

Growth-arresting Activity of Acmella Essential Oil and its Isolated Component D-Limonene (1, 8 P-Mentha Diene) against *Trichophyton rubrum* (Microbial Type Culture Collection 296)

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ABSTRACT

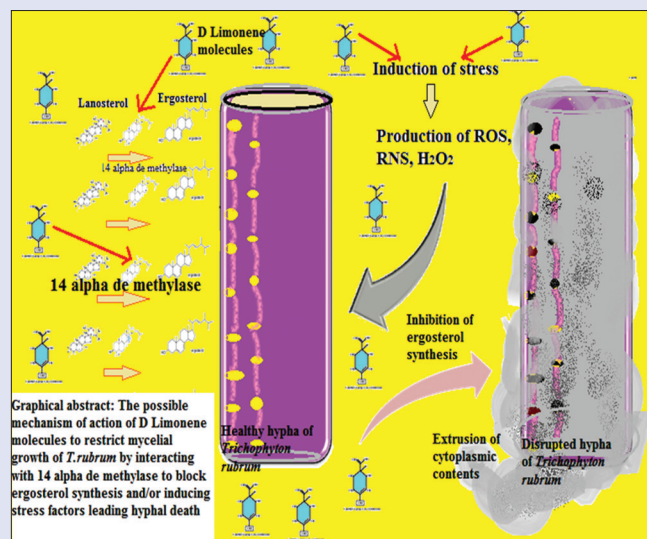
Background: *Spilanthes acmella* is used as a remedy in toothache complaints by the tribal people of Western part of Odisha, India. **Objective:** The objective of this study was to study the growth-arresting activity of an indigenous Acmella essential oil (EO) (*S. acmella* Murr, *Asteraceae*) and its isolated component, d-limonene against *Trichophyton rubrum* (microbial type culture collection 296). **Materials and Methods:** The EO was extracted from flowers of indigenous *S. acmella* using Clevenger's apparatus and analyzed by gas chromatography–mass spectrometry (GC-MS). High pressure liquid chromatography (HPLC) was carried out to isolate the major constituent. The isolated fraction was subjected to fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). The antidermatophytic activity was screened for using “disc diffusion” and “slant dilution” method followed by optical, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) studies. The molecular dockings were made between d-limonene with cell wall synthesis-related key enzymes (14 methyl deaminase and monooxygenase). **Results:** The GC-MS analysis EO had inferred the presence of 7 number of major ($\geq 2\%$) components. The component with highest peak area (%) was found to be 41.02. The HPLC-isolated fraction was identified as d-limonene (1,8 p-Mentha-diene) by FTIR and NMR. Qualitative and quantitative assays had suggested the growth inhibitory activity of Acmella EO and its component. Shrinkage, evacuation, cell wall puncture, and leakage of cellular constituents by the activity of Acmella oil and d-limonene were evidenced from optical, SEM, and TEM studies. The computer simulation had predicted the binding strengths of d-limonene and fluconazole with dermatophyte cell wall enzymes. **Conclusion:** There could have been synergistic action of all or some of compounds present in indigenous Acmella EO.

Key words: Acmella essential oil, cellular disruption, d-limonene (1,8 p-menthadiene), traditional herbal medicament, *Trichophyton rubrum*

SUMMARY

- There was presence of seven number of (d-limonene, ocimene, β -myrcene, cyclohexene, 3-(1, 5-dimethyl-4-hexenyl)-6-methylene, β -caryophyllene, and β -sesquiphellandrene and β -phellandrene) major components in the indigenous Acmella essential oil
- The d-limonene content was 41.02% in the indigenous oil

- The antidermatophytic activity of Acmella essential oil could have been attributable to its chemotypes.



Abbreviations used: °C: Degree centigrade; w/v: Weight/volume; TS: Transverse section; min: minute; Hz: hertz; h: Hr.

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INTRODUCTION

Dermatophytosis is an infection of the hair, skin, or nails caused by a dermatophyte, which is most commonly of the trichophyton genus and less commonly of the microsporium or *Epidermophyton* genera^[1] are challenging to treat. The therapeutic options for invasive fungal infections are quite limited and include only three structural classes of drugs: polyenes, azoles, and echinocandins. An attractive antifungal drug target is the fungal cell wall because the structure is absent from host cells, and thus, molecules that inhibit its synthesis are likely to have low human toxicity.^[2]

The growing appreciation of functional assays and phenotypic screens may further contribute to a revival of interest in natural products for

drug discovery. The reemergence of natural products for drug discovery in genomics era have been extensively reviewed.^[3] Historically, drugs

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were discovered through identifying the active ingredient from traditional remedies or by serendipitous discovery. The traditional or classical approach seeks first to identify the active compounds generally from large compound libraries and conducting standardized assays against etiological agents most importantly clinical isolates. The second approach is computer simulation studies in which the objective is to initially identify the broadly represented targets in fungal pathogens. Mechanism of action studies (MOA) is the next in the drug development process to ensure that the active component inhibits a fungal cell target not targets of host cell. Research in aromatic and medicinal plants, and particularly their essential oils (EO), has attracted many investigators.^[4-8] Many studies have concluded that these herbal products have huge potentiality to inhibit growth of fungal strains.

The members of genera *Spilanthes* are known by various names and are widely used in traditional medicine in various cultures. This genus belongs to the family *Asteraceae* (formerly *Compositae*) and has more than 300 species, generally distributed in the tropics.^[9] *Spilanthes acmella*, a well-known antitoothache plant with high medicinal usages, has been recognized as an important traditional medicinal plant and has an increasingly high demand worldwide. The plant is enriched with remarkable diuretic, antibacterial, and anti-inflammatory activities.^[10] While the name “toothache plant” comes from the numbing properties, it produces when the leaves and flowers are chewed. The oral use of plant extracts has been a subject of consideration in drug designing processes. Therefore, researchers have taken interest to extract the bioactive constituents using suitable extraction methods such as solvent extraction and steam distillation. From the literature survey, it was learned that volatile oils are promising antifungal agents.^[11-14] As *S. acmella* is one of the oil-rich species, we had taken interest in steam distillation method to extract its EO.

The neat oil was subjected to gas chromatography–mass spectrometry (GC-MS), fourier transform infrared spectroscopy (FTIR), and high pressure liquid chromatography (HPLC) phytochemical screening. The GC-MS analysis had inferred about the presence of seven number of components, of which d-limonene was present highest in amount. Hence, the consequential studies were focused on d-limonene only. Mention may be made here that d-limonene is an oral dietary supplement form of family of hydrocarbons containing a natural cyclic monoterpene. This phytoconstituent is widely used as a flavor and fragrance and is listed to be generally recognized as safe in food by the Food and Drug Administration (21 CFR 182. 60 in the Code of Federal Regulations, USA) with low toxicity. In the field of drug discovery studies, it is very logical to carry out experiments, when the active constituent is low toxic and copiously available in nature.

Here, we are reporting the growth inhibitory property of isolated d-limonene compound, against a strain of *Trichophyton rubrum* (Microbial Type Culture Collection [MTCC] 296). Although attempts were made to evaluate the antidermatophytic activity of d-limonene before, this study had included indigenous d-limonene.

MATERIALS AND METHODS

Dermatophytic strain and media

The dermatophytic strain used in this study was a strain of *T. rubrum* (MTCC 296) availed from MTCC, Chandigarh, India. Sabouraud dextrose agar and broth, procured from Hi-Media, Mumbai, India, were used for the cultivation of the strains. The supplied culture was revived on agar plates and slants and identified by following Kaminski's identification scheme.^[15]

Plant material

The inflorescence part of an indigenous aromatic and medicinal plant, namely, Akarakara [Figure 1] was collected from peripherals of a



Figure 1: The indigenous *Spilanthes acmella* Murr. (*Asteraceae*) plant

remote Kumbhari village, Western Odisha, India, during the month of December. The identification and authentication of the collected plant material were made by the Botanical Survey of India, Central National Herbarium, Howrah, India. Further, the herbal extracts were subjected to pharmacognostical analysis (data are not shown here).

Hydrodistillation of essential oil

The fresh flowers of plant were subjected to hydrodistillation to extract EO for 5 h using a Clevenger apparatus^[16] in the Department of Pharmacognosy, Barpalli Pharmacy College, Barpalli, Odisha, India. The hydrodistilled EO was dried over anhydrous sodium sulfate, filtered, and stored at +4°C.

Phytochemical analysis

The hydrodistilled EO was analyzed for GC-MS at Indian Institute of Chemical Biology (CSIR), Kolkata, in a GC-MS system (SHIMADZU-QP5050A) using column DB5MS (30 mm × 0.25 mm, 0.25 μm film thickness). The respective parameters used were 70 eV EI, source temperature: 200°C, injection temperature: 220°C, and interface temperature: 300°C. The carrier gas was helium, with flow rate 1.0 ml/min, in constant flow mode and split less injection. The column temperature program was 60°C for 2 min, then raised to 300°C at a rate of 10°C/min and was made isothermal at this point for 20 min. The eluted plots were identified as individual components of EO, were matched by Wiley 229. Lib and NIST 107.Lib database.

High pressure liquid chromatography analysis

The HPLC analysis of *Spilanthes* flower EO was performed at Sophisticated Analytical Instrument Facility (SAIF, DST), CSIR-Central Drug Research Institute (CDRI), Lucknow, India, using Waters HPLC Model-515 with PDA detector. The chromatographic column used for the analysis was Inertsil CN-3, (250 mm × 4.6 mm, 5 μm as particle size). An isocratic separation was conducted with mobile phase composition of n-hexane and isopropanol (1:15). The flow rate was 1.0 ml/min. The process was monitored at wavelength (λ) 250 nm.

Infrared study

The chief component isolated from HPLC was found to be limonene (1-methyl-4-(prop-1'-en-2'-yl) cyclohex-1-ene), which was subjected to IR analysis at Central Instrumental Facility, School of Chemistry, Sambalpur University, Odisha. IR spectra were recorded on Shimadzu

FTIR Prestige-21 spectrophotometer in potassium bromide (KBr) using diffuse reflectance system technique. The wavelength was indicated in cm^{-1} .

Nuclear magnetic resonance spectral analysis

The nuclear magnetic resonance (NMR) spectra of the HPLC isolated fraction of limonene were recorded on a DRX-300 MHz Bruker, Switzerland (300 MHz for ^1H , 75 MHz for ^{13}C), NMR spectrometer in CDCl_3 . Chemical shifts were expressed in ppm downfield from TMS taking as an internal standard. Data are given in the following order: δ value, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), number of protons, coupling constants J is given in Hz.

Anti-dermatomycotic assays

The anti-dermatomycotic assays were made using methods of Pattnaik *et al.*;^[17] terbinafine was taken as referred antifungal drug. The hydrodistilled Acmella EO as well as the HPLC isolated fraction were screened for anti-trichophyton activity. Both qualitative and quantitative assays were carried out using “disc diffusion” and “slant dilution” methods, respectively. The “disc diffusion” method had inferred about the degree of sensitivity based on the zone of inhibition (diameter in mm) on agar plates whereas “slant dilution” method had determined the minimum inhibitory concentration (MIC) of limonene against the test strain of trichophyton. Sodium taurocholate salt was taken as diluent at a concentration of 0.4% (w/v).

Qualitative assay

For the purpose of qualitative assay, Sabouraud agar plates were inoculated with 7-day old culture of *T. rubrum* (MTCC 296) mycelia. Both the neat and diluted form of Acmella EO (sodium taurocholate salt at a concentration of 200 $\mu\text{g}/\text{ml}$ [W/V] was used as diluent) as well as isolated d-limonene impregnated discs were put on plates in triplicates. The plates were incubated at $30^\circ\text{C} \pm 2$ for 7 ± 2 days. The radius of respective zones was measured and the mean (μ) value of triplicates was calculated to correct statistical error (if any).

Quantitative assay

The quantitative assay was carried out using “slant dilution” method. MIC and minimum fungicidal/fungistatic concentration (MFC) of drug against the test trichophyton strain was determined. The Acmella EO as well as the component, d-limonene were assorted with media using “pour plate method” at a range of concentration (0.5–10.0 $\mu\text{l}/\text{ml}$, V/V). Further, the fungal inoculums (7 days old) were placed at the middle of each drug incorporated slants. The slants were incubated at 30°C for 7 days. Proportionality of mycelia growth on each of slant surfaces was correlated with the respective drug concentrations. The minimum drug concentration inhibiting the mycelia growth was determined as MFC. Further, appearance of mycelia with growth/no growth on subculture slants was considered as fungistatic/fungicidal activity of test drugs, respectively.

Microscopic studies

The mycelia of test trichophyton strain grown in slants with SIC of Acmella EO and d-limonene were subjected to light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The SEM studies were carried out at materials and metallurgy (M and M) laboratory, National Institute of Technology (NIT), Rourkela, Odisha, whereas the TEM studies were carried out in the Department of Anatomy, All India Institute of Medical Sciences, New Delhi. Hyphal culture of 7 days old, grown on solid agar plates were sprayed with neat Acmella EO and limonene at a concentration of 1 $\mu\text{l}/\text{cm}^2$ and reincubated. The

mycelia in their transverse sections were processed for TEM at different time intervals (2, 4, 6, and 8 h) and were studied.

Molecular docking studies

Computer-simulated molecular dockings were made between the d-limonene (1,8 p-Mentha diene) with the cell wall key enzymes 14 demethylase^[18] and its precursor enzyme monooxygenase (PDB5J7X).^[19] The PDB files of d-limonene and the said enzymes were retrieved from Gnu PDB (www.molecules.gnu-darwin.org) and NCBI RSCB pdb databases (www.ncbi.nlm.nih.gov), respectively. In this study, fluconazole and terbinafine were taken for 14α -demethylase and squalene monooxygenase target enzymes, respectively, as reference drugs. The PDB files of fluconazole (DB00196) and terbinafine (DB00857) were extorted from drugBank database. The files were opened and molecular dockings were made using the Hex 8.0.0 software (LORIA, France) offline.^[20] The putative fungal cell wall-associated enzymes were categorized as receptor and the drug molecules as ligands, respectively. The docking control parameters were set using standardized parameters. The degree of binding strength of the docked molecules were autocalculated as “E total” values. The E values were evaluated based on immensity of negative scores. The more negative score, the greater was binding affinity between the receptor and ligand molecules.

RESULTS

From the gas chromatography–mass spectrometry (GC-MS) analysis of the Acmella EO, it was found that the oil was constituted with of 14 components [Figure 2]. The major components (those of $\geq 2.4\%$) were further fragmented by MS analysis. The component of the EO which eluted in the form of 1st peak, with a percentage of 2.48, had characteristically significant peak which was agreeable with the structure of β -phellandrene (β -3-Methylene-6-(1-methylethyl) cyclohexene). Another major component (12%) eluted in the GC analysis detected as β -myrcene (7-Methyl-3-methylene-1,6-octadiene). Further a component which was eluted in the form of 3rd peak (41%) convincingly, the mass fragmentation pattern was agreeable to the limonene structure (1-methyl-4-(prop-1'-en-2'-yl) cyclohex-1-ene). In addition, a component which was eluted in the form of 4th peak (20.39%) was perceived as Ocimene (3,7-dimethyl-1,3,6-octatriene). Further, a component (7th eluted peak, 5%) having characteristic fragmentation pattern was agreeable with β -caryophyllene, trans-(1R,9S)-8-Methylene-4,11,11-trimethylbicyclo undec-4-ene. Likewise, a component with 3.71% eluted peak had a mass spectrum and fragmentation pattern which was comparable with to β -sesquiphellandrene (R)-3 β -[(S)-1,5-dimethyl-4-hexenyl]-6-methylenecyclohexene. The 14th eluted peak was identified as the cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene.

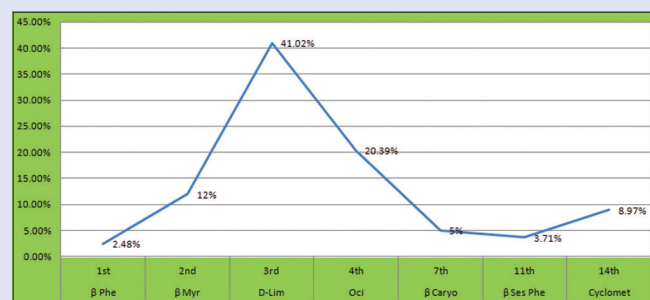


Figure 2: The gas chromatography–mass spectrometry compositional analysis major components (%) of indigenous Acmella essential oil

The HPLC isolated fraction of Acmella EO was collected, and then, the solvent was removed under vacuum to obtain the isolated component.

The GC mass spectral characteristics of d-limonene (1,8 p-mentha diene) being was supported by FTIR analysis [Figure 3]. The appearance of sharp peaks at 2918, 2851, 2833, and 1613/cm in IR region corresponds to $-C=C-H$, $C-H$, and $C=C$ stretching frequencies, respectively. Two separate sharp singlets at δ 1.65 and 1.72 were attributed to two CH_3 groups at different environments. Appearance of two different distinct multiplets was between δ 1.39-1.47 and 1.79-2.11 for the proton at C-5 and a group of six protons of C-3, C-4, C-5, and C-6, respectively. In addition, two different sharp characteristic peaks at δ 4.70 and 5.39 were assignable to a couple of olefinic protons at C-1' and a single proton at C-2 were agreeable to the structure of the isolated d-limonene. IR spectrum of ENF in KBr pellet exhibited peaks at 2918/cm ($-C=C-H$), 2854, 2833/cm ($C-H$), 1643/cm ($C=C$), 1436/cm ($=CH_2$), and 887/cm ($=C-H$).

The appearance of sharp peaks at 2918, 2851, 2833, and 1613/cm in IR region corresponds to $-C=C-H$, $C-H$ and $C=C$ stretching frequencies, respectively.

The 1H NMR spectrum [Figure 4] of electronegative frequency was taken as 300 MHz operating frequency in $CDCl_3$ and spectrum of electronegative frequency displayed signals corresponding to a the limonene nucleus at δ ppm: 5.39 (1H , s, Ar-H), 4.70 (2H, s, non-Ar-H), 1.75–2.10 (6H, d, Ar-H), 1.72 (3H, s, Me), 1.64 (3H, s, Me), and 1.43–1.53 (1H , d, Ar-H).

In the ^{13}C NMR spectrum [Figure 5], the peaks appearing chemical shift at δ ppm: 150.15 (C-2'), 133.67 (C-Ar, C-1), 120.75 (C-Ar, C-2), and 180.46 (C-1') carbons, respectively. In contrast to this, characteristics peaks appearing chemical shift at δ ppm: 41.19 (C-Ar, C-4), 30.90 (C-Ar, C-6), 30.68 (C-Ar, C-3), and 28.01 (C-Ar, C-5) along with two well-separated sharp peaks at δ ppm 23.48 (C-Me, 1C) and 20.81 δ ppm (C-Me, 1C) for methyl carbon connected to the ring and C-3' carbon.

Qualitative test

Evidenced from the results obtained from the qualitative test, it was observed that the Acmella EO and its major component d-limonene had mycelia growth inhibitory activity.

Quantitative test and fungicidal/fungistatic activity

The MIC of the Acmella EO and limonene against *T. rubrum* was observed to be 1 μ /ml (V/V) and 2 μ /ml (V/V), respectively. The MIC values were fungistatic as which was evident from subcultured slants. However, the elevated MIC values (4 and 6 μ /ml) were determined as fungicidal concentrations, respectively.

Microscopic studies

Light microscopy

The light microscopy of *T. rubrum* mycelia exposed with sublethal dose of Acmella EO and d-limonene revealed the presence of two forms of hyphae. One form was observed to be transparent while other forms of hyphae were heavily laden with cytoplasm [Figure 6].

Scanning electron microscopy

The SEM images taken for Acmella oil and d-limonene treated *T. rubrum* mycelia, had displayed some slender and thin thread-like hyphae in comparison to normal hyphae [Figure 7a]. In addition, an individual hypha (with its characteristic rough surface having pyriform-shaped microconidia) was observed to be intact while another hypha was found in ruptured condition [Figure 7b and c].

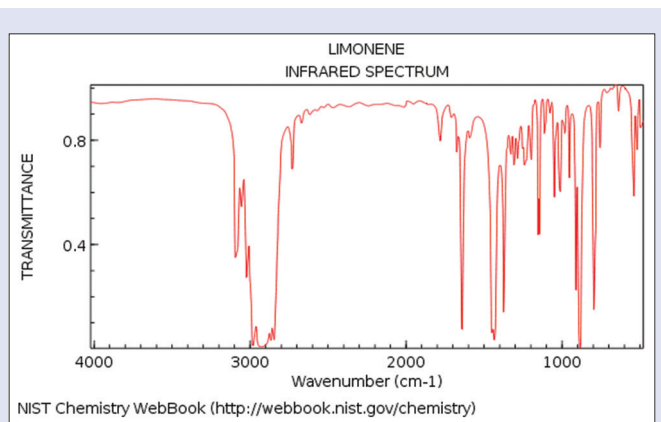


Figure 3: The Fourier transform infrared spectroscopy profile of d-Limonene

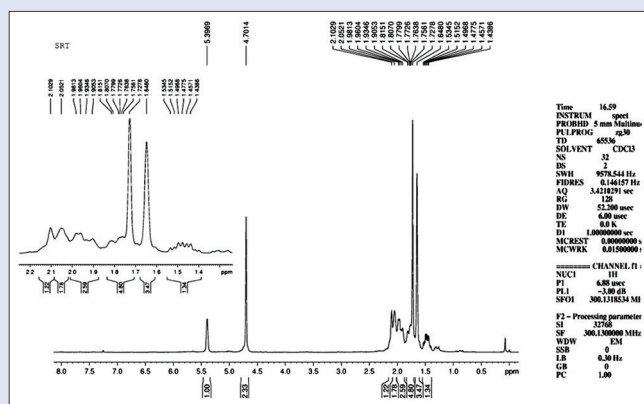


Figure 4: The proton nuclear magnetic resonance profile of d-limonene

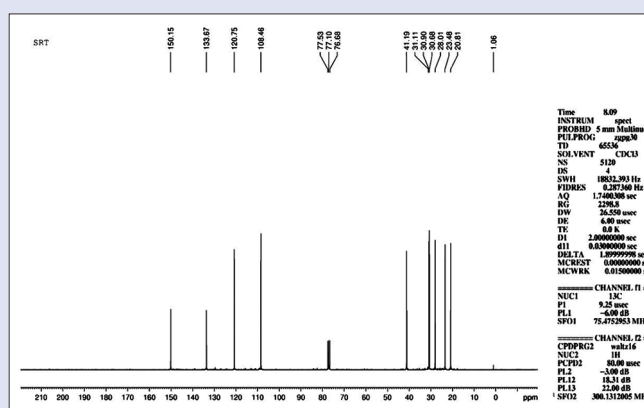


Figure 5: The carbon nuclear magnetic resonance profile of d-limonene

Transmission electron microscopy

A more detailed ultrastructural observation was made from TEM studies. After 1st h of exposure to Acmella EO and d-limonene, adsorption of oil droplets was observed along the periphery of call wall of test trichophyton strain [Figure 8a]. In addition, the hyphae (TS) were observed with extruded intracellular material [Figure 8b]. After 8th hour of exposure to Acmella oil and d-Limonene, there was absolute incision of cell membrane along with cell wall of test trichophyton strain [Figure 8c].

Molecular docking studies

The snapshots taken from molecular dockings [Figure 9] were demonstrating the binding of receptor molecules (lanosterol 14 α -demethylase and squalene monooxygenase) with ligands (fluconazole, terbinafine, and d-limonene). There was comparable intermolecular binding strength in terms of E total value was observed with reference drug fluconazole (-278.14) and d-limonene (-230.87) with enzyme 14 α demethylase. In contrast, there was differential intermolecular binding strength observed between terbinafine (-1516.30) and d-limonene (-437.85) with receptor squalene monooxygenase. d-limonene had better affinity toward 14 α -methylase than squalene monooxygenase.

CONCLUSION

From GC-MS, FTIR, and moreover FTIR analysis, it was evident that the indigenous variety of Acmeilla EO consists seven number of traceable components. IR spectroscopy provided a fingerprint of the drug through which we could identify the nature of bonding and types of functional groups in the samples. All values of IR and NMR were in the decreasing order. The spectral parameters were comparable with 1-methyl-4-(prop-1'-en-2'-yl) cyclohex-1-ene, i.e., d-limonene [Figure 10].

It was interesting to note here that, the amount of d-limonene (1,8 p-mentha diene) in the indigenous EO was highest (%). d-limonene has been designated as a chemical with low toxicity based on lethal dose (LD50) and repeated-dose toxicity studies when administered orally to animals.^[21] In sensitivity assays, it was observed that both Acmeilla EO and d-limonene were effective to restrict the growth of *T. rubrum* mycelia at lower concentrations. Further, the effect was perpetual because the remnants of oil-treated mycelia were unable to grow when subcultured to fresh media. Therefore, both the EO and its component had sufficient fungicidal activity in their test concentrations. As the mycelia of the test, trichophyton strain was unable to grow, this MIC value was considered as MFC (Minimum fungicidal concentration) of test herbal hydrodistillates. The light microscopic observation was clearly illustrating the evacuation of mycelial contents could be due to effect of Acmeilla EO and its component d-limonene. Evidently, there was a causal link between the antifungal action of Acmeilla EO (and d-limonene) and the abnormal cells, including cell wall and plasma membrane disruption. The Acmeilla EO as well as d-limonene oil exposed hyphae were observed to be distorted. The slenderness appeared in the oil-exposed hyphae could be due to induction of stress.^[22] The appearance of intracellular globular bodies around the ruptured cell wall of fungal cells could be lipid droplets,^[23] escaped from ruptured

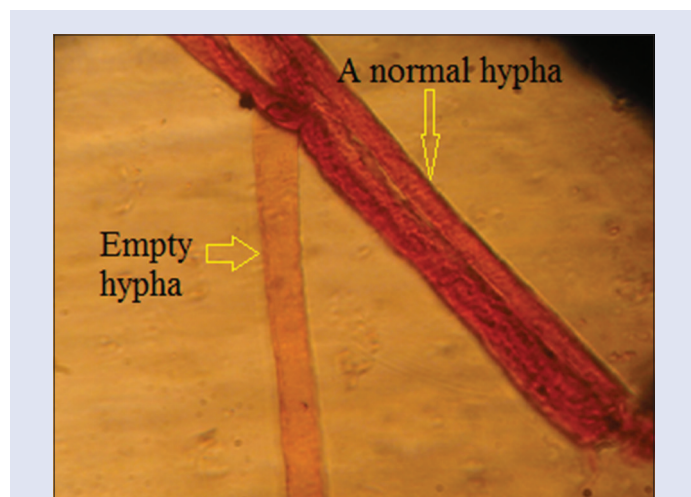


Figure 6: The optical microscopy of Acmeilla essential oil exposed hyphae of *Trichophyton rubrum* (Microbial Type Culture Collection 296). The arrows are indicating the empty hypha and adjacent normal healthy hypha

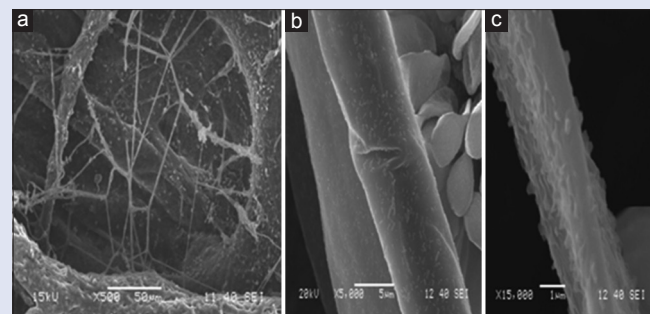


Figure 7: The scanning electron microscopy images of hyphae of *Trichophyton rubrum* (microbial type culture collection 296) exposed with d-limonene at a subinhibitory fungicidal concentration. (a) The mycelial network comprising both slender and healthy hypha. (b) One single healthy hypha magnified. (c) One ruptured hypha magnified

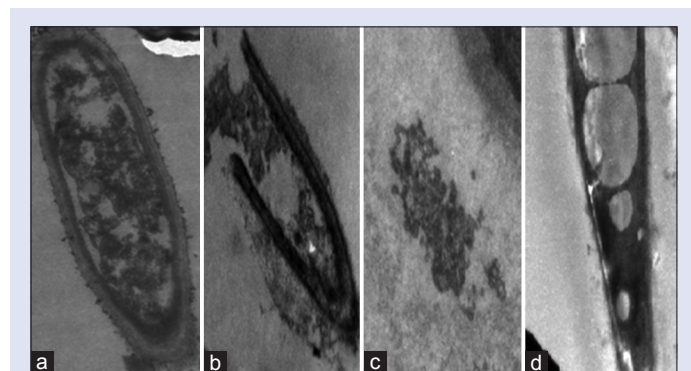


Figure 8: The transverse sections of d-limonene exposed single hypha. (a) A normal cell with intact cell membrane and cell wall. (b) a cell with ruptured cell membrane and cell wall. (c) Extruded cytoplasmic material. (d) Adsorption of lipid globules On the hyphal cell surfaces

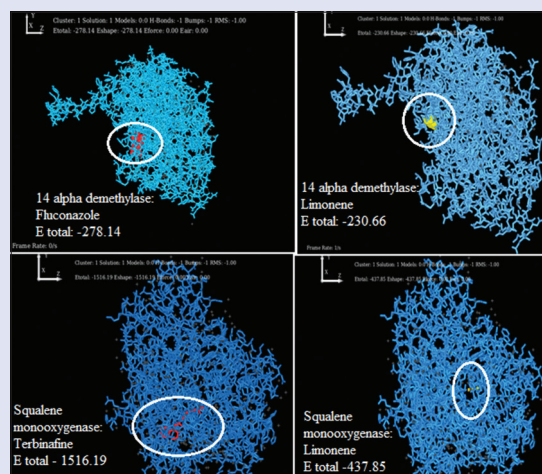


Figure 9: Computer-simulated docking images of receptors (fungal cell wall target enzymes) and ligands (drugs); Upper panel: 14 alpha demethylase: fluconazole and d-limonene, respectively; Lower panel: Squalene Monooxygenase: Terbinafine and d-limonene, respectively; the docking sites are circled

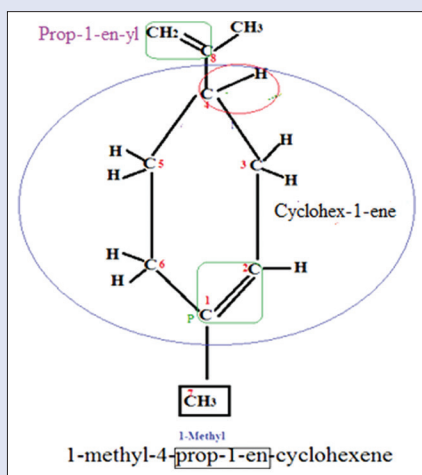


Figure 10: The structure of d-limonene generated from phytochemical analysis of indigenous Acmella essential oil

cells. The computer simulation studies were quite supportive about these observations. This is relevant to state about the computer-simulated interactions between ligand and receptor are most often described using van der Waals and electrostatic energy terms. These scoring functions are fit to reproduce experimental data, such as binding energies and/or conformations, as a sum of several parameterized functions. In a docking experiment, one usually looks for a ligand which can bind with desired receptor molecule with preferred positive Gibbs free energy. In this context, pertinent work^[24,25] had shown that the monoterpenes had an affinity for ergosterol relating their MOA to cell membrane destabilization.

In a nutshell, the Acmella EO being an indigenous variety, available at a remote place of India, and having a higher amount of limonene content was efficient to restrict the growth of mycelia of *T. rubrum* (MTCC 296). The major isolated component, d-limonene had also a demonstrable trichophyton mycelia growth inhibitory activity like reference drug, fluconazole. Further, it may be suggested here that the anti-dermatomycotic activity of Acmella EO might not be due to a mechanism of a single component but could have resulted from the effect of different compounds on cell targets. The previous study^[26] had included commercially procured d-Limonene.^[27] The problem in taking the commercially availed active constituent is pervasiveness of uncertainty in the process of genotypic characterization of plants. This may be added here that we had already studied the *in vivo* efficacy of Acmella EO on the *T. rubrum*-induced infections in symptomatic rat models^[28] and had inferred about therapeutic activity of this indigenous EO.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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