4.1. Isolation, characterization and identification of fungi

In the present study, a total of 29 strains of yeasts including clinical and reference strains and belonging to *Candida albicans* (21), non-albicans *Candida* (6), *Cryptococcus neoformans* (02), and eight strains of filamentous fungi viz. *Aspergillus* spp (03), *Trichophyton rubrum* (02), *Alternaria solani* (01), *Fusarium oxysporum* (01), *Mucor rouxii* (01) were isolated/obtained from various sources. All the clinical and reference strains of yeasts were subjected to morphological, cultural and biochemical characterization to check their identity. The characteristics of yeasts are presented in the tables 1.a,b and 2.a,b and figures 1,2,3. Similarly, filamentous fungi were identified and confirmed on the basis of their cultural, morphological and microscopic examination of vegetative and reproductive structures as given in table 3, figures 4,5. Further, molecular characterization of *C. albicans* 04 was performed using 18S rRNA gene sequencing. These sequences were compared with similar sequences of the reference organisms using Basic Local Alignment Search Tool (BLAST; a genome database of the National Center for Biotechnology Information). Our sequences showed 99.9% conformity with the 18S rRNA gene sequences of *C. albicans* isolate Kw946 (GenBank accession number FN652297.1) (figure 6).

4.2. Virulence factors in test fungi

As presented in table 4 and figure 7, out of 21 strains of *C. albicans* tested for extracellular production of phospholipase, proteinase and haemolysin on solid medium, a maximum number of strains (15) were positive for proteinase production followed by production of haemolysin (11) and phospholipase (7). However, higher activity index (0.35) was recorded for phospholipase production in both *C. albicans* 03 and 04 strains. Among 6 strains of non-albicans *Candida* (NAC) production of phospholipase, proteinase and haemolysin was recorded in 3 different strains with the highest activity index (0.31) for phospholipase production in *C. tropicalis* 01 (figure 8).

Cell surface hydrophobicity (CSH), germ tube formation (GTF), and production of proteinase and haemolysin in liquid medium among the strains of *C. albicans* is depicted in table 5. As expected, all the test strains of *C. albicans* produced germ tubes in the range of 52.33% to 89.33%. However, 76% of test strains were positive for CSH (in the range of 24.76% to 66.21% CSH). Production of proteinase was recorded in 75% of the
test strains of *C. albicans*. Seven strains form different clinical origin and three reference strains exhibited higher proteolytic activity with absorbance values (>1.0). Haemolysin production was recorded in 52% of the *C. albicans* strains. Interestingly, 4 strains of *C. albicans* (01, 03, 06 and 09) produced significant amount of both proteinase and CSH whereas strain *C. albicans* 01 in addition also produced significant amount of haemolysin (41.52%). Among the NACs, three strains out of 6 were hydrophobic with % CSH ranging from 41.22 to 65.39. Strain *C. glabrata* 01 was maximum hydrophobic with % CSH of 65.39. Four test strains exhibited haemolysin production in the range of 15.96 to 60.42%. Strain *C. tropicalis* 01 produced maximum haemolysin. Moreover, three strains exhibited production of proteinase. Strain *C. tropicalis* 01 was highly proteolytic with absorbance values of 0.98. All the strains producing protease on solid medium could also produce in liquid medium except three strains *C. albicans* MTCC12983, *C. glabrata* 01 and *C. tropicalis* 02. Similar variation could also observe in the production of haemolysin on solid and liquid medium (table 5).

As evident from table 6, out of eight strains of filamentous fungi, *A. niger* IOA3 and *T. rubrum* IOA9 showed the extracellular production of lipase, phospholipase, gelatinase and proteinase enzymes on solid medium whereas, strains *A. solani* MTCC2101, *F. oxysporum* MTCC284, *M. rouxii* MTCC386 could not produce these enzymes. When the production of elastase and keratinase was evaluated in liquid medium, only *A. niger* IOA3 could produce elastase (OD495 of 0.085) and keratinase was produced by *T. rubrum* IOA9 and *T. rubrum* MTCC296.

### 4.3. Biofilm forming ability of Candida spp

Out of 18 clinical and 3 reference strains of *C. albicans*, 13 strains formed moderate to strong biofilms as determined by safranin staining assay using polystyrene tubes. Three strains produced weak biofilms and two strains could not form biofilms (table 7 and figure 9). *C. albicans* NRRLY12983 and *C. albicans* SC5314 strains formed strong biofilms. Strain visually exhibiting strong biofilms showed absorbance values of >1.0 when assessed by XTT reduction assay in 96 well microtiter plates (figure 10). No strain of non-albicans *Candida* forms biofilm except *C. tropicalis* 01 which formed weak biofilm. In overall, 27 strains of *Candida* spp tested for the production of virulence factors, the order of expression of various virulence factors was maximum for biofilms.
(74.07%) followed by CSH (70.37), proteinsae (66.66), haemolysin (51.85) and phospholipase (37.03) as presented in table 8.

4.4. Susceptibility of fungal strains to antifungal drugs

Table 9 shows the susceptibility behavior of strains of *C. albicans*, NACs and other yeasts to six antifungal drugs as determined by disc diffusion method. Zone of inhibition of fluconazole, itraconazole and clotrimazole varied from nil to <16 mm against test strains of *C. albicans*. Whereas, nystatin, amphotericin B and ketoconazole could show zone of inhibition ranging from ≥16 mm to 32 mm against two, seven and nine strains, respectively. A varying level of sensitivity behavior in terms of zone of growth inhibition of test NACs and *Cryptococcus* strains against six antifungal drugs was recorded. No or marginal zone of inhibition was recorded for itraconazole against test strains except *C. albicans* NRRLY12983 (12.33 mm) and *C. albicans* SC5314 (29.33 mm). Based on the antifungal sensitivity behavior maximum number of yeasts strains were resistant to azoles in the order of fluconazole = itraconazole > ketoconazole > clotrimazole.

Further MIC and MFC of amphotericin B, ketoconazole, itraconazole and fluconazole was determined against these strains using the broth macrodilution method to assess the level of resistance. The strains were considered resistant at the MIC value ≥ 1.0 µg/ml for itraconazole, and ≥ 64.0 µg/ml for fluconazole as established by CLSI breakpoints. Due to the lack of consensus on MIC breakpoints for amphotericin B and ketoconazole, the MICs for these drugs were established following the criteria suggested by other workers. The limit of resistance was considered as ≥ 2.0 µg/ml for amphotericin B (Yang *et al.* 2005) and ≥ 1.0 µg/ml for ketoconazole (Milan *et al.* 1998). As presented in table 10 and 11, all test strains of *C. albicans* were resistant to fluconazole (MIC in the range of 128-256 µg/ml), itraconazole (4-128 µg/ml) and ketoconazole (2-256 µg/ml). Thirteen strains were resistant to amphotericin B with the MIC ranging from 32-128 µg/ml, whereas susceptible strains exhibited an MIC range of 0.25-1.0 µg/ml. Moreover, MIC<sub>50</sub> of ketoconazole, amphotericin B, itraconazole and fluconazole was found to be 32, 64, 128 and 256 µg/ml, respectively, against test strains. These data further indicated the higher level of resistance in the test strains of *C. albicans* against azoles and surprisingly, co-resistance to amphotericin B was also evident. The strains of NACs and other yeasts were considered resistant at the MIC value as described for *C. albicans*, however, breakpoints
for C. neoformans were determined as described by Perfect and Cox (1999). As presented in table 10, all the strains were resistant to itraconazole (MIC range of 16-128 µg/ml). Strains C. tropicalis 01, 02 and C. neoformans MTCC4424 were susceptible dose dependent (8-32 µg/ml) and others were resistant (128-512 µg/ml) to fluconazole. Moreover, strains of C. tropicalis 01, 02 and C. neoformans 01 were susceptible (0.25-0.5 µg/ml) and others were resistant (16-128 µg/ml) to ketoconazole. In general, 51.72% isolates of yeasts tested were multi-drug resistant to amphotericin B, ketoconazole, itraconazole and, fluconazole (table 12).

Susceptibility of filamentous fungi to antifungal drugs is presented in table 13. All the test strains were resistant to fluconazole as well as itraconazole showing zone of inhibition ≤9.0 mm. Nystatin was most effective followed by ketoconazole and clotrimazole. The MIC and MFC data of antifungal drugs amphotericin B, ketoconazole, itraconazole and fluconazole against test fungi is presented in table 14. The level of resistance was higher against fluconazole compared to itraconazole with MIC and MFC values in the range of 100-200 µg/ml and 200-400 µg/ml, respectively. A. niger IOA-3 showed the highest level of resistance to fluconazole, with MIC and MFC at 800 and 1600 µg/ml, respectively. All the test fungi were resistant to both three or four different antifungal drugs and displayed multi-drug resistant behavior (table 15).

4.5. Physico-chemical properties of essential oils

Test oils displayed physico-chemical properties such as colour, refractive indices, specific gravity, optical rotation and solubility in alcohol in the range of quality reference for respective oils. The data obtained for some of the most active essential oils/compounds is given in annexure-1. The test oils of C. copticum, C. martini, S. aromaticum, cinnamaldehyde, citral and eugenol appeared to be pale yellow in colour and others were reddish brown, yellow or colourless. Refractive indexes for test oils were in the range of 1.48 to 1.54 and specific gravity in the range of 0.88 to 1.05. S. aromaticum and C. copticum exhibited optical rotation of -0.153 and +05.25, respectively. All these test oils were soluble in 95% alcohol.

Furthermore, the major active ingredients of oil of C. copticum were ♯-cymene (33.67%), thymol (22.82), ♯-terpine (21.61) and ♯-pinene (12.30) and α-pinene (4.59) as analyzed by GC and GC-MS (figure 11 and table 16). Major constituents of Oil of C. citratus was
mainly comprised of α-citral (43.95%) and β-citral (28.87%) as presented in (figure 12, table 17). Geraniol (43.84%) and geranyl acetate (26.91) were the most prominent constituent of C. martini (figure 13, table 18). Cinnamaldehyde was the predominant component (79.10%) of C. verum (figure 14, table 19). Oil of S. aromaticum was predominately composed of eugenol (74.32%) and Caryophyllenes (27.97%) (figure 15, table 20). Oil of T. vulgaris was primarily comprised of thymo (44.71%), γ-terpinene (26.01) and α-cymene (21.22) (figure 16, table 21). Minimum assay for active compounds citral and geraniol were 81% and 85 %, respectively (figures 17,18).

4.6. Susceptibility of fungal strains to essential oils
The screening result for antifungal activity of essential oils and active compounds against the drug-resistant strains of yeasts using a disc diffusion method is given in table 22 and figure 19.a,b. Out of the 23 tested essential oils and active compounds, cinnamaldehyde and oil of C. martini were highly active against all the test strains followed by others in the order of citral >eugenol >C. verum >C. citratus >S. aromaticum =C. coticum =geraniol >T. vulgaris, exhibiting zone of inhibition >20 mm against majority of strains. Oils of Eucalyptus sp, M. piperita, C. sinensis, C. paradisi and C. limon were least active against majority of the strains exhibiting zone of inhibition <16 mm. Oils of A. graveolens, F. vulgare, P. crispum, R. officinalis, S. album, Z.mays, M. fragrans and Z. officinale were found inactive at tested concentrations.

Further, a total of ten essential oils and active compounds exhibiting the highest activity against the test yeasts strains by disc diffusion method were assessed for their potency of inhibitory activity in terms of MIC using broth macrodilution method. As evident from table 23, test oils were highly active in the order of C. martini (MIC50 90 µg/ml) >cinnamaldehyde >citral =C. citratus =C. coticum >eugenol =S. aromaticum =C. verum >T. vulgaris =geraniol.

As shown in table 24 and figure 19.c,d, essential oils, namely C. coticum, C. verum, S. aromaticum, C. martini, and T. vulgaris exhibited strong and broad spectrum activity against all the strains of filamentous fungi with a zone of inhibition ranging from 11.00 mm to 38.66 mm. Cinnamaldehyde, citral, eugenol and geraniol showed broad- spectrum antifungal activity with a zone of inhibition ranging from 21.66 mm to 42.66 mm against
all eight test strains. Cinnamaldehyde exhibited the highest zone of inhibition (42.66 mm) against *T. rubrum* MTCC296.

Further, antifungal activity of essential oils and active compounds were determined in terms of MIC and MFC presented in table 25. Oils of *C. copticum*, *C. verum*, *S. aromaticum*, *C. martini* and *T. vulgaris* exhibited strong inhibitory activity against the test strains (MIC range of 72–288 µg/ml and MFC range of 144–576 µg/ml). Peppermint oil was strongly active against *A. fumigatus* MTCC2550 and moderately active against *T. rubrum* MTCC296 (MIC of 288 and 576 µg/ml, respectively). Active compounds namely cinnamaldehyde, eugenol and geraniol, showed activity higher than essential oils with MICs ranging from 40 to 160 µg/ml. Cinnamaldehyde was most active against *A. solani* MTCC2101, *T. rubrum* IOA-9 and *T. rubrum* MTCC296 strains, with MICs and MFCs of 40 and 80 µg/ml, respectively.

4.7. Time-kill assay for fungal strains

The potency of killing the strains of *C. albicans* by most active essential oils viz. *S. aromaticum*, *C. copticum*, *C. verum*, *C. citratus*, *C. martini*, *T. vulgaris* and active compounds namely eugenol, cinnamaldehyde, citral, and geraniol was evaluated against 4 strains namely *C. albicans* 01, *C. albicans* 09, *C. tropicalis* 01 and *C. neoformans* 01. Amphotericin B and fluconazole were used as positive controls. The time dependent killing of *C. albicans* 01 by the essential oils and active compounds revealed a decrease of >1log_{10} in the viable count compared to the control at 8 h by citral, cinnamaldehyde and eugenol followed by *C. verum* oil (between 9-10 h), geraniol and *C. martini* (between 19-20 h), and *C. citratus*, *S. aromaticum* (24 h). Amphotericin B showed a similar effect by 34 h and fluconazole could not even up to 48 h (figures 20.a,b). However, citral was most active against *C. albicans* 09 by reducing the viable count >1log_{10} in a 6 h followed by cinnamaldehyde and eugenol (8 h) (figure 20.c). Oils of *C. verum* and *S. aromaticum* exhibited activity similar to as against *C. albicans* 01 (figure 20.d). Oils of *C. martini* and *C. citratus* produced the same effect in 18 h and geraniol in 22 h, whereas, treatment with amphotericin B resulted similar effect in 44 h and fluconazole could not up to 48 h (figures 20.c,d).

The time dependent killing of *C. tropicalis* 01 by the test oils and compounds revealed a decrease of >1log_{10} in the viable count compared to the control at 6 h by geraniol and
eugenol, whereas C. verum and cinnamaldehyde showed such effect in 7-8 h. Oils of C. citratus, citral and amphotericin B showed a similar effect by 20 h (figures 21.a,b). Eugenol and cinnamaldehyde were most fungicidal against C. neoformans 01 by reducing the viable count by >1 log₁₀ in 6-8 h followed by geraniol and C. verum in 8-10 h. Other test oils exhibited similar effect in 18-20 h including amphotericin B (figures 21.c,d).

The ability to kill fungal strains by most active essential oils and active compounds against A. fumigatus MTCC2550 and T. rubrum IOA-9 is given in figure 22. The time-dependent killing of A. fumigatus MTCC2550 by essential oils and active compounds revealed a difference of > 1 log₁₀ in viable counts compared to the control between 6 and 8 h for cinnamaldehyde (figure 22.b). In contrast, treatment with the oils of C. verum, S. aromaticum, C. martini and T. vulgaris (figure 22.a) and eugenol, geraniol and citral (figure 22.b) showed a difference of > 1 log₁₀ in viable counts compared to the control between 8 and 10 h. Fluconazole as a positive control showed no difference of > 1 log₁₀ up to 10 h. A similar pattern of results was also observed against T. rubrum IOA-9 (figures 22.c,d).

4.8. Inhibition of fungal biomass and radial growth in filamentous fungi by essential oils

Essential oils and active compounds exhibiting strong antifungal activity were tested for their ability to inhibit production of biomass and radial growth in test fungi. The oils tested exerted concentration dependent inhibitory effects on the production of fungal biomass (tables 26,27) and mycelial radial growth (tables 28,29) and were highly active even at lower concentrations (18 to 40 μg/ml). On the basis of ability to test fungi cinnamaldehyde was most inhibitory resulting in 95.30% reduction in the biomass and 98.11% reduction in the radial growth in T. rubrum IOA-9 at 40 μg/ml. Similarly, 76.27% reduction in biomass and 80.74% in radial growth was recorded against A. fumigatus MTCC2550 at 80 μg/ml of cinnamaldehyde. The other test oils exerted antifungal activity in the order of eugenol >geraniol =C. verum >citral >S. aromaticum> C. citratus> C. martini, against both the test fungi Reference drug fluconazole also exhibited concentration dependent inhibition in biomass and radial growth of test fungi up to 84.89% and 87.08%, respectively at 200 μg/ml (tables 27,29).
4.9. Toxic effect of essential oils on fungal hyphae

The concentration and time dependent toxic effects of oils *C. verum* and *S. aromaticum* were evaluated against the test fungi and morphological changes if any was observed under light microscopy. As shown in figure 23, the treatment with 20 μg/ml *C. verum* resulted in the formation of chlamydoconidia and also, autolysis of the hyphal cytoplasm in 20 h in *T. rubrum* IOA-9. Formation of chlamydospores is considered as an indicator of stress conditions produced in the presence of oils. Several morphological alterations such as hyphal shrinkage, autolysis of cytoplasm and necrosis seems to occur in 24 h with the treatment of 80 μg/ml *S. aromaticum*.

4.10. Effect of essential oils against ungerminated and germinated conidia growth

It was observed that oils of *C. verum*, *S. aromaticum*, cinnamaldehyde and eugenol inhibited significantly (*P*<0.05) both ungerminated and germinated conidia of *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 at a concentration range from 5 to 80 μg/ml as depicted in figures 24,25,26,27. Oil of *C. verum* and its major active constituent cinnamaldehyde exhibited edge over *S. aromaticum* and eugenol in activity against both ungerminated and germinated conidia of these fungi at sub-inhibitory concentrations.

4.11. Effect of essential oils on cellular and hyphal morphology using scanning electron microscopy

To explore the possible mechanism of interaction of essential oils/compounds with fungal cell wall, membrane and cellular content, the strains of *C. albicans* 04 and *A. fumigatus* MTCC2550 were subjected to scanning electron microscopic studies after treatment with sub-inhibitory concentration of cinnamaldehyde and eugenol. The electron micrographs for *C. albicans* 04 obtained from scanning microscopy observations showed important morphological damage (figure 28). Untreated cells appeared to be oval in shape and with smooth cell surface and polar bud scar (figures 28.a,b). Cells treated with cinnamaldehyde at 50 μg/ml revealed deformed and interconnected cells with shrinkage of cell surface, non polar bud scars and lysis of cytoplasmic material (figures 28.c-e). Similar observation was also recorded with treatment of eugenol at 200 μg/ml (figures 28.f-i).

For, *A. fumigatus* MTCC2550, as clearly evident from SEM observations, the healthy and continuous hyphae are produced in untreated control sample (figures 29.a), whereas
treated sample (cinnamaldehyde 40 µg/ml or eugenol 160 µg/ml) exhibited unusual pattern of hyphal growth as well as alterations in cell shapes and sizes such as blistering and necrosis of hyphae (figures 29.b-h). Severely collapsed and squashed hyphae were evident due to the lack of cytoplasm.

4.12. Effect of essential oils on ultrastructure of fungal cell using transmission electron microscopy

The effect of sub-inhibitory concentrations of most active compounds (i.e. cinnamaldehyde and eugenol) on cell constituents of *C. albicans* 04, *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 was observed by transmission electron microscopy. In untreated sample of *Candida* cells, organelles such as nuclei, mitochondria and nucleus are appeared to be normal (figure 30.a). Treated sample exhibited several changes including thickening of cell wall, stretching of cell membrane, expansion of endoplasmic reticulum, leakage of cell wall and cell membrane, and abnormal distribution of polysaccharides leading to deterioration of cytoplasmic contents (figures 30.b-l). For filamentous fungi, in control specimens mycelial cells showed long strands of hyphae with smooth cell walls and cell membranes with other cytoplasmic organelles intact (figures 31.a, 32.a). In a hyphal specimen treated with cinnamaldehyde or eugenol, the ultrastructural changes were indicated as lysis of cell walls and plasma membranes, expansion of endoplasmic reticula near the cell membrane and excessive vacuolization along with disintegration of cell walls were observed. Other changes like disintegration of mitochondria, plasma membranes, and the disorganization of cytoplasmic content due to the abnormal distribution of polysaccharides were seen. Disintegration of nuclear as well as cytoplasmic contents and leakage of cytosolic content were also observed (figures 31.b-j, 32.b-j).

4.13. Effect of essential oils on fungal cell wall integrity in the presence of sorbitol

Cell wall damaging effects of an antimicrobial compound can be reversed in the presence of an osmo-protectant compound such as sorbitol. Subsequently MIC of antimicrobial is increased several folds in the test medium containing such compounds. Therefore, to determine the effect of these agents on fungal cell wall, MIC of *C. verum, S. aromaticum*, cinnamaldehyde, eugenol and fluconazole was evaluated against *C. albicans* 04 and *A. fumigatus* MTCC2550 in the presence and absence of sorbitol.
As presented in table 30, MIC of eugenol and cinnamaldehyde was increased up to two-folds against *C. albicans* 04 and there was no increase in MIC of *C. verum* and *S. aromaticum* upto 7 days. Data presented in table 31 shows that there were two fold increase in MIC of *C. verum*, *S. aromaticum* and eugenol whereas four-fold increase was observed for cinnamaldehyde against *A. fumigatus* MTCC2550.

4.14. Effect of essential oils on cell permeability

Effect on cell permeability in terms of cell leakage can be assessed by measuring intracellular component release to the medium from washed cells in buffer. Cellular component which absorb light at 260 nm represent one class of leakage components, primarily nucleotides of which uracil containing compounds exhibit strongest absorbance. The amount of K$^+$ leaked out to the external environment is also an indicator of interference with membrane lipid fluidity or integrity. Therefore, to ascertain the effect of these oils onto the cell membrane, the 260 nm absorbing material and K$^+$ leakage was determined. Further, these membrane damaging effects were confirmed by flow cytometry.


Figure 33.a shows that the release of cellular content in *C. albicans* 04 in the presence of test oils is increased in terms of the fungal agent concentration. Eugenol was most effective by exhibiting OD$_{260}$ of 0.470 at 4X MIC followed by other oils and amphotericin B (as a positive control ) exhibiting OD$_{260}$ in the range of 0.403-0.429. The treatment with 4X MIC of test agents induced consistent leakage of intracellular content with the increase in time. All the test oils and amphotericin B showed significant release of 260 nm absorbing material (OD value in the range of 0.450-0.522 at 120 min) with eugenol being at edge (figure 33.b). The similar pattern was also observed for *A. fumigatus* MTCC2550 (figures 33.c,d).

4.14.2. Membrane damage in terms of K$^+$ leakage

At 1X MIC, among the test agents, eugenol induced maximum leakage (13.64 n moles/mg dry weight of cell) in *C. albicans* 04 followed by amphotericin B > *C. verum* > *S. aromaticum* > cinnamaldehyde and further exhibited increase in K$^+$ leakage with increasing concentration (figure 34.a). As evident from figure 34.b, the time dependent increase in K+ leakage was also observed for test agents at 4X MIC with the maximum
leakage by eugenol (13.90). Similar pattern of concentration and time dependent increase in K+ leakage was also recorded for A. fumigatus MTCC2550 (figures 34.c,d).

4.14.3. Membrane integrity assay by flow cytometry
Propidium iodide is a nucleic acid binding fluorescent probe commonly employed to evaluate the effect of drugs on cell membranes. Cells with severe membrane lesion leading to inherent loss of viability will internalize PI, resulting in an increase in red fluorescence. The PI penetration in Candida cells treated with various concentrations of test compounds and positive control is shown in figures 35,36,37. Our results showed eugenol being most effective in damaging the cell membrane of Candida cells in treatment of 1 h. At 4X MIC, 50.98% cells were nonviable as indicated by red fluorescence in density plots and cell density recorded in histograms by making gate (figures 35.a-d, e-h). Eugenol and amphotericin B at this concentration resulted in uptake of PI by 40.21 and 30.42% cells, respectively (figures 36.a-d, e-h and 37.a-d, e-h). Substantial morphological changes were observed on scattergram of cells upon treatment with test agents as shown in figures 35.i-l, 36.i-l, 37.i-l. The production of membrane lesion by test agents was increasing with increase in concentration as presented in figure 38 and the efficacy was at par with the membrane damaging effects shown by amphotericin B (figure 39).

4.15. Ergosterol quantitation in the presence of essential oils
Table 32 and figure 40 have summarized the effect of test oils and active compounds on ergosterol biosynthesis in C. albicans 04 compared to positive control fluconazole. A decrease in ergosterol biosynthesis with the increasing concentration of test agents was observed. Eugenol and cinnamaldehyde were most effective in reducing ergosterol biosynthesis exhibiting 59.61% and 58.96% reduction, respectively compared to untreated control at 0.25X MIC. At 1X MIC maximum reduction of 97.07% was recorded for eugenol followed by cinnamaldehyde > S. aromaticum > C. verum >fluconazole.

4.16. Ergosterol binding assay
The ergosterol binding assay revealed a two- to four-folds increase in MIC of test agents against C. albicans 04 in the presence of 100 μg/ml and 200 μg/ml of ergosterol.
respectively (table 33). This effect was similar to as shown by positive control amphotericin B.

4.17. Interaction of essential oils with antifungal drugs against C. albicans, A. fumigatus and T. rubrum

The combinational effects of the oils of C. copticum, C. citratus C. martini, C. verum, S. aromaticum, T. vulgaris, and some of their major active compounds like eugenol, cinnamaldehyde, citral and geraniol with fluconazole or amphotericin B against C. albicans 01, C. albicans 02, C. albicans 09 and C. albicans NRRLY12983 strains are given in tables 34,35. Majority of the tested essential oils and active compounds showed varying level of interaction with fluconazole or amphotericin B against the test strains. T. vulgaris and eugenol showed synergy and C. copticum indifference with both fluconazole and amphotericin B. Oil of S. aromaticum showed synergistic interaction with only amphotericin B against all the test strains except C. albicans 01. Citral, cinnamaldehyde and oils of C. verum and C. citratus exhibited either synergy or no interaction responses in combination with amphotericin B. Geraniol displayed a varying level of synergistic interaction with both fluconazole and amphotericin B against test strains except C. albicans NRRLY12983. Among all the tested combinations with fluconazole, eugenol (C. albicans 02, 09) and citral (C. albicans 01) exhibited the highest synergy by reducing the MIC of fluconazole and their own by 16- to 32-fold. In combination with amphotericin B, cinnamaldehyde exhibited the highest synergy by reducing the MIC of amphotericin B by 32-fold and its own by 16-fold whereas, eugenol showed reduction of 8- and 16-fold, respectively. No combination was found to be antagonistic against the test strains.

Whereas, interactive effects of these oils in combination with fluconazole against A. fumigatus MTCC2550 and T. rubrum IOA-9 are given in table 36. All the tested essential oils and active compounds except C. copticum showed significant levels of synergistic interaction with fluconazole against T. rubrum IOA-9 (FICI values 0.312, 0.281, 0.257, 0.250 and 0.156). The oils S. aromaticum, T. vulgaris, eugenol, citral and cinnamaldehyde exhibited synergistic interactions with fluconazole against A. fumigatus MTCC2550 but no interactions were observed for the oils of C. martini and geraniol with fluconazole. The maximum level of synergy was detected between cinnamaldehyde and fluconazole against both T. rubrum IOA-9 (0.156) and A. fumigatus MTCC2550 (0.187).
Cinnamaldehyde was most effective in combination, showing the strongest synergy with fluconazole and reducing the MIC of fluconazole up to 8-fold against both *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 and a reduction in its own MIC up to 16- and 32-fold, respectively. The highest reduction in MIC (i.e. 128-fold) was recorded for oil of *S. aromaticum* in combination with fluconazole against *T. rubrum* IOA-9. No combination was found to be antagonistic against the test fungi.

4.18. Anti-virulence activity of essential oils and antifungal drugs against test fungi

Six essential oils namely *C. copticum, C. citratus*, *C. martini, C. verum, S. aromaticum, T. vulgaris* and four active compounds viz. eugenol, cinnamaldehyde, citral and geraniol; exhibiting promising antifungal activity were assessed for their anti-virulence activity at sub-MICs. Antifungal drugs amphotericin B and fluconazole were used as control. Six strains of *C. albicans* (04, 05, 07, 16, MTCC183, SC5314) and one strain *C. tropicalis* 01 were selected, being producers of significant amount of virulence factors, for inhibition assays. As given in annexure-9, there was no significance (P<0.05) decrease observed in log cfu in the test strains of *C. albicans* samples treated with 0.25X and 0.5X MICs of test agents compared to untreated control.

4.18.1. Effect on GTF, CSH, proteinase and haemolysin production in *Candida* spp

Oils *C. copticum, C. verum, S. aromaticum, T. vulgaris*, eugenol and cinnamaldehyde were highly effective at both 0.25X and 0.5X MICs by inhibiting >50% of GTF in strain *C. albicans* 04. The order of GTF in the presence of 0.5X MIC of these oils was *C. copticum* (6.86%) $<$*T. vulgaris* =eugenol $<$cinnamaldehyde $<$*S. aromaticum* $<$*C. verum* (15.60%). Oils of *C. citratus* and *C. martini* showed similar reduction only at 0.5X MIC (figure 41). Other test oils and fluconazole were least effective at tested sub-MICs in inhibiting GTF. Similar effect of test oils was also recorded against strain *C. albicans* SC5314.

As evident from table 37, treatment of test strains to sub-MICs of oils influenced the CSH in varying capacity. *C. copticum* at both 0.25X and 0.5X MICs was significantly (P<0.05) effective in reducing the CSH in all the test strains with a maximum in *C. tropicalis* 01 (reduction in %CSH from 60.97 to 6.69%) followed by cinnamaldehyde, citral and eugenol. Oils *T. vulgaris, S. aromaticum, C. verum* and *C. citratus* at tested sub-MICs significantly reduced the % CSH in test strains. Geraniol at both the sub-MICs
produced significant effect in strain *C. albicans* 07 (up to 12.90% CSH) and *C. albicans* MTCC183 (up to 19.90% CSH). Oil *C. martini* was significantly effective only at 0.5X MIC in reducing the CSH in test strains except *C. albicans* 04 and *C. tropicalis* 01. A similar trend of CSH inhibition was recorded with fluconazole and amphotericin B at 0.5X MIC against test strains.

Each of test oils at 0.5X MIC reduced the production of proteinase by ≥ 70% in one or other test strains (figures 42, 43, 44). Percent reduction in the proteinase production by *C. albicans* 04 at 0.5X MIC of oils was recorded in the order of *T. vulgaris* > *S. aromaticum* > *C. verum* = *C. copticum* > *C. citratus* > geraniol > eugenol > citral > *C. martini* > cinnamaldehyde. Similar effect was also observed in *C. tropicalis* 01 by these oils at 0.25 X MIC. However, cinnamaldehyde and geraniol were less effective at this concentration. Whereas, a maximum reduction of 46.94% was recorded for fluconazole at 0.5 X MIC against strain *C. albicans* 09.

The oils *C. copticum*, *C. verum*, *T. vulgaris*, cinnamaldehyde and eugenol at 0.5X MIC showed haemolysin reduction of ≥ 60% in test strains. Other test oils excluding *C. martini* and cinnamaldehyde and eugenol at 0.5X MIC showed reduced production in the range of 30 - ≤ 60%. Similar inhibition was exhibited by oils of *S. aromaticum*, *C. verum*, eugenol, cinnamaldehyde and geraniol at 0.25 X MIC. The oils *C. martini*, citral, amphotericin B and fluconazole were ineffective at tested sub-MICs (figures 45, 46, 47).

### 4.18.2. Effect on elastase and keratinase production/activity in filamentous fungi

More than 50% reduction in elastase production by *A. niger* IOA-3 was recorded in oils of *C. copticum*, *C. verum*, *S. aromaticum*, *T. vulgaris*, cinnamaldehyde and eugenol at 0.5X MIC (figure 48.a). A maximum reduction in elastase production (61.84% and 90.53% at 0.25X MIC and 0.5X MIC, respectively) was shown by *T. vulgaris*. Oils of *C. martini* and geraniol showed >50% reduction in the keratinase production in *T. rubrum* IOA-9 (figure 48.b). Other test oils and fluconazole were less effective in reducing the production of proteinases.

As shown in table 38, cinnamaldehyde showed highest reduction (95.56%) over untreated control in elastase activity followed by *C. martini*, *C. verum*, and *T. vulgaris*. Highest inhibition (97.31%) over untreated control in keratinase activity was recorded for geraniol followed by *C. martini*, and citral. Among the known inhibitors tested, MgSO₄
showed 94.10% and 39.77% inhibition in elastase and keratinase activities, respectively. Similarly, EDTA and PMSF inhibited elastase activity by 72.63%, 70.06%, and keratinase activity by 54.48% and 5.70%, respectively.

4.19. Anti-biofilm activity of essential oils and antifungal drugs against *C. albicans*

The oils which were used to assess their anti-pathogenic activity were also tested for their potential to eradicate established biofilms and inhibition of formation of biofilms in *C. albicans* strains. Antifungal drugs amphotericin B and fluconazole were used as reference drug. Strains *C. albicans* 04 and *C. albicans* SC5314 displayed strong ability to form biofilm when tested using XTT reduction assay showing OD\(_{492}\) of 1.36±0.66 and 1.09±0.01, respectively and therefore selected for biofilm inhibition assays.

4.19.1. Effects of essential oils and antifungal drugs on planktonic and sessile cells

As evident from table 39, sessile MIC (SMIC) of amphotericin B and fluconazole were increased to 512- and 1024-folds against above test strains. MIC of oils and compounds against planktonic cells was ranged from 90-400 and 45-360 µg/ml against *C. albicans* 04 and *C. albicans* SC5314, respectively. SMIC of all the test oils and compounds except *C. verum* were in the range of 200-400 µg/ml against *C. albicans* 04 and 100-360 µg/ml against *C. albicans* SC5314. Eugenol, *C. citratus* and *T. vulgaris* showed similar MIC against both the planktonic and sessile cells of *C. albicans* 04 and *C. albicans* SC5314, respectively.

4.19.2. Killing of sessile cells by essential oils and antifungal drugs

As depicted in figures 49.a,b,c,d more than 80% reduction in viable count of sessile cells of both the test strains was exhibited by *C. copticum*, *T. vulgaris*, eugenol and geraniol in 10-12 h. Cinnamaldehyde and citral produced similar effects in 30 h and 48 h, respectively against both the strains. Amphotericin B and fluconazole did not show this effect up to 48 h.

4.19.3. Inhibition of biofilm formation by essential oils and antifungal drugs

As presented in table 40, varying level of attenuation in the biofilm formation by planktonic *C. albicans* cells was observed in the presence of tested (assayed) agents. Biofilm formation was inhibited maximally by *T. vulgaris* at 0.5X MIC followed by *C. citratus* >eugenol >*C. copticum* >cinnamaldehyde in both of the test strains. Other test oils/compounds were least effective in inhibiting the biofilm formation. Fluconazole and
amphotericin B at 0.5 X MICs led to 48.16 and 67.59% formation of biofilm, respectively in *C. albicans* 04. Similar pattern of inhibition of biofilm formation was also recorded for test oils/compounds and drugs against *C. albicans* SC5314.

### 4.19.4. Light microscopy of sessile cells formed in the presence of essential oils

Concentration dependent inhibition of biofilm formation in *C. albicans* 04 at sub-MICs of eugenol, cinnamaldehyde, *C. citratus* and *T. vulgaris* was studied using light microscopy. Control untreated biofilm of 48 h exhibited dense network of multilayered yeast cells and hyphae with extracellular materials (figure 50.a). Treatment with 0.125 X MIC of eugenol, seemingly resulted in loss of polysaccharide materials and also reduction in the formation of hyphae (figure 50.b). Whereas, scattered aggregation of yeast cells was observed at 0.25 X MIC, and loosening of yeast cells and a total disruption of biofilm occurred at 0.5 X MIC (figures 50.c,d). Similar effect was also observed with the treatment of sub-MICs of cinnamaldehyde, *C. citratus* and *C. copticum* (Figures 50.e-j).

### 4.19.5. Scanning electron microscopy of sessile cells formed in the presence of essential oils

Further, observations with light microscopy were confirmed by SEM analysis. Untreated cells resulted in intact biofilm formation with dense matrix and multilayered network of yeast cells and hyphae leading to a compact three dimensional structure (figure 51.a). The cells treated at 50 and 100 μg/ml of cinnamaldehyde and eugenol, respectively, exhibited loosening of cells and disappearance of matrix. Filamentation was inhibited and amount of biofilm formed was also reduced leading to the destruction of three dimensional structure (figures 51.b,c). Untreated sessile cells showed smooth cell membrane (figure 51a. inset) whereas treatment with oils exhibited shrinkage in cell membranes of sessile cells (figure 51b. inset1 and inset2). Similar effects of *C. citratus* and *T. vulgaris* were also observed on sessile yeast cells (figure 51.c-e).

### 4.19.6. Interactive effect of active compounds and antifungal drugs on established biofilms

The combinational effects of the eugenol and cinnamaldehyde with fluconazole or amphotericin B against biofilms of *C. albicans* 04 and *C. albicans* SC5314 are given in table 41. The test compounds showed varying level of interaction with fluconazole or
amphotericin B against the biofilms of test strains. Eugenol and cinnamaldehyde were synergistic with fluconazole by exhibiting FIC1 value of 0.25 and 0.312 respectively, against the biofilms of *C. albicans* 04. Among all the tested combinations with fluconazole, eugenol exhibited the highest synergy with FIC1 value of 0.140 against *C. albicans* SC5314. Whereas, interaction of test compounds with amphotericin B was indifferent against both the strains.