1-71.1% (*C. reticulata*), 66.8-80.9% (*C. sinensis*) and 50.8-65.5% (*C. paradisii*)[23]. However, Neng-Guo & Yue-Jin et al also obtained Twenty-one components such as limonene (89.96 ± 1.64%), followed by β-myrcene (4.49 ± 0.38%), α-pinene (0.63 ± 0.05%), 3-carene (0.48 ± 0.04%), caryophyllene (0.47 ± 0.04%)[24]. Serially, Vasudeva and Sharma also reported fourteen different components including limonene (89.089%), β-myrcene (2.933%), linalool (2.927%), α-pinene (0.865%), (E)-citral (0.749%) as major part of *Citrus grandis* essential oil[17]. In Algeria, Abderrezak et al. reported the linalool (18.6%), Cis-linalool oxide (8.1%), trans-carveol (11.9%), endo-fenchyl acetate (5.5%), carvone (5.8%) and γ-terpinene (6.9 %) in essential oils of *Citrus aurantium*[25]. Darjazi et al. reported the Aldehydes ranged from 0.14% to 0.28% as oxygenated flavor components from pummelo oil[26]. Subsequently Ou et al. conducted a study to compare difference with in chemical composition *C. grandis* oils extracted using cold-pressed and distilled techniques and found that a greater amount via distilled 55.74% in compare to cold-pressed 32.63% respectively[27]. The major constitution was β-pinene with following to linalool, β-citral and α-citral, β-pinene and limonene in oil of *C. grandis*. Recently Dar et al. also confirmed the presence of limonene (93.50%), β-pinene (2.979%) and α-pinene (0.792%) as main components in the essential oil[28]. Sajid et al. also characterized the essential oils from *Citrus pseudolimon* and *Citrus grandis* peels and

reported thirty six in *C. pseudolimon* and thirty three total components in *C. grandis* including limonene in range 47.07% and 71.48% as the major component[29]. So from according to presented data, these components in essential oils depend on relation to climate, soil composition, and part of the plant with age of the plant[30]

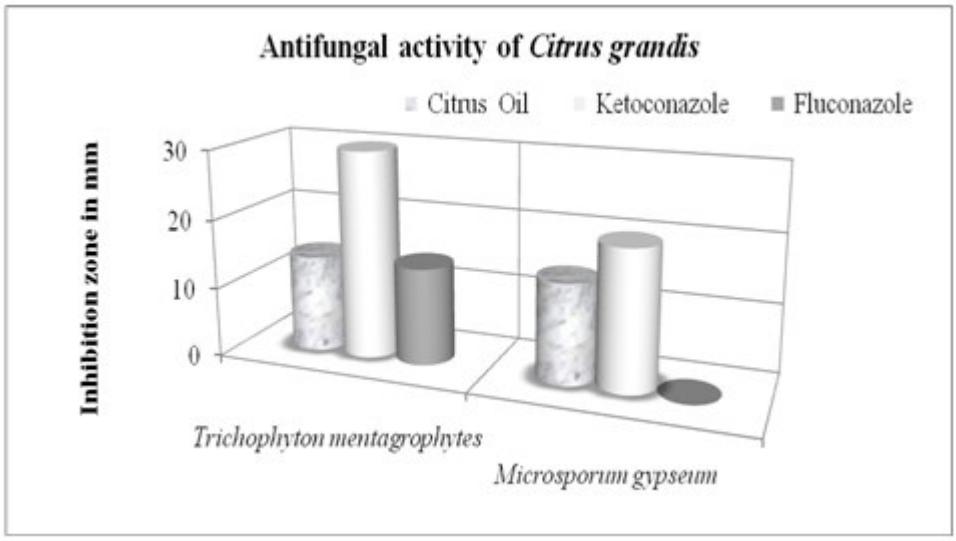

Figure 2
Graphical representation of comparative antifungal activity of Citrus grandis essential oil with standards against Dermatophytes.

The present results of Anti-Dermatophytic activity of *C. grandis* essential oil by disc diffusion methods showed the agreement with Vasudeva and Sharma who reported The highest antifungal activity of Citrus species essential oil against *Fusarium oxysporum* (10.23 mm) followed by *Aspergillus niger, Aspergillus fumigatus* with zone of inhibition of 9.9 mm and 9.32 mm respectively[17]. The MIC ranging from 3.12 µl/ml to50 µl/ml was also recorded in study. Here we applied the same procedure for evaluate the antifungal potential against dermatophytic strains. Subsequently in study of Dhiman et al., 2010, The *C. sinensis* fruit peel methanolic extract exhibited appreciable antifungal activity with minimum inhibitory concentration of 12.5 µg/ml against *C. albicans* and *A. niger* [31].

CONCLUSION

The Citrus Essential oils have aromatic compounds that possess wide spectra of antimicrobial activity. The results showed that the essential oil of *C. grandis* leaves was also able to inhibit the fungi growth used in this study with different degrees of inhibition. Further, pharmacological studies are required to construct actual herbals in treating various infections and skin diseases.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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