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Chemical composition and additive inhibitory activity of combination of *Mentha piperita, Cinnamonum vernum & Jasminum officinale* essential oils as an alternative therapy against Dermatophytosis

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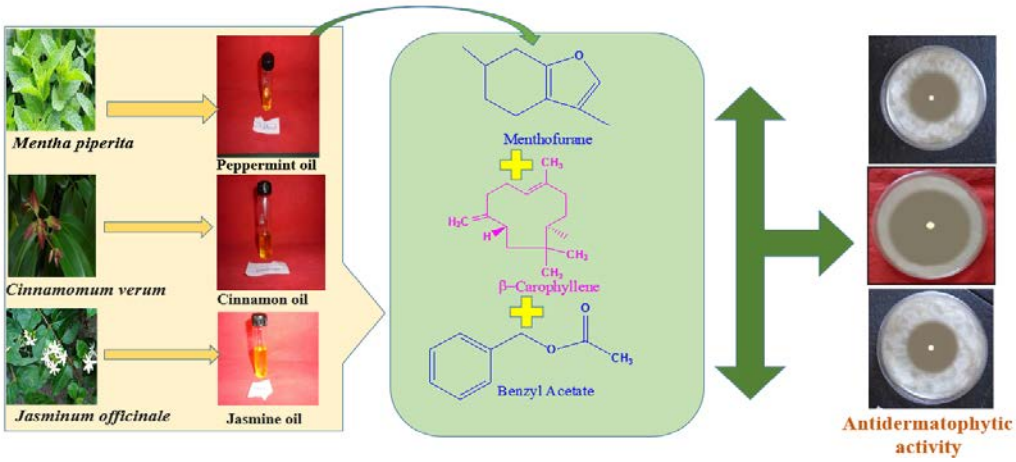
Article

ABSTRACT



The antifungal potential of Peppermint, Cinnamon & Jasmine essential oils alone and in combination, against common causes of superficial fungal infections in humans was investigated via *in-vitro* investigations, in order to determine a suitable dosage for use in clinical trials. Antimycotic activity of three plant derived Essential oils (EOs) namely *Mentha piperita*, *Cinnamonum vernum* & *Jasminum officinale* were evaluated against *Trichophyton equinum* and *Microsporum canis*, causative agent of zoonotic dermatophytosis by Disc diffusion method alone and in mixture and further determination of Minimum Inhibitory Concentration (MIC) by modified Microdilution method. Chemical compositions of *M.piperita, C.vernum & J. officinale* essential oil were determined by Gas chromatography Mass spectrometry (GC-MS). In *M.piperita* essential oil, thirteen compounds, *C.vernum*, ten compounds and *J. officinale*, fifteen compounds were identified by GC-MS. The excellent antidermatophytic activity of mixture of oils was found against *M.canis* & *T.equinum* as compared to single oils and standard drugs used. The results concluded that the combination of three essential oils showed remarkable and excellent inhibitory activity against fungal pathogens and can be used as an alternative topical therapy for the treatment of dermatophytosis after undergoing clinical trials and also regarded as an environmentally safe mode of diseases control.

Keywords: Additive, Essential oils, Chemical, GC-MS, Dermatophytosis

INTRODUCTION

Dermatophytosis is most prevalent form of fungal infections found in India because of the tropical climatic and humid conditions. The infection is caused by a group of keratinophilic fungi called dermatophytes. Fungi belong to the three genera namely, *Trichophyton*, *Microsporum* and *Epidermophyton.* Dermatophytosis can transfer from soil and animals to humans and cause infection on many parts of the body.[1-3] Dermatophytosis, an integumentary, cosmopolitan mycotic disease, is important from public health as well as economic point of view and is prevalent both in sporadic and epidemic forms over 145 countries of the world including India. It is an important occupational mycozoonoses of dairymen, animal handlers, livestock farmers, pet owners, veterinarians, etc.[4] *Trichophyton spp*. and *Microsporum spp.* cause skin diseases in animals, such as *T. equinum* and *M. canis*, which are known as zoophilic dermatophytes. The transmission of these zoophilic dermatophytes occurs via infective arthrospores coming from the hair coat of infected animals or the environment.[5] Poor unhygienic conditions and management of pets transmitted the infection to other animals followed by infection in humans irrespective of any age where they colonize outermost keratinized tissues of the skin.[6] Dermatophytes infections in humans occur after contact with contaminated products or specimens, such as soil, hair, or crust on the epidermal layer of infected animals.[7]

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Predisposing factors affecting dermatophytosis include the number of infective spores, frequency of transmission, health conditions, and the physiological stress experienced by animals This infection occurs either directly through contact with sick cats or indirectly through blankets, bed covers, toys, cages, clothes, and other objects contaminated with spores. Various previous researchers have shown that the *Mentha piperita* essential oil possess antiviral, antibacterial, antifungal, antibiofilm formation, radioprotective, antioedema, analgesic and antioxidant activities of the EO because of its main components, Menthol and Menthone.[8-10] Similarly *Cinnamonum vernum* EOs is a good source of new antimicrobial agents. With regards to volatile components, the chemical composition of cinnamon essential oils depends on the part of the plant from which they are extracted.[11-13] *J. officinale* belongs to Oleaceae family and is an attractive vine. It contains sweet smelling flowers that yield fragrant essential oil.[14] It is also used to treat fever, diabetes, diarrhoea, ringworm, ulcers and eruptions in mouth.[15] *J. officinale var. grandiflorum* contains alkaloids, triterpenoids, flavonoids, tannins, steroids, glycosides, terpenes, salicylic acid and resins. Various components have been reported to be present in the jasmine essential oil.[16] The benefit of essential oil combinations against fungal pathogens is highlighted, as the majority of combinations resulted in noteworthy antifungal activity, and several displayed synergistic interactions against each of the reference strains respectively. This highlights the potential of essential oil combinations, not only to act as an antifungal, but also for their ability to permeate into lower skin levels where conventional antifungals are unable to reach. An additional advantage of using essential oils is that not only have they been shown to be beneficial against the fungal pathogens, they are also able to inhibit bacterial pathogens, thereby potentially preventing secondary bacterial infections. The aim of this work was to analyze the chemical composition of three essential oils and to evaluate the antifungal activity of *M. piperita*, *C. vernum* & *J. officinale* alone and their mixtures against dermatophytes and their antifungal results compared with two synthetic, commercially available antibiotics.

RESULTS AND DISCUSSION

The emergence of antifungal resistant strains of various fungi has prompted researchers to develop new strategies for fighting fungal infections[17]**.** Natural antimicrobials are of utmost importance due to safety issue and availability. Hence, search for new, cheaper antimycotic from natural sources is an urgent need. The data obtained in the present investigation proves the antidermatophytic activity of essential oils. The response of dermatophytes to treatment with various plants products varied from organism to organism. Essential oils have been used since ancient times in aromatherapy and disease control. In the present study, extraction of essential oils from the fresh leaves of *M. piperita*, bark of *C. vernum* & flower of *J. officinale* were carried out by standard hydrodistillation method, Clevenger's apparatus in a laboratory and all operations were carried out at room temperature (Figure 1). The chemical composition of essential oils *M.piperita*, *C.vernum* & *J. officinale* essential oil and antidermatophytic activity of essential oil (alone and in combination) was screened against dermatophytes which are causal organisms of Zoonotic Dermatophytosis or ringworm infection, by using disc diffusion method. In the present study, *T. equinum* and *M. canis* were isolated from horse and cat habitat of soil samples and these two etiological dermatophytes were responsible for Zoonotic dermatophytosis from animals to human beings hence antidermatophytic studies were carried out on *T.equinum* and *M.canis*. Isolated dermatophytes were identified from Institute of Microbial Technology (IMTECH), Chandigarh, India. These dermatophytes were identified as *Trichophyton equinum* (MTCC 570) and *Microsporum canis* (MTCC 761) on the basis of phenotypic characteristics.

Table 1. Chemical components (%) of the essential oils distilled from *Mentha piperita*

Compounds	**RI**	**% oil**
α-Pinene	930	0.32
Sabinene	970	0.26
β-pinene-1	978	0.58
β-pinene-2	1035	6.69
Ci$-sabinene hydrate	1072	0.50
Menthone	1155	2.45
Menthofuran	1168	11.18
Neomenthal	1166	2.79
Menthol	1178	53.28
Neomenthy acetate	1274	0.65
Menthyl acetate	1298	15.10
Isomenthyl acetate	1308	0.61
β -Bourbonene	1389	0.37

Several antimycotic drugs are available in the market, but due to various side-effects, long duration of treatment and high cost of the drugs, treatments have not been successful in some cases. Thus, a search for new drugs with better and cheaper substitutes from plant resources is natural choice. These findings promoted us to explore other plant products which could be exploited as effective antifungals. In the present study, chemical composition of selected three essential oils was determined by GC-MS (Table 1-3). GC/MS analyses of *M. piperita* essential oil showed that the main constituent of the *M. piperita* EO was Menthol (53.28%) followed by Menthyl acetate (15.1%) and Menthofuran (11.18%) as shown in Table 1.

In the present study, thirteen (13) compounds representing 99.37% area of the oil were identified. The composition of the EOs might be affected by the developmental stages of the plant. Some authors reported alpha terpinen as the dominant component of *M. piperita* EO (19.7%) while other previous studies identified Menthol as one of the main constituents of the EO.[18,19]

Table 2. Chemical components (%) of the essential oils distilled from *Cinnamonum vernum*

Compounds	RI	% oil
E-cinnamaldehyde	15.22	7.2
α-Pinene Linalool	5.66	1.3
Linalool	9.86	8
β-Caryophyllene	18.58	7.4
Eucalyptol	8.08	6.4
Eugenol	16.90	5.6
α-Humulene	19.47	1.7
δ-Cadinene	20.97	1.4
p-Cymene	7.82	1.9
Limonene	7.93	1.2

Our findings coincide with Camele *et al* who also reported the chemical analysis of peppermint oil by using GC-MS and identified the main components were menthol (70.08%) and menthone (14.49%) followed by limonene (4.32%), menthyl acetate (3.76%) and β-caryophyllene (2.96%).[20.] Several research projects reported the antifungal efficacy of plant EOs against postharvest fruit pathogens, being considered natural, safe and biodegradable alternatives.[21,22] Similarly, second oil i.e. *C.vernum,* chemical constituents was identified by GC-MS analysis resulted in the identification of ten (10) chemical compounds for *C.vernum* essential oil, as indicated in Tables 2. (*E*)-cinnamaldehyde (7.2%), linalool (8.00%), *β*-caryophyllene (7.40%), eucalyptol (6.40%) and eugenol (5.60%) were the main components of the *C. vernum* essential oil. In accordance with our results, several studies have reported that cinnamaldehyde is the major chemical compound of *C. zeylanicum* bark essential oil.[23,24] Behbahani et al reported same findings, (E)-cinnamaldehyde (71.50%), linalool (7.00%), β-caryophyllene (6.40%), eucalyptol (5.40%), and eugenol (4.60%). *C. zeylanicum* essential oil contained remarkable levels of phenolic and bioactive compounds with outstanding ability to scavenge free radicals and inhibit β-carotene oxidation.[25] Third essential oil, *J. officinale*, more than 15 chemical constituents were identified by GC-MS as seen in Table 3.

GC-MS analysis of *J. officinale* essential oil obtained through hydrodistillation method indicated the presence of various constituents including benzyl acetate, benzyl benzoate, isobutylene epoxide, hydroperoxide pentyl, benzyl alcohol, 5-hexene-2-one, phytol, linalool, isophytol, eugenol, geranyl linalool, methyl linoleate, cis-jasmone, indole and methyl palmitate. Among the identified compounds, benzyl alcohol has been reported in *J. officinale* and *J. sambac*.[26] These results are in accordance with Rao & Rout in 2002 & 2003 reported more than 100 constituents have been found in various jasmine samples with the main chemical components being benzyl acetate, linalool, benzyl alcohol, indole, benzyl benzoate, cis-jasmone, geraniol, methyl anthranilate, α-terpineol, cis-3-hexenyl benzoate, eugenol, nerol, farnesol, p-cresol, benzoic acid, benzaldehyde, nerolidol, isophytol, phytol and phytyl acetate[27-29] while others Toyoda et al (1978) identified some volatile N-heterocycles and fatty acid esters have also been found either in low concentrations or as trace components.[30]

Table 3. Chemical components (%) of the essential oils distilled from *Jasminum officinale*

Compounds	RI	% oil
Benzy acetate	17.27	7.6
Benzyl benzoate	7.66	4.5
Isobutylene epoxide	10.88	6.6
Hydroperoxide pentyl	11.58	6.8
Benzyl alcohol	9.09	5.5
5-hexene-2-one,	8.55	4.5
Phytol	7.67	4.0
Linalool	6.66	4.1
Isophytol	6.83	4.2
Eugenol	5.82	3.9
Geranyl linalool	5.94	3.0
Methyl linoleate	4.64	2.8
Cis-jasmone	4.65	1.9
Indole	4.61	1.8
Methyl palmitate	4.62	1.2

The antidermatophytic activity of *M. piperita*, *C. vernum* & *J. officinale* essential oil were screened against *T. equinum* and *M. canis* by Disc diffusion method as shown in Table-4. In this study, the EOs of *Mentha piperita* exhibited strong antimycotic activities against *T. equinum* (67mm) & *M.canis* (60mm) at 100% concentration of pure essential oil than standard drugs (Table-4). Many studies have highlighted the promising antibacterial and antifungal activity of peppermint EO against some human and phytopathogens.[21,31] The antifungal activity of *M. piperita* was tested against the following postharvest fungi such as *Botrytis cinerea, Monilinia fructicola, Penicillium expansum and Aspergillus niger* and found excellent results against all tested fungi.

Antimycotic activity of *C. vernum* essential oil was also evaluated by disc diffusion method and found excellent inhibition of zone at 100% concentration of pure essential oil against *T. equinum* (58mm) & *M. canis* (62mm) as compared to standard antifungal drugs (Table 4). Previous studies have reported antifungal activity of cinnamon oil against filamentous fungi [8] and human pathogenic fungi.[9] In a similar study, Denkova-Kostova et al. found that cinnamon EO exhibited good antifungal effect on *A. flavus* and *A. niger*, which might be attributed to cinnamaldehyde, the main antifungal component of cinnamon EO.[32] It was also described that cinnamaldehyde could perform strong antifungal activity against *A. niger* and *Fusarium*

sambucinum by disrupting the integrity of their cell membrane.[33] Achar et al. found that 500–2000 ppm clove EO could inhibit not only the growth of *A. flavus* but also the production of aflatoxin.[34] Studies have also shown that EO can act as enhancers of commercial antifungal agents to enhance the antifungal activity, and EOs are potential alternatives to synthetic fungicides.

Jasmine essential oils showed moderate activity against *T. equinum* (49mm) & *M.canis* (45mm) respectively (Table 4). *J.officinale* pharmacological activities as anti-microbial, anti-viral, and anti-spasmodic, cytotoxic, in addition to wound promoter.[35] Jasmine oil was demonstrated to have antimicrobial properties against many types of bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Bacillus subtilis* and fungus such as *Candida albicans* and *Saccharomyces cerevisiae*. The plants are leading candidates in the development of novel medications because of their extensive range of biological activities, which are influenced by the presence of several phytocompounds.[36] Further, in the current study, combination of three essential oils were also prepared and screened against *T. equinum* & *M. canis.* The diameter of inhibition zone of three mixtures of oils was found to be 78mm & 76mm against *T. equinum* and *M. canis* respectively as seen in Table 4.

Table 4: Antifungal activity of *essential* oils alone & their mixture against *T. equinum* & *M.canis*

Oil	Test strain	IZ of sample
M.piperita	*T. equinum*	67 mm
	M.canis	60 mm
C.vernum	*T. equinum*	58 mm
	M.canis	62mm
J.officinale	*T. equinum*	49 mm
	M.canis	45 mm
Mixture of three oils	*T. equinum*	78mm
	M.canis	76mm

Here, IZ= Inhibition zone (in mm) including the diameter of disc (6mm). IZ of Ketoconazole & Clotrimazole (10mcg/disc) is 32mm & 30mm against *T. equinum.* IZ of Ketoconazole & Clotrimazole (10mcg/disc) is 34mm & 31 mm against *M. canis.*

These results confirms that mixture of oils have excellent antidermatophytic activity as compared to single oils and standard antifungal drugs. Mixture of oils showed additive and synergistic inhibitory action against fungal pathogens. According to our study, mixture of oils was found to be more effective in inhibiting the growth of *T. equinum* and *M.canis* than single oils and standard drugs used against *T.equinum* and *M.canis*. Our results was in agreement with Sharma et al (2011)[37] who also reported *Curcuma longa* (turmeric), *Zingiber officinale* (ginger) and mixture of oils possess potential inhibitory activity against *T.rubrum* and *M.gypseum* as compared to single oils and standard antifungal drugs and concluded that combination of *Curcuma longa* & *Zingiber officinale* essential oils showed excellent additive, synergistic and antidermatophytic activity as compared to single oils and references antibiotics i.e. Clotrimazole and Ketoconazole. These results conclude that mixture of oils can be used as an excellent antidermatophytic agent for the treatment of zoonotic dermatophytosis.[37] Our findings are similar to Prasad et al who found synergistic antifungal efficacy of essential oils of *Cymbopogon martini*, *Chenopodium ambrosiodies* and their combinations against dermatophytes and some filamentous fungi *in vitro.*[38] Our work also coincides with the findings of Casella *et al* reported the antifungal potential of tea tree and lavender essential oils alone and in combinations against *T. rubrum* and *T. mentagrophytes* and effective inhibition by mixture of oils than a single oils alone.[39] The present results were coincides with Sharma et al (2011) who reported the antifungal potential of *Curcuma longa* (turmeric) and *Zingiber officinale* (ginger) essential oils alone and in combinations, against common causes of Pityriasis versicolor infections and concluded that antifungal activity of combinations of two essential oils indicated their additive, synergistic or antagonistic effects against individual microorganism tested [40]**.** Several previous studies also focused on antifungal activity of mixture of oils in vitro. Studies have shown that the antifungal effect of combined EOs is better than that of single EOs by combining two or more EOs to create a synergistic effect.[41]

The MIC of *M. piperita*, *C.vernum*, *J.officinale* and mixture of oils were determined by microdilution method against *T. equinum* and *M.canis* have been presented in Table 5. The results show that the *M. piperita* oil, *C. vernum* & *J. officinale* exhibited inhibitory action at 0.8 µl/ml, 0.6 µl/ml, 0.9 µl/ml, 1.1 µl/ml & 1.4 µl/ml, 1.3 µl/ml against *T. equinum* & *M. canis* respectively. These results are in agreement with Tullio et al reported the same findings, higher antifungal activity of *M. piperita* EO was also detected against dermatophytes (MIC = 0.125%, v/v for Microsporum species) and other non-Candida yeasts, such as *Saccharomyces cerevisiae*, and *Pichia carsonii.*[42] *M.piperita* EO showed higher activity against these dermatophytes than azole drugs and displayed fungistatic activity in agreement with a study by Ibrahim et al. with *Mentha x piperita* L. EO.[43] MIC of *C. vernum* EO was reported at very low concentrations and shows fungicidal activity are in agreement with Liu et al also reported the same findings.[44] Similarly, results of MIC of Jasmine EO were in agreement with other researchers, reported the same findings at low concentrations of jasmine oil showed inhibitory activity against *C.albicans* and bacteria. MIC of mixture of oils (*M.piperita* oil + *C.vernum* +*J.officinale*) was found to be 0.05 µl/ml and 0.07 µl/ml to 2 µl/ml against *T. equinum* and *M.canis*. Even after 4, 8 and 12 days interval, no growth was observed at that low concentrations and control taken without oil showed 100 % growth of *T. equinum* and *M.canis*. MIC values of combination of essential oils were observed at very low concentrations (Table 5).

Our results are in agreement with other studies which indicated that the fungal pathogens are highly susceptible to the essential oil combinations and were found to display the strongest inhibition with MIC values at very low concentrations.[37]

Table 5: MIC of essential oils & their mixtures against *T. equinum* & *M.canis*

Oil	Test strain	MIC
M.piperita	*T. equinum*	0.8 µl/ml
	M.canis	0.6 µl/ml
C.vernum	*T. equinum*	0.9 µl/ml
	M.canis	1.1 µl/ml
J.officinale	*T. equinum*	1.4 µl/ml
	M.canis	1.3 µl/ml
Mixture of oils	*T. equinum*	0.05 µl/ml
	M.canis	0.07 µl/ml

Mentha piperita

Cinnamonum vernum

Jasminum officinale

Figure 1. Extracted Essential oils from plants

Finally, this study confirms that mixture of oils possesses higher antifungal activity and can be used to cure dermatophytic infections and may potentiate the efficacy of chemotherapeutics and play important role as herbal, traditional medicine in pharmaceuticals for the treatment of superficial fungal infections of the skin.

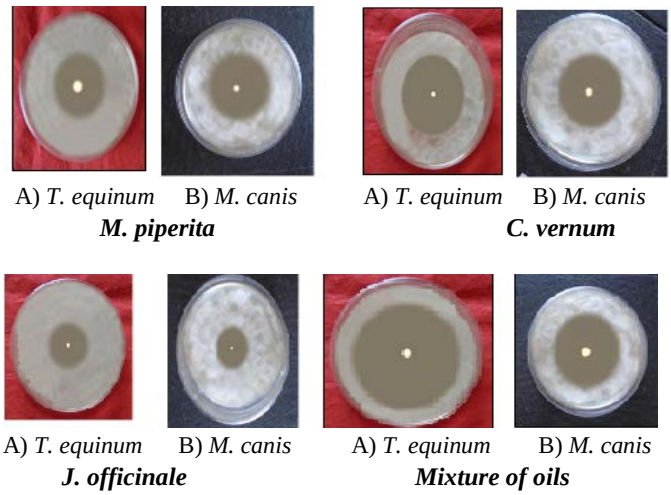

Figure 2. Antidermatophytic activity of essential oils at 100% concentrations against selected fungal strains

On the basis of present findings, recommended this mixture of essential oils can be used as a topical treatment for ringworm infection in animals and human as alternative natural plant derived drug to the conventional antifungal agents for treatment of dermatophytosis.

EXPERIMENTAL

Collection & Isolation of Dermatophytes from Soil samples by Hair Bait Technique

For this present study, different soil samples were collected from animal habitat in sterile polyethylene bags and brought to the laboratory for further microbiological analysis. Keratin substrates (Ks) were collected from different sources such as chicken feather, buffalo hairs, goat hairs, dog hairs, pig hairs, ship hairs, and horse hairs in clean sterilized plastic bags and transferred to the laboratory. Samples were transmitted to the laboratory as early as possible for further analysis like pH, temperature, humidity, salinity, total dissolved solids. Isolation of keratinophilic fungi was done by Kotwal & Sumbali, 2016 [45]. Moist chamber were prepared using sterile soil samples. 2-3 cm short strands of sterilized defatted baits were spread over soil samples. 10-15 ml of sterile water was added to the soil to facilitate germination of fungal spores. Petri dishes were then incubated at room temperature at 28°C for 15-20 days. Plates were examined periodically for the development of mycelium. Pure cultures of isolated dermatophytes were maintained on Sabouraud Dextrose Agar (SDA) and Dermatophyte Test Media, Hi-Media, both supplemented with chloramphenicol and cycloheximide. Cultures were incubated aerobically at room temperature (25°C) for up to 4 weeks.

Identification of Isolated Dermatophytes

Positive cultures were examined both macroscopically (color of the surface and reverse, topography, and texture) and microscopically (two types of conidia, small unicellular microconidia and larger septate macroconidia) for species identification, pigment production and further microscopy by Lactophenol Cotton Blue Staining. Isolated dermatophytes were further identified from Institute of Microbial Technology

(IMTECH), Chandigarh, India on the basis of phenotypic characteristics.

Collection of different plant parts for extraction of Oil

Fresh leaves of *M. piperita*, bark of *C. vernum* & flower of *J. officinale* were purchased from Aadhunik Ayurveda Vitals Khasra no 79, Mohbaewala Industrial area Dehradun–Uttrakhand. Identification of *M. piperita*, *C. vernum* & *J. officinale* were done from Herbarium office, all herbarium previous specimen acts as a reference material for identification of above selected plants, besides this on the basis of taxonomy, biodiversity, ecology from Department of Botany, University of Rajasthan, Jaipur.

Extraction of Oil

Extraction of oil from the *M. piperita*, *C. vernum* & *J. officinale* were carried out by standard hydrodistillation method, Clevenger's apparatus and all operations were carried out at room temperature.[46] The fresh leaves of *M. piperita*, bark of *C. vernum* & flower of *Jasminum* were washed to remove soil and cut into small pieces. Small pieces of fresh material of *M. piperita*, *C. vernum* & *J. officinale* (250 gm) were placed in a separate flask together with distilled water (1L). After 5-6 hours, oil was collected from the apparatus, anhydrous with sodium sulphate for removal of water traces, then this 100 % pure essential oil were dispensed into dark bottles and stored at 4°C until used. The essential oils thus obtained were subjected to antidermatophytic activity.

Screening of Essential Oils by Disc Diffusion Method

The Disc diffusion method was used for screening of essential oils.[47] Essential oils of *M. piperita*, *C. vernum* & *J. officinale* alone as well as their mixtures were screened against *T. equinum* and *M. canis*. Standard size Whatman No.1 filter paper discs, 6.0 mm in diameter, sterilized by dry heat at 140°C in an oven for one hour were used to determine antifungal activity. Sabouraud's Dextrose Agar medium (Hi media) for disc diffusion test was prepared. Composition of SDA Media for 1 litre media is Peptone (10gm), Dextrose (20gm), Agar (20gm) and Distilled water (1000 ml). After sterilization, it was poured into sterilized petriplates and allowed to solidify. A suspension that was just turbid by visual inspection was prepared by suspending in 0.9 % NaCl solution and the homogeneous suspension was used for inoculation and test inoculum was maintained at $1\text{-}5\times10^6$ CFU/ml. The spore suspension of each of the fungi was prepared from 8 to 10-day-old cultures separately. The suspension was vortexed and 0.1 aliquots were spread over the respective agar medium plates Sterilized filter paper discs were soaked in neat, undiluted (100%) concentration of single oils and their mixtures (*M.piperita* + *C.vernum* + *Jasminum officinale*). Each oil-saturated disc contain 100 µl concentration of pure essential oil was placed on an agar plate containing fungal spore suspension. Similarly, combination of three essential oils was prepared by adding 1ml of each *M.piperita*, *C.vernum* & *J. officinale* pure essential oil and each disc saturated with 100 µl concentration of mixture of all three oils. Similarly, solutions of standard antibiotics (Clotrimazole (Sigma) and Ketoconazole (Sigma) of 10 mcg/disc concentration) for antifungal activity were prepared and impregnated in the filter-paper discs. These discs were then placed over the plates preceded with respective fungal strain. The plates were incubated at 28-30°C for 48-72 hours. Three replicates were kept in each case and average values were calculated. After incubation, the petriplates were observed for formation of clear inhibition zone around the disc, indicating the presence of antifungal activity. The diameter of the inhibition zones was measured in mm. Intensity of antifungal activity was interpreted on the basis of diameter of Inhibition zone. More, the diameter of inhibition zone, more the intensity of essential oil to inhibit the growth of fungal strain used and the activity index was calculated on the basis of inhibition zone of essential oil & standard drug to determine the activity of oils. The activity of oils was measured by the following formula:-.

Activity Index=Inhibition zone of sample / Inhibition zone of standard drugs

Determination of Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) of *M. piperita*, *C.vernum* and *J.officinale* oil against *T.equinum* and *M. canis* were determined by Micro dilution method with slight modification.[48] Sterilized Brain heart infusion agar were poured into the sterilized culture tubes and allowed to solidify. In this media, 10% agar concentration was used for 1 litre media to make the semisolid. Brain Heart infusion agar media was selected for MIC to deep insert the inoculum inside the media to observe the visual growth. Test inoculum was prepared in 0.9% NaCl solution, the suspension was vortexed properly. Different concentrations of *M.piperita*, *C.vernum* & *Jasminum* alone & mixture of oil (*M.piperita* + *C.vernum* + *J.officinale)* were added in media containing culture tubes, afterwards a standard platinum loopful (~0.005 ml, Himedia, Flexiloop) of the inoculum suspension was inserted deep into each tube of medium containing a different concentration of oils, as well as oil-free control. The culture tubes were then incubated at 28°C for 48-72 hours to determine the MIC. MIC was defined as the lowest concentration that did not yield visual growth after the inoculation period.[49] All experiment was performed in triplicate.

Gas chromatography Mass Spectrometry (GC-MS) analysis of *M. piperita, C.vernum* & *J.officinale* essential oils.

Quantitative and qualitative analysis of the essential oils were performed using a GC-MS apparatus through Clarus 600 GC-MS system (Perkin Elmer, USA).[50] The compounds were separated on Elite-5MS column. For the GC-MS detection, an electron ionization system with ionization energy of 70eV was used. Nitrogen gas was used as carrier gas with a flow rate of 1.21ml/min. The column was raised from 50ºC to 320ºC at a rate of 3 ºC min. The relative percentage of the oil constituents was expressed as percentage by peak areas and the identification of oil components was based on their retention time with available literature values.

CONCLUSION

The present study clearly demonstrates that the essential oil of *M. piperita, C. vernum, J. officinale* and their mixtures have good promise as an antifungal agent that could be used as a therapeutic remedy against pathogenic fungi on account of its various *in vitro* antifungal properties, viz., strong fungicidal action, long shelf-

life, its tolerability of heavy inoculum density, thermo stability, broad range of antidermatophytic activity and absence of any adverse effects. Further, in current research, the potential of essential oil combinations as being beneficial in inhibiting the growth of fungal pathogens as compared to synthetic antifungal drugs. Thus there is a need for an alternative therapy that is safe, cheaper, economical & easily available so wide range of essential oils from plants are still unexplored for their antifungal activity. Hence, the present study conclude that to explore new antifungal agents alternative to synthetic drugs for the treatment of dermatophytic & other fungal diseases. This research study will be beneficial for the human health, human economy, environment safety and antimicrobial drug resistance for proper and timely treatment of fungal infections in immunocompromised patients.

Declarations: The authors declare that the above research work is original piece of work, not published previously anywhere.

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