

Research Paper

Carum copticum and *Thymus vulgaris* oils inhibit virulence in *Trichophyton rubrum* and *Aspergillus* spp

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Abstract

Emergence of drug-resistant strains has demanded for alternative means of combating fungal infections. Oils of *Carum copticum* and *Thymus vulgaris* have long been used in ethnomedicine for ailments of various fungal infections. Since their activity has not been reported in particular against drug-resistant fungi, this study was aimed to evaluate the effects of oils of *C. copticum* and *T. vulgaris* on the growth and virulence of drug-resistant strains of *Aspergillus* spp. and *Trichophyton rubrum*. The gas chromatography-mass spectrometry analysis revealed thymol constituting 44.71% and 22.82% of *T. vulgaris* and *C. copticum*, respectively. Inhibition of mycelial growth by essential oils was recorded in the order of thymol > *T. vulgaris* > *C. copticum* against the tested strains. RBC lysis assay showed no tested oils to be toxic even up to concentration two folds higher than their respective MFCs. Thymol exhibited highest synergy in combination with fluconazole against *Aspergillus fumigatus* MTCC2550 (FICI value 0.187) and *T. rubrum* IOA9 (0.156) as determined by checkerboard method. Thymol and *T. vulgaris* essential oil were equally effective against both the macro and arthroconidia growth (MIC 72 µg/mL). A > 80% reduction in elastase activity was recorded for *A. fumigatus* MTCC2550 by *C. copticum*, *T. vulgaris* oils and thymol. The effectiveness of these oils against arthroconidia and synergistic interaction of thymol and *T. vulgaris* with fluconazole can be exploited to potentiate the antifungal effects of fluconazole against drug-resistant strains of *T. rubrum* and *Aspergillus* spp.

Key words: anti-elastase activity, arthroconidia, synergy, thymol, virulence.

Introduction

Fungal infections caused by various pathogenic and opportunistic strains are on the rise in the different parts of the world. This is primarily due to growing number of high risk patients particularly immunocompromised. Filamentous fungi including *Aspergillus fumigatus* and dermatophytes are common reported pathogens (Vermount *et al.*, 2008; Dagenais and Keller, 2009). Invasive aspergillosis caused by *Aspergillus* spp. in immunocompromised hosts may cause morbidity and mortality in a range from 40 to 90% in high risk populations (Dagenais and Keller, 2009). Whereas, increased incidence of dermatophytoses, infections of hair, skin and nails, caused by *Trichophyton* spp., have been reported in recent years especially in the tropical countries (Vazquez, 2003). Such infections are not

life-threatening; however, both immunocompetent and immunosuppressed persons are affected. Such infections have increased considerably among pediatric and geriatric populations (Monod, 2008) and can become serious in immunocompromised patients resulting in invasive infections (Sokovic *et al.*, 2008). Hence, the management of these fungal infections would be a definite challenge to mankind.

In spite of introduction of newer antifungal drugs, such fungal infections have been threatened by the development of drug resistant strains, host toxicity and variable drug bioavailability (Barker and Rogers, 2006). Also, these drugs are costly and not affordable to a larger section of human population across the globe (Barker and Rogers, 2006; Baddley and Pappas, 2007; Ahmad *et al.*, 2010). Therefore,

alternative strategies have to be explored and exploited. One of the approaches is to search for novel broad spectrum antifungal compounds from natural products including medicinal plants which can be used alone or in combination with known antifungal drugs. Combination therapy has potential advantages over monotherapy in terms of reducing dose related toxicity and emergence of drug resistance (Baddley and Pappas, 2007). However, in addition to killing fungi, attenuation of virulence factors has been considered as novel antifungal drug action. Targeting virulence attributes is an attracting attention to develop antifungal agent based on anti-pathogenic drug principle (Gauwerky *et al.*, 2009). Establishment of infection by fungi depends on the host cell interaction with complex interplay of secretion of virulence factors mainly proteinases including elastinases, keratinases, gelatinases and lipases such as esterases and phospholipases. These extracellular enzymes have been identified as putative virulence factors in *Aspergillus* spp. and *Trichophyton* spp. by assisting these organisms to degrade structural barrier and immune cells, and to obtain nutrient and in establishing infections (Voltan *et al.*, 2008). In addition, arthroconidia are considered as the primary cause of infection and pathogenesis by dermatophytes and often produced under *in vivo* condition (Yazdanparast and Barton, 2006). Arthroconidia are dormant fungal conidia that are metabolically active cells enriched with lipid-containing vacuoles and intracellular organelles and are highly resistant to some antifungal agents and adverse environmental conditions (Yazdanparast and Barton, 2006; Barros *et al.*, 2007). This variation in drug sensitivity due to vegetative or arthroconidial form in *T. rubrum* is the main reason for failure of dermatophytosis treatment in clinical practice.

Plant products traditionally being used in ethnomedicine have been expected to deliver newer antifungal compounds. In particular, oils of *C. copticum* and *T. vulgaris* have been well documented for ethnomedicinal values in traditional medicine (Pina-Vaz *et al.*, 2004; Bairwa *et al.*, 2012). Antifungal activities of these essential oils against *Aspergillus* spp. and *Trichophyton* spp. have been reported by several workers (Zacchino *et al.*, 1999; Cavaleiro *et al.*, 2006; Tullio *et al.*, 2007; Bajpai *et al.*, 2009; Khan and Ahmad, 2011). But interaction of these oils with antifungal drugs has not been explored. Moreover, the influence of these oils on fungal virulence factors synthesis and activity are also not yet explored or poorly known.

In lieu of this, the present study was aimed to determine *in vitro* growth inhibition of *T. rubrum* and *Aspergillus* spp. by the oils of *C. copticum* and *T. vulgaris* and their major component thymol. These agents were also assessed for their synergistic interaction with antifungal drug fluconazole. Further, to explore anti-pathogenic drug principle of these oils, inhibition of elastase and keratinase enzymes was examined.

Materials and Methods

Plant essential oils and drugs

Essential oils (*C. copticum*; ajowan and *T. vulgaris*; thyme) were purchased from Aroma Sales Corporation, New Delhi. Thymol (98% purity) was obtained from Himedia Ltd., Mumbai. The drug powder of fluconazole was purchased from Pfizer, Mumbai, India. Stock solution of fluconazole was prepared in dimethyl sulphoxide (DMSO) at a concentration of 25 mg/mL and stored at -20 °C until used. The purity of oils and active compounds was determined by physico-chemical analyses such as specific gravity, refractive index, optical rotation and solubility in alcohol at Fragrance and Flavour Development Centre, Kannauj, India. The suspensions of essential oils were prepared by diluting ten times in 1% DMSO and used in assays at concentrations ranging from 4.5 to 2304 µg/mL.

Fungal strains

Aspergillus fumigatus MTCC2550 was purchased from Microbial Type Culture Collection, India; *A. niger* IOA3 and *T. rubrum* IOA9 were collected from Jawaharlal Nehru Medical College and Hospital, AMU, Aligarh, India and are maintained at the departmental culture collection. These fungal strains were resistant to azoles including fluconazole and itraconazole (Khan and Ahmad, 2011).

Gas chromatography and gas chromatography-mass spectrometry analysis

The percentage composition of oils of *C. copticum* and *T. vulgaris* was determined by GC-FID and the compounds were identified by GC-MS. GC analysis was carried out on a Shimadzu 2010 Gas Chromatograph equipped with an FID and 25 m x 0.25 mm x 0.25 µm WCOT column coated with diethylene glycol (AB-Innowax, 7031428, Japan). Injector temperature was set at 270 °C and detector at 280 °C. Nitrogen was used as a carrier gas at a flow rate of 3.0 mL/min at a column pressure of 74.9 kPa. 0.2 µL of sample were injected into column with a split ratio of 90.0. The linear temperature program of 60 °C to 230 °C set at a rate of 3 °C/min with hold time at 230 °C for 10 min. The samples were then analyzed on the same Shimadzu instrument fitted with the same column and following the same temperature program as above. MS parameters used were: ionisation voltage (EI) 70eV, peak width 2 s, mass range 40-600 amu and detector voltage 1.5 V. Results were based on GC-FID. Peak identification was carried out by comparison of the mass spectra with database of NIST05 and Wiley8 libraries. Identification of compounds was confirmed by comparison of their relative retention indices with literature values (Davies, 1990).

Assays for determination of antifungal activity

Antifungal effect of essential oils was determined in terms of MIC and inhibition of biomass in liquid medium and mycelial radial growth on solid medium as described below.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration of oils of *C. copticum*, *T. vulgaris*, thymol and fluconazole was determined as described elsewhere (Khan and Ahmad, 2011).

Inhibition of mycelial biomass production

Method of Shafique *et al.* (2011) with slight modification was used to assess the effect of essential oils and drugs on the production of mycelial biomass by tested fungi. Briefly, 50 mL Sabouraud dextrose broth (SDB) containing different concentrations of essential oils was inoculated with 500 μ L of freshly prepared conidial suspension ($\sim 1.5 \times 10^6$ cfu/mL). In the corresponding control an equal amount of distilled water was added. The flasks were incubated at 28 ± 2 °C for 5 days. Thereafter, mycelial biomass from triplicate samples for each treatment was collected on pre-weighed Whatman filter paper No.1. Mycelial yield was determined after drying the mycelial mat at 80 °C for 24 h. The experiments were performed three times and mean percent loss in mycelial dry weight was calculated over untreated control.

Inhibition of mycelial radial growth

Inhibition of mycelial radial growth by tested oils and drugs was determined by the method of Quiroga *et al.* (2004) with little modification. Briefly, a 5 mm diameter disc of inoculum of the tested fungi was cut from the periphery of an actively growing culture and placed onto the SDA Petri plates amended with oils and thymol. The SDA plates without oils and thymol and served as untreated controls. All the inoculated plates were incubated at 28 ± 2 °C for 5 days. Three replicates for each combination of tested fungi and oils/thymol concentrations were used. The experiment was performed three times and the mean diameter of the radial growth of the fungi was recorded at the end of the incubation period and percent growth inhibition was calculated in comparison with untreated controls.

RBC lysis assay

The toxicity of essential oils or active compounds was evaluated by the red blood cell (RBC) lysis assay as adapted by Luize *et al.* (2005) with some modifications. The freshly obtained RBCs of sheep blood were washed with 1 mL of PBS (pH 7.0) and 4 mL was added to 5% (w/v) glucose solution to obtain 4% RBC suspension. 750 μ L of PBS containing the desired concentration of test agent was mixed with 750 μ L of RBC suspension in Eppendorf tubes and incubated at 37 °C for 2 h. Triton X-100 (0.1% (v/v) in PBS) was used as a positive control whereas 1% DMSO and PBS were used as negative controls. Tubes were centrifuged at 2000 rpm for 10 min and the absorbances of supernatant were read at 540 nm. Percent haemolysis was calculated as: $[(A-B)/(C-B)] \times 100$. Where A and B are the absorbance values of supernatant from the test

sample and PBS (solvent control) respectively and C is the absorbance value of supernatant from the sample after 100% lysis. Each experiment was performed three times in triplicate and the mean values were considered for calculation of percent haemolysis.

Interaction of essential oils with fluconazole

A checkerboard microtiter test was performed to evaluate the interaction of *C. copticum*, *T. vulgaris* oils and thymol with fluconazole against *A. fumigatus* MTCC2550 and *T. rubrum* IOA9. The series of two-fold dilutions, in eight numbers, of each agent were made in SDB to obtain four times the final concentration being achieved in the microtiter well. Furthermore, 50 μ L of each dilution of oils/compounds was added to the 96 well microtiter plates in the vertical direction, while 50 μ L of each dilution of fluconazole was added in the horizontal direction, so that various combinations of essential oils or active compounds and fluconazole could be achieved. Also, 100 μ L of conidial suspension were added to each well and plates were incubated at 30 °C for 2 days. The nature of interaction was defined quantitatively by means of fractional inhibitory concentrations (FIC) that were calculated as the MIC of the combination of essential oil or active compound with fluconazole divided by the MIC of essential oil or active compound or fluconazole alone. An FIC index (FICI) was obtained by adding both FICs. The combination result was interpreted as follows: $FICI \leq 0.5$, synergistic; $> 0.5-4.0$, no interaction; > 4.0 , antagonistic as described by Odds (Odds, 2003).

MIC Determination against macroconidia and arthroconidia in *T. rubrum*

Firstly, for the preparation of the macroconidia suspensions, cultures were grown on malt extract agar (MEA), pH 5.6, at 30 °C for 7 days. For arthroconidia formation, a standard pH 7.5 was employed for MEA and cultures were grown for 15 days on 5% CO₂ at 37 °C in a CO₂ incubator (Shel Lab, USA). The microscopic examinations of macroconidial and arthroconidial suspensions were conducted. Further, susceptibility of macroconidia and arthroconidia of *T. rubrum* IOA09 strain to *C. copticum*, *T. vulgaris* oils, thymol and fluconazole was tested in terms of MICs. Briefly, colonies were covered with sterile saline containing 1% Tween 80, macroconidia or arthroconidia were harvested by sterile scraping and the solution was filtered through glass wool to remove residual hyphae or long chains of arthroconidia. The number of macroconidia or arthroconidia was estimated microscopically and the conidial suspensions were diluted in RPMI 1640 (Sigma) buffered with MOPS to obtain density of 0.5×10^5 cfu/mL. The tests were performed in 96-well microtitre plates. Each microdilution well containing 100 μ L of the diluted (2 x) test agent concentrations was inoculated with 100 μ L of the

diluted (2 x) macroconidial or arthroconidial suspensions. Microtitre plates were incubated at 28 °C and MICs were recorded after 5 days of incubation. MIC was defined as the lowest concentration resulting in total inhibition of visible growth. All the tests were performed three times in triplicates and mean values were considered for calculation of MICs.

Assays for determination of anti-virulence activity

Detection and inhibition of elastase activity

The method of Kothary *et al.* (1984) was adapted to detect the production of elastase among tested isolates; the elastase activity was determined by colorimetric assay of Sachar *et al.* (1955) with some modifications employing elastin congo red (Sigma) as a substrate. Briefly, 250 µL each of 50 mM sodium borate buffer (pH 8.5) and elastin congo red (20 mg/mL in 50 mM sodium borate buffer, pH 8.5) was mixed and vortexed at room temperature for 5 min. Next, 250 µL of each fungus was added and incubated at 37 °C for 3 h at 180 rpm. Test sample was replaced by 250 µL of buffer as control. After incubation, 750 µL of 10% trichloro acetic acid was added to stop the reaction and kept for 30 min on ice. Insoluble material in the assay mixture was removed by centrifugation at 5,000 rpm, 30 min and the absorbance was read at 495 nm using a double beam uv-vis spectrophotometer (UV5704SS, India).

Inhibition of elastase activity by fungal agents (oils at 1:10 dilution) was evaluated using the modified method of Okumura *et al.* (2007). The known inhibitors of proteinases namely, ethylene diamine tetra acetic acid (EDTA), 5 mM; and metal ion Mg²⁺ (MgSO₄), 1 mM; were also used in the study. In the assay, 200 µL of test sample was mixed with 50 µL of inhibitor solution and incubated at 37 °C for 45 min before adding to elastin congo red substrate mixture. Further, procedure was followed as performed for elastase activity. Test sample without exposure to inhibitor was run as control. All the experiments were done three times in triplicate and the mean test absorbance value was subtracted from the mean untreated control absorbance value to obtain the percent reduction in elastase activity.

Detection and inhibition of keratinase activity

Estimation of keratinase activity in tested fungi was performed using the modified method of Muhsin and Aubaid (2000). Briefly, 500 µL of cell free supernatant was mixed with 50 mg of guinea pig hair in 5 mL of 0.03 M phosphate buffer (pH 7.8) and incubated at 37 °C for 3 h, 150 rpm. Test sample was replaced by buffer to run the control. Reaction mixture was stopped by adding 5 mL of 10% TCA and kept on ice for 30 min and centrifuged at 5,000 rpm for 30 min. The hair was removed by filtration and the absorbance was read at 280 nm using a double beam uv-vis spectrophotometer (UVS504SS, India).

Inhibitory effect of test agents was evaluated by the method of Okumura *et al.* (2007) with some modifications, and employing known inhibitors of proteinases as used in elastase inhibition assays. In the assay, 400 µL of test sample was mixed with 100 µL of inhibitor solution and incubated at 37 °C for 45 min. The control was run without inhibitors. Each experiment was performed three times in triplicate and inhibition of keratinase activity was calculated in terms of percent reduction in mean absorbance value of test sample compared with untreated control.

Statistical analysis

All the experiments were performed three times with three replicates per experiment and data are expressed as mean ± standard deviation. Statistical significance of the differences was determined by the one way ANOVA test using Minitab (V.11.0 for Windows). % reduction in elastase and keratinase activity in test strains in the presence of oils/inhibitors was compared to untreated control by one way ANOVA using Duncan's method. P-values of ≤ 0.05 were considered as statistically significant.

Results

GC-GC/MS analysis

GC and GC-MS analysis of essential oils revealed the presence of various major and minor compounds. Main *C. copticum* oil components were p-cymene (33.67%), thymol (22.82%) and γ-terpinene (21.61%). Thymol was the main constituent (44.71%) of *T. vulgaris* followed by γ-terpinene (26.01%) and α-cymene (21.22%).

Antifungal activity

MIC of fluconazole was found to be 200 µg/mL against *A. fumigatus* MTCC2550 and *A. niger* IOA3 whereas 800 µg/mL against *T. rubrum* IOA9. Both the tested oils exhibited MIC of 72 µg/mL and 144 µg/mL against *T. rubrum* IOA9 and *A. fumigatus* MTCC2550, respectively. MICs of *T. vulgaris* and *C. copticum* were 288 µg/mL and 576 µg/mL against *A. niger* IOA3, respectively. Thymol exhibited MIC of 192 µg/mL against the strains *A. fumigatus* MTCC2550 and *T. rubrum* IOA3 whereas 384 µg/mL against *A. niger* IOA3.

Furthermore, tested oils exerted concentration dependent inhibitory effects on the production of fungal biomass (Table 1) and mycelial radial growth (Table 2). Thymol was the most inhibitory resulting in reduction in the biomass as well as radial growth from 94.07% to 99.75% in tested strains at 72 µg/mL. Reference drug fluconazole also exhibited concentration dependent inhibition in biomass and radial growth of tested fungi up to 84.89% and 87.08%, respectively at 200 µg/mL.

Table 1 - Concentration dependent inhibition of dry biomass production in *A. fumigatus* MTCC2550, *A. niger* IOA3 and *T. rubrum* IOA9 by essential oils/active compounds in comparison with fluconazole.

Conc.(µg/mL)	Percent inhibition in dry biomass over untreated control											
	<i>C. copticum</i>			<i>T. vulgaris</i>			Thymol			Fluconazole		
	AF	AN	TR	AF	AN	TR	AF	AN	TR	AF	AN	TR
4.5	21.34 ± 2.45	22.64 ± 1.91	19.90 ± 1.86	24.51 ± 2.45	22.11 ± 1.45	17.90 ± 1.27	19.56 ± 1.73	22.23 ± 3.45	19.251.09	12.32 ± 1.81	11.41 ± 3.24	11.41 ± 3.24
9	30.79 ± 2.19	32.99 ± 3.34	30.5 ± 3.45	35.88 ± 2.19	33.12 ± 2.64	33.55 ± 3.36	33.89 ± 1.22	30.34 ± 3.18	3.251.66	21.29 ± 2.56	21.26 ± 1.41	21.26 ± 1.41
18	41.67 ± 2.34	46.37 ± 3.13	57.44 ± 4.16	47.54 ± 2.08	41.23 ± 3.34	54.44 ± 4.28	44.96 ± 2.72	47.23 ± 4.35	49.12 ± 4.42	30.90 ± 2.54	44.54 ± 2.81	44.54 ± 2.81
36	52.56 ± 3.45	59.77 ± 2.47	66.02 ± 4.78	59.37 ± 4.68	55.23 ± 3.91	69.02 ± 5.50	68.02 ± 5.39	72.33 ± 6.56	64.95 ± 4.04	54.31 ± 6.13	53.33 ± 3.41	53.33 ± 3.41
72	61.45 ± 1.66	64.55 ± 2.69	80.27 ± 2.45	71.79 ± 2.29	70.12 ± 2.39	82.27 ± 2.02	95.50 ± 6.92	94.38 ± 5.23	99.753.25	66.62 ± 3.47	65.70 ± 1.81	65.70 ± 1.81
144	76.34 ± 3.40	79.81 ± 4.22	100	86.02 ± 1.40	81.24 ± 4.56	100	-	-	-	77.63 ± 1.90	77.71 ± 4.16	77.71 ± 4.16
288	90.67 ± 3.40	93.33 ± 3.30	-	98.42 ± 1.40	95.34 ± 5.65	-	-	-	-	85.32 ± 0.47	84.89 ± 3.19	84.89 ± 3.19

AF- *A. fumigatus* MTCC2550; AN- *A. niger* IOA3; TR- *T. rubrum* IOA9.

-; indicates no biomass.

Table 2 - Concentration dependent inhibition of mycelial radial growth in *A. fumigatus* MTCC2550, *A. niger* IOA3 and *T. rubrum* IOA9 by essential oils/active compounds in comparison with fluconazole.

Conc.(µg/mL)	Percent inhibition in radial growth over untreated control											
	<i>C. copticum</i>			<i>T. vulgaris</i>			Thymol			Fluconazole		
	AF	AN	TR	AF	AN	TR	AF	AN	TR	AF	AN	TR
4.5	12.56 ± 1.53	19.23 ± 2.03	20.45 ± 3.39	11.56 ± 1.73	14.34 ± 1.45	17.251.39	20.73 ± 1.15	21.44 ± 2.43	13.04 ± 1.37	10.56 ± 1.81	12.34 ± 1.47	12.34 ± 2.69
9	21.89 ± 1.42	28.67 ± 3.13	35.45 ± 2.66	25.89 ± 1.42	22.38 ± 2.34	30.251.66	30.70 ± 2.57	32.34 ± 3.23	23.89 ± 1.34	17.251.27	19.35 ± 1.81	19.92 ± 3.41
18	30.46 ± 2.12	40.13 ± 3.91	50.12 ± 6.32	40.46 ± 2.12	39.23 ± 2.45	45.12 ± 4.22	41.79 ± 2.20	42.23 ± 3.20	37.70 ± 2.33	23.04 ± 2.86	25.23 ± 2.86	29.03 ± 4.93
36	59.32 ± 5.49	55.34 ± 5.23	79.15 ± 5.23	59.02 ± 5.39	55.35 ± 4.45	68.15 ± 4.23	59.42 ± 4.38	68.34 ± 5.35	63.70 ± 4.10	36.39 ± 2.92	35.44 ± 3.24	53.384.58
72	67.27 ± 3.10	69.33 ± 5.39	98.46 ± 5.13	73.27 ± 3.10	71.89 ± 3.91	95.13 ± 3.15	97.26 ± 2.94	96.34 ± 6.56	94.07 ± 3.64	66.47 ± 4.53	67.44 ± 5.45	61.87 ± 6.18
144	96.70 ± 4.42	95.33 ± 5.13	-	98.20 ± 6.32	95.34 ± 5.34	-	-	-	-	71.04 ± 3.57	75.45 ± 4.67	70.67 ± 5.60
288	-	-	-	-	-	-	-	-	-	87.08 ± 2.98	89.49 ± 5.36	86.00 ± 4.64

AF- *A. fumigatus* MTCC2550; AN- *A. niger* IOA3; TR- *T. rubrum* IOA9.

-; indicates no biomass.

Cellular toxicity assay

Tested agents showed no haemolysis *in vitro* at their respective MFCs. Only at a concentration two to four times higher of MFCs (2304 µg/mL) partial haemolysis (10-25%) was observed. Complete haemolysis was shown at 0.1% (v/v) Triton X-100 (positive control) and no haemolysis was observed by 1% DMSO and PBS (solvent controls) (Table 3).

Synergy of essential oils with fluconazole

T. vulgaris and thymol showed significant levels of synergistic interaction with fluconazole against *T. rubrum* IOA9 and *A. fumigatus* MTCC2550, whereas, *C. copticum* interacted indifferently with fluconazole against the tested fungi (Table 4). Thymol exhibited the highest synergy with FICI values of 0.187 and 0.156 against *A. fumigatus* MTCC2550 and *T. rubrum* IOA9, respectively.

Susceptibility of macroconidia and arthroconidia

As shown in Table 5, the MICs of eugenol and *T. vulgaris* were found to be 72 µg/mL against both macro and arthroconidia and MICs of *C. copticum* was only 2-fold higher against arthroconidia, in comparison with macro-

Table 5 - Effect of essential oils and active compounds in terms of MIC against arthroconidia compared to macroconidia in *T. rubrum* IOA9.

Test agents	MIC (µg/mL)	
	Macroconidia	Arthroconidia
Essential oils		
<i>Carum copticum</i>	72	144
<i>Thymus vulgaris</i>	72	72
Active compounds		
Thymol	72	72
Antifungal drugs		
Fluconazole	200	1600

conidia. Control drug fluconazole was not much effective, as MIC against arthroconidia was 8-fold higher compared with macroconidia.

Inhibition of elastase and keratinase activity

Since *A. niger* IOA3 and *T. rubrum* IOA9 showed relatively higher production of elastase and keratinase, respectively, the essential oils were only tested for their anti-elastase and anti-keratinase activity against these two strains. As shown in Table 6, thymol showed the highest re-

Table 3 - Haemolytic properties of *C. copticum*, *T. vulgaris* oils and thymol.

Test agents	Percent haemolysis of RBC (Mean ± SD)						
	Concentration of essential oils/active compounds (µg/mL)						
	18	36	72	144	288	576	2304
Essential oils							
<i>C. copticum</i>	1.48 ± 0.14	1.84 ± 0.14	2.44 ± 0.19	3.51 ± 0.07	4.27 ± 0.17	4.62 ± 0.27	21.03 ± 0.30
<i>T. vulgaris</i>	1.38 ± 0.14	1.84 ± 0.14	2.74 ± 0.19	3.21 ± 0.07	4.09 ± 0.17	4.92 ± 0.27	19.11 ± 0.40
Active compounds							
Thymol	1.64 ± 0.19	2.59 ± 0.07	3.45 ± 0.12	3.99 ± 0.05	4.68 ± 0.17	5.66 ± 0.20	16.34 ± 0.23

Table 4 - FIC and FICI for combination of essential oils or active compounds with fluconazole against *A. fumigatus* MTCC2550 and *T. rubrum* IOA9.

Test combinations	<i>A. fumigatus</i> MTCC2550				<i>T. rubrum</i> IOA9			
	MIC _a	MIC _c	FICI	T	MIC _a	MIC _c	FICI	T
<i>C. copticum</i> with fluconazole								
<i>C. copticum</i> (µg/mL)	144	9	0.562	I	72	18	0.75	I
Fluconazole (µg/mL)	200	100			200	100		
<i>T. vulgaris</i> with fluconazole								
<i>T. vulgaris</i> (µg/mL)	144	18	0.250	S	72	9	0.250	S
Fluconazole (µg/mL)	200	25			200	25		
Thymol with fluconazole								
Thymol (µg/mL)	192	12	0.187	S	192	6	0.156	S
Fluconazole (µg/mL)	200	25			200	25		

MIC_a, MIC of one agent alone; MIC_c, MIC of agent in most effective combination; I- indifferent; S- synergy.

Table 6 - Effect of essential oils and active compounds on elastase and keratinase activities in *A. niger* IOA3 and *T. rubrum* IOA9, respectively.

Test agents	Percent reduction in absorbance at 495 nm over untreated control for elastase activity	Percent reduction in absorbance at 280 nm over untreated control for keratinase activity
Essential oils		
<i>C. copticum</i>	81.24 ± 3.89	24.33 ± 1.24
<i>T. vulgaris</i>	90.75 ± 3.45	15.96 ± 1.23
Active compounds		
Thymol	95.56 ± 4.09	13.90 ± 1.22
Inhibitors		
EDTA	72.63 ± 1.26	54.48 ± 2.20
MgSO ₄	94.10 ± 1.63	39.77 ± 1.06

duction (95.56%) in comparison with untreated controls in elastase activity followed by *T. vulgaris* (90.75%) and *C. copticum* (81.24%). On the contrary, tested oils were less effective in reducing the keratinase activity.

Discussion

The present *in vitro* study explored efficacy of oils of *C. copticum* and *T. vulgaris* and their major active constituent thymol in inhibiting growth and virulence against the azole-resistant strains of *Aspergillus* spp. and *T. rubrum*. Tested oils/compound exerted concentration dependent inhibitory effects on the production of fungal biomass and mycelial radial growth. Thymol was the major active ingredient of both the oils as revealed by GC/GC-MS analysis. This indicates that inhibitory activity of the oils of *C. copticum* and *T. vulgaris* may be due to the thymol. However, minor constituents may also play a key role in the biological activities of these oils. Further, oils of *C. copticum*, *T. vulgaris* and thymol were tested for their toxicities to sheep erythrocytes. An alternative approach to combat drug-resistant strains is the use of antifungal agents in combination to reduce host toxicity and emergence of resistance. The promising *in vitro* antifungal activity of tested agents along with their non-toxicity to RBCs, prompted us to investigate *in vitro* combinations of these essential oils and thymol with fluconazole to increase efficacy of this drug against the azole-resistant strains. To our knowledge, no investigation for these combinations has been carried out before.

T. vulgaris and thymol showed significant levels of synergistic interaction with fluconazole against tested fungi, whereas, *C. copticum* interacted indifferently. Thymol exhibited highest synergy with fluconazole against *A. fumigatus* MTCC2550 (FICI values of 0.187) and *T. rubrum* IOA9 (0.156), respectively. Level of synergy of *T. vulgaris* with fluconazole was comparatively lesser, with the FICI value of 0.250. This is probably due to the pres-

ence of α -cymene (21.22%) and γ -terpinene (26.01%) that has diluted the synergistic effect of thymol (44.71%) compared to pure compound. The amount of thymol in *C. copticum* is 22.82% that is almost two fold lesser compared to amount present in *T. vulgaris* and it could have probably further reduced the synergistic effect of thymol and resulted in indifference type of interaction with fluconazole. Thymol reduced the MIC of fluconazole up to 4-fold and its MIC decreased up to 16- and 32-folds against *A. fumigatus* MTCC2550 and *T. rubrum* IOA9, respectively. Treatment of fungal infections caused by the drug-resistant fungi may require higher doses in monotherapy and if fluconazole is used in higher concentration it can lead to adverse side effects such as hepatotoxicity (Groll *et al.*, 1998). In these perspectives, these data suggests that thymol could be quite effectively used in combating dose-related toxicity and drug-resistance against fluconazole in the treatment of these fungi.

Infections caused by filamentous fungi are characterized by the presence of hyphal elements in host tissues, however, formation of arthroconidia constitute the primary mode of transmitting dermatophytic infections such as onychomycosis in humans and animals (Malten and Thiele, 1973; Gupta *et al.*, 2003). Arthroconidia are more resistant to antifungals and may be one of the causes of therapeutic failure, mainly in patients whose lesions contain abundant arthroconidia (Miyazi and Nishimura, 1971). Generally, *in vitro* testing evaluates the responses of microconidia, macroconidia or hyphae (Arrese *et al.*, 2001; Yazdanparast and Barton, 2006) but under *in vivo* conditions infectious entity is mostly found to be arthroconidia (Yazdanparast and Barton, 2006). Hence, there is a need of susceptibility testing for antifungal agents against arthroconidia in order to determine their effectiveness at infection sites. Therefore, we compared MIC of tested oils and fluconazole against macroconidia and arthroconidia in *T. rubrum* IOA9. Tested oils/compounds showed inhibitory activity against arthroconidia at par compared with fluconazole. Arthroconidia and macroconidia of *T. rubrum* IOA9 were equally susceptible to *T. vulgaris* and thymol. However, arthroconidia was found to be more tolerant to fluconazole in comparison with macroconidia. These data account for the effectiveness of thymol and oil of *T. vulgaris* containing higher amount of thymol (44.71%) in treating dermatophytic infections. In our knowledge, efficacy of these oils and compounds against arthroconidia has not been reported before.

The systematic development of active pharmaceutical ingredients addressing virulence factors as a prime target could provide entirely novel therapeutic options for the treatment and/or prevention of localized or systemic fungal disease. Targeting virulence factors is now gaining interest as an alternative strategy to develop new anti-infective agents. The increasing trend of screening of plant products for their anti-virulent activity against fungi prompted us to

evaluate some of these oils for their anti-pathogenic efficacy. Production of elastases and keratinases are reported to aid in the pathogenesis of *Aspergillus* spp. and *Trichophyton* spp. (Okumura *et al.*, 2007, Vermout *et al.*, 2008) and, therefore inhibition of elastase and keratinase activities in *A. fumigatus* and *T. rubrum* was determined by these oils. Tested oils showed > 80% reduction in the elastase activity of *A. fumigatus* MTCC2550. However, these oils appeared to be less effective in reducing the keratinase activity. Since proteinases contribute to fungal virulence by destroying host tissues and digesting immunologically important proteins such as antibodies and complement factors (Santos *et al.*, 2007), the inhibition of these enzyme activities in particular elastase and anti-arthroconidial activity may effectively reduce the pathogenesis of *A. fumigatus*.

Conclusions

In our study, the novel anti-proteinase activity of the tested oils in addition to mycelial growth inhibition, and effectiveness against drug tolerant arthroconidia are indicative of their efficacy as potential antifungal drugs. The data obtained have highlighted the *T. vulgaris* as more inhibitory compared with *C. copticum* and this activity appeared to be correlated to the presence of high amount of thymol in the *T. vulgaris* oil. Moreover thymol and *T. vulgaris* containing higher amount of thymol also exhibited strong synergistic interactions with fluconazole against the tested fungi. This could highlight the potential exploitation of thymol in combination therapy for treatment of drug-resistant strains of *A. fumigatus* and *T. rubrum*. The current studies draw attention for developing new compounds especially thymol as antifungal agents in the management of aspergillosis and dermatophytosis. However, *in vivo* evaluations are needed to unfold the therapeutic potential of these agents in combating such fungal diseases.

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Conflict of interest

Authors declare no conflict of interest.

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