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Huma Jafri Iqbal Ahmad Prof

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Thymus vulgaris essential oil and thymol inhibit biofilms and interact synergistically with antifungal drugs against drug resistant strains of *Candida albicans* and *Candida tropicalis*

Huma Jafri, Iqbal Ahmad*

Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-2020022

***Corresponding author:**
Prof. Iqbal Ahmad
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University,
Aligarh 202002, India
e.mail:*ahmadiqbal8@yahoo.co.in*

ABSTRACT

Role of biofilm in disease development and enhance tolerance to antifungal drugs among *Candida* species has necessitated search for new anti-fungal treatment strategy. Interference in pathogenic biofilm development by new antifungal compounds is considered as an attractive anti-infective strategy. Therefore, the objective of this study was to evaluate *Thymus vulgaris* essential oil and its major active compound, thymol for their potential to inhibit and eradicate biofilms alone and in combination with antifungal drugs against *Candida* spp. with especial reference to *Candida tropicalis.*

Anti-candidal efficacy of *T. vulgaris* and thymol in terms of minimum inhibitory concentration (MIC) was first determined to select the sub-MICs against *C. albicans* and *C. tropicalis*. Biofilm formation in the presence and absence of test agents was determined in 96-well microtiter plate by XTT reduction assay and effect of essential oils at sub-MICs of the test agents on biofilm development on glass surface was analysed by light and scanning electron microscopy. Synergistic interaction between essential oils and antifungal drugs were studied by checkerboard method.

Effect of sub-MIC of *T. vulgaris* (0.5 × MIC) and thymol (0.5 × MIC) on biofilm formation showed a significant reduction (p <0.05) in biofilms. Light microscopy and SEM studies revealed disaggregation and deformed shape of *C. albicans* biofilm cells and reduced hyphae formation in *C. tropicalis* biofilm cells at sub-MICs of thymol. Significant effect of *T.*

vulgaris and thymol was also recorded on pre-formed biofilms of both *C. albicans* and *C. tropicalis. T. vulgaris* and thymol also showed synergy with fluconazole against both in planktonic and biofilm mode of growth of *C. albicans* and *C. tropicalis.* However, synergy with amphotericin B is clearly evident only in planktonic *Candida* cells. Thyme oil and thymol alone or in combination with antifungal drugs can act as promising antibiofilm agent against drug resistant strains of *Candida* species and needs further *in vivo* study to synergise its therapeutic efficacy.

Keywords: Antibiofilms; *T. vulgaris*; Thymol; Synergy; *C. albicans*; *C. tropicalis;* synergistic interaction; biofilm inhibition; antifungal drugs

1. Introduction

Candida albicans and non-albicans *Candida* species are associated with superficial and systemic infections in critically ill patients with weakened immunity [1]. *Candida* species have been emerged as a significant cause of morbidity and mortality and accounting for approximately 72% of all nosocomial fungal infections, 15% of all hospital acquired infections and blood stream infections (8-15%) [2]. According to Brazilian Network of Candidemia study, about 40.9% cases of infections are caused by *C. albicans* followed by *C. tropicalis* (20.9%), *C. parapsilosis* (20.5%) and least with *C. glabrata* (4.9%) [3]. *C. albicans* is very common opportunistic pathogen producing oral, vaginal and or systemic candidiasis [4,5] and also the most common agent of hospital acquired bloodstream infections [6,7]. About 8 to 9% of all the blood stream infections with the crude mortality rate of 40% [8]. Other species of *Candida* like *C. tropicalis*, *C. parapsilosis* and *C. glabrata* are also increasingly reported responsible for candidemia and other conditions [3].
Furthermore, biofilm formation by *Candida* species has been documented on variety of medical devices such as catheters, dialysis, joint devices etc [4]. Fungal implant infections are less common than bacterial infections but tend to be more serious or problematic. In the biofilm mode of growth, yeast cells display characteristic traits different with planktonic state. Biofilms provide several folds increase in resistance to antifungal drugs as well as resist host defence which lead to failure of conventional antifungal therapy [9]. Antifungal drugs mainly polyenes and azoles are commonly used to treat *Candida* infections. However, efficacy of these drugs is limited in many cases due to development of resistance, poor penetration power in biofilm and undesirable side effects [9,10].

In this perspective, search for alternative mode of therapy and discovery of new anti-candidal compounds/combinations with improved mode of action with no or least toxicity from various sources including medicinal plants are needed.

Plant derived products and essential oils are used against various ailments including infectious diseases since long in traditional system of medicine [11]. *T. vulgaris* essential oil and its active constituent, thymol have been used as an antioxidant, anti-inflammatory, local anaesthetic, antiseptic, antibacterial and antifungal agent [12]. Antibiofilm activity of essential oils have been subject of recent investigation [13,14, 15]. However, little work has been reported on *T. vulgaris* and thymol against drug resistant *Candida* species especially *C. tropicalis*.

In this study, the antibiofilm activity was investigated against the strong biofilm forming strains of *C. albicans* and *C. tropicalis* at sub-MICs *in vitro*. Furthermore, combination of oils with antifungal drugs (fluconazole and amphotericin B) was determined to explore the synergistic interaction against the strains of *C. albicans* and *C. tropicalis*.

2. Materials and methods

2.1. Candida strains used

Four clinical isolates of *Candida* were obtained from Department of Microbiology, King George Medical University, Lucknow, India, two *C. albicans* strains [CAJ-01, CAJ-12 (KGMU028)] and two strains of *C. tropicalis* (CT-03 and CT-04) were further characterized and identified in our laboratory. Reference strains such as *C. albicans* MTCC3017 and *C. tropicalis* NRLLY12968 were obtained from Microbial Type Culture Collection, CSIR, IMTECH, Chandigarh, India and Fungal Culture Collection of the Agricultural Research Service, USDA at Peoria, USA respectively. The strains were tested for their morphological and biochemical characteristics such as growth on Hicrome *Candida* differential agar, germ tube formation, nitrate reduction and urease production using standard method [16. 17]. All strains of *Candida* were maintained in the laboratory on Sabouraud dextrose agar (SDA) slants at 4°C. The strain CAJ-01(Accession number: KY884676), CAJ-12 (KGMU028) (Accession number: MN263238) and CT-04 (Accession number: KY033482) were characterized by 18S rRNA gene sequence analysis by Macrogen, South Korea.
Medium, Sabouraud dextrose broth/agar (SDB/SDA) and Hicrome *Candida* differential agar were obtained from Hi-Media Laboratory, Mumbai, India.

2.2. Antifungal drugs and essential oils

Drug powder of amphotericin B (Hi-Media, India), fluconazole (Pfizer Co., India), ketoconazole (Hi-Media, India) and itraconazole (Jansen Co., Mumbai, India) were tested for antifungal activity against the test strains. Stock solutions of antifungal drugs were prepared in dimethyl sulphoxide (DMSO) at a concentration of 25 mg/ml and stored at -4°C until use. Essential oils of *Thymus vulgaris* and thymol (99% purity) were purchased from Aroma Sales Corporation, New Delhi, India and Hi-Media Laboratory, Mumbai, India respectively. DMSO (1%) was used to dilute essential oil and thymol. Fresh antifungal drug solution was prepared in DMSO before use.

2.3. Gas chromatography and high resolution gas chromatography-mass spectrometry analysis of plant essential oils

The composition of *T. vulgaris* oils was identified by GC-HRMS analysis. GC-MS analysis was carried out on JEOL AccuTOF GCV equipped with FID detector and separation was attained in column of 30 m × 0.22 mm × 0.25 µm (Fischer Scientific, UK) at IIT Bombay, Mumbai India. Helium gas was the carrier gas and the flow rate of mobile phase was set at 1.21ml/min. The sample was injected into the column with a split ratio of 1:10. The linear temperature was set at 60 ºC to 230 ºC with the hold time at 60 ºC for 2 min. The peak of the samples was identified by using the above system with the reference database NIST libraries. The relative retention indices of the compounds were compared with the reports in the literature to identify the compound.

2.4. Determination of minimum inhibitory concentration (MIC) of antifungal drugs and essential oils against planktonic growth

The Clinical Laboratory Standards Institute (CLSI) method M27-A3 [18] with some modifications was used to determine MIC/MFC of antifungal drugs and essential oils against *Candida* strains. Briefly, overnight grown culture of yeast strains (0.5 McFarland) prepared in SDB. A hundred microliter of two-fold dilution of test agent were made in RPMI 1640 medium (Sigma, India) and 100 µl of inoculum (2.5×10^3 CFU/ml) was added in each well. Plates were incubated for 48 h at 37°C. Agent free control was included. MIC was defined as the lowest concentration of agents inhibited the visible growth of *Candida* strains. Minimum fungicidal concentration (MFC) was defined as the concentration completely inhibited the growth of *Candida*. Based on MIC values, the strains were designated as resistant if MIC values are ≥1.0µg/ml, ≥64 µg/ml, ≥2.0 µg/ml and ≥1.0 µg/ml for ketoconazole, fluconazole, amphotericin B and itraconazole respectively [15].

2.5. Biofilm formation assay using microtiter plate

Biofilm forming ability of test isolates was screened by 96-well microtiter plate method as previously described [19]. In brief, *Candida* strains were grown in SDB (glucose 8% w/v) at 37°C for 24 h. Harvested *Candida* cells were re-suspended in RPMI 1640 medium containing L-glutamine and bicarbonate absence. A hundred microliter of *Candida* cell suspension (1.5×10^6 CFU/ml) after standardization was added to the wells of microtiter plates and incubated at 37°C for 48 h followed by gently aspiration of the medium. The wells were washed three times to remove non-adherent cells. Next, XTT was used to assay biofilm formation as described earlier [15] by taking absorbance at 490 nm using MTP reader. Each experiment was conducted at least two times in triplicate and data was recorded as the mean absorbance values.

2.6. Inhibition of biofilm formation

The biofilm cells of test strains were treated with different concentrations of test agent in 96-well microtiter plate was analysed as described above [15]. Briefly, biofilm was formed using RPMI 1640 medium in the presence and absence of sub-MICs of test agents (essential oil, thymol and antifungal drugs). On the basis of MIC value of *Candida*, sub-MICs (0.25 × MIC and 0.5× MIC) of test agents (fluconazole, amphotericin B, *T.vulgaris* and thymol) were diluted in RPMI 1640 medium to prepare final concentrations. Then, 0.1 ml of test agents (2 × final concentrations) and 0.1 ml of standardized cell suspension were added to each well of microtiter plates. The mixture was incubated at 37°C for 48 h. Test agent free wells and biofilm free well serve as a positive and negative control respectively. Subsequently non adherent cells were removed by washing wells with PBS. The experiment was performed three times in triplicates and mean absorbance values were used to measure the inhibition of biofilm formation as follows: (mean OD_{490} of treated well/ mean OD_{490} of untreated control well) × 100

2.7. Light microscopy of biofilms developed on glass surface

C. albicans (CAJ-01) and *C. tropicalis* (CT-04) were allowed to grow in the presence of thymol on cover slip in 12-well tissue culture plate using similar conditions as described for microtiter plate method [15]. In brief, *Candida* strains was grown in SDB (glucose 8% w/v) at 37°C for 24 h. *Candida* cells were harvested and re-suspended in RPMI 1640 medium. Two-fold serial dilution of thymol were prepared in RPMI 1640 medium and one ml was added to each well of plate containing sterile glass coverslips (diameter 15 mm). Subsequently, 1 ml of standardised cell suspension was inoculated and incubated at 37°C for 48 h. At the end of incubation, medium was discarded and glass coverslips were washed 2-3

times with sterile PBS and stained with 0.1% crystal violet and incubated at 37°C for 10 min. The glass cover slip was viewed under light microscope (Olympus, Japan).

2.8. Scanning electron microscopy of biofilms developed on glass surface

C. albicans (CAJ-01) and *C. tropicalis* (CT-04) biofilms were formed on glass coverslips at sub-MICs of thymol at 37°C for 48 h as described above. Biofilm cells were washed with PBS and fixed with 5% glutaraldehyde in cacodylate buffer in a graded concentration of ethanol (25, 50, 75, 95 and 100%), immersed in hexamethyldisilazane and dried under air for overnight at room temperature. The glass coverslips were then mounted on aluminium stubs with silver paint, sputter coated with gold and subjected to SEM analysis (JSM 6510, LV, JEOL, JAPAN).

2.9. Determination of biofilm eradication by essential oils and antifungal drugs

Candida biofilms were allowed to form in 96-well microtiter plate as mentioned above. Next, 0.1 ml of two-fold serial dilutions of test agents (fluconazole, amphotericin B, *T. vulgaris* and thymol) made in RPMI 1640 medium were added to each biofilm well of microtiter plates and further incubated at 37℃ for 48 h. A series of drug-free wells and biofilm-free wells (medium broth) were also included to serve as positive and negative control respectively. Biofilm eradication was determined as sessile MIC (SMIC) by XTT reduction assay. Each experiment was conducted at least two times in triplicate and SMIC was determined by comparing the reduction in the mean absorbance of the test agents treated biofilm to the untreated control and expressed as the MIC of agent that eradicated ≥ 80% of the sessile cells.

2.10. Kinetics of inhibition of sessile cells

Time dependent killing assay was performed to determine the potency of essential oils and antifungal drugs using a standardized method [19]. Briefly, pre-formed biofilm in 96-well plate was challenged with 2 × MIC of test agents. After incubation wells were washed to remove non-adherent cells and biofilm mass was scraped off the well using a sterile scalpel. Subsequently, the biofilm cells were added to PBS and vortexed gently to disrupt the aggregates, serially diluted in normal saline solution (NSS) and spread on SDA plates. The plates were incubated at 37°C for 24 h. Viable count of *Candida* was determined and data was presented as $\log_{10}$ CFU/ml.

2.11. In vitro synergy assay between essential oil and antifungal drugs in planktonic mode of growth

A checkerboard microtiter assay was adopted to assess the synergy between test agents against the test strains of *C. albicans* (CAJ-01, CAJ-12 (KGMU028) and *C. albicans*

MTCC3017) and *C. tropicalis* (CT-03, CT-04 and *C. tropicalis* NRLLY12968) by using the method as described by Vitale et al. [20] with little modifications. Briefly, two-fold serial dilutions of test agents were prepared in RPMI 1640 medium in 96-well microtiter plate. Further, 50 µl from each dilution of essential oils were added to the 96-well microtiter plates in the vertical direction and same amount of antifungal drugs were added in horizontal direction to obtain the various combinations of test compounds. Subsequently, 100 µl of inoculum suspension (0.5 McFarland) of *Candida* strains was added to each well followed by incubation at 37°C for 48 h. The interaction was determined as fractional inhibitory concentrations index (FICI) which was calculated as follows MIC of the combination of essential oils or active compounds with fluconazole or amphotericin B divided by the MIC of essential oils or active compounds or fluconazole or amphotericin B alone. FICI was determined by adding both FICIs. The FICI result was interpreted as follows: FICI ≤ 0.5: synergistic, > 0.5-4.0: no interaction, > 4.0: antagonistic.

2.12. In vitro synergy assay between essential oils and antifungal drugs in sessile mode of growth

A checkerboard microtiter assay was performed to evaluate the interaction of thyme oil, thymol with fluconazole and amphotericin B against the test *Candida* strains. In brief, biofilms of test strains were formed in the wells of microtiter plates and treated with various combinations of test agents (essential oils and drugs) by adding 50 µl of each prepared dilution of essential oils and drugs in the vertical and horizontal direction of plate. The plates were incubated at 37°C for 48 h.The extent of synergy was determined in terms of FICI index as described above.

3. Statistical analysis

Statistical analysis was determined by one way ANOVA using Duncan's method (IBM SPSS Statistics, version 20). The data with p value <0.05 was considered significant.

4. Results

4.1 Phytochemical analysis of essential oil by GC-MS analysis

The chemical composition of *T. vulgaris* essential oil is presented in table 1 and figure 1. Various volatile compounds mainly mono-terpenes and sesqui-terpenes were identified. The major components of *T. vulgaris* essential oil were thymol (54.73%), carvacrol (12.42%), terpineol (4.00%), nerol acetate (2.86%) and fenchol (0.5%).

4.2. Minimum inhibitory concentration of antifungal drugs and essential oils

MIC and MFC of antifungal drugs were determined against *C. albicans* and *C. tropicalis* as presented in Table 2. MICs of fluconazole and itraconazole ranged from 8-1024 µg/ml and 256-1024 µg/ml respectively. In contrast, MICs of amphotericin B and ketoconazole was found to be in the range of 2-16 µg/ml and 16-1024 µg/ml respectively. Based on the MIC values of antifungal drugs presented in table 2 it is evident that all strains of *C. albicans* and *C. tropicalis* showed variation in MIC values against antifungals being maximum tolerant to itraconazole. While CAJ-12 (KGMU028) also exhibited high MIC (1024 µg/ml) against fluconazole. Antifungal activity of *T. vulgaris* and thymol are presented in table 3 against the *Candida* strains. Planktonic MIC (PMIC) of *T. vulgaris* and thymol were exhibited ranging from 1.56-50 µg/ml against the test strains of *C. albicans* and *C. tropicalis*. MICs of *T. vulgaris* essential oil were 25, 3.12 and 1.56 µg/ml against the *C. albicans* (CAJ-01), CAJ-12 (KGMU028) and *C. albicans* MTCC3017 respectively. In contrast, MICs of thyme oil were 25, 50 and 25 µg/ml against CT-03, CT-04 and *C. tropicalis* NRLLY12968 respectively.

4.3. Biofilm formation by Candida strains

The biofilm forming ability on polystyrene microtiter plate was determined on the basis of absorbance in XTT reduction assay. The strains were divided as strong (OD_{490} >0.800), moderate (OD_{490} >0.4 to 0.8) and weak (OD_{490} <0.4) [15, 21]. All the strains of *C. albicans* (CAJ-01, CAJ-12 (KGMU028) and *C. albicans* MTCC3017) and *C. tropicalis* (CT-04 and *C. tropicalis* NRLLY12968) formed strong biofilms except CT-03. The biofilm forming ability in terms of absorbance was found 1.369 ± 0.02, 1.145 ± 0.08, 0.973 ± 0.13, 0.433 ± 0.03, 1.338 ± 0.02 and 0.931 ± 0.03 for CAJ-01, CAJ-12 (KGMU028), *C. albicans* MTCC3017, CT-03, CT-04 and *C. tropicalis* NRLLY12968 respectively (Table 4).

4.4. Inhibition of biofilm formation

Data presented in table 5 shows ability of *T. vulgaris* and thymol to inhibit biofilm development in *Candida* strains. At 0.5 × MIC of *T. vulgaris* (12.5 µg/ml) and thymol (3.12 µg/ml), the biofilm formation in CAJ-01 was found to be 26.30 and 16.93% respectively. Similarly, CAJ-12 (KGMU028) cells also displayed noticeable reduction in biofilm formation at sub-MICs of *T. vulgaris* and thymol. At 0.5 × MIC of thymol, the biofilm forming ability of *C. tropicalis* (CT-04) strain was 20%. Similarly, *T. vulgaris* also showed significant (p <0.05) reduction in the biofilm formation.

4.4.1. Light microscopy of biofilm cells

Inhibition of biofilm formation by thymol was also analysed on glass coverslips and visualized under light microscope. Untreated control *C. albicans* (CAJ-01) and *C. tropicalis* (CT-04) biofilms of 48 h exhibited multi-layered yeast cells with substantial amount of

extracellular matrix. *C. tropicalis* also formed hyphae in the sessile mode (Fig. 2A). Biofilm formation was inhibited to varying extent at sub-MICs of thymol. CAJ-01 and CT-04 biofilm showed disaggregation of cells and reduced matrix production (Fig. 2). At sub-MIC (1.56 µg/ml) of thymol, there were reduction in hyphae production in CT-04 strain.

4.4.2. Scanning electron microscopy of biofilm cells

The above study also analysed the ultra structural changes in the thymol treated biofilm cells by electron microscopy. Distinct morphological changes were also observed in the sessile cells of CAJ-01 and CT-04 at sub-MICs of thymol (Fig. 3 and 4). Untreated CAJ-01 cells exhibited multilayer of yeast cells with substantial amount of matrix whereas CT-04 exhibited dense network of cells with hyphae formation. Thymol treated CAJ-01cells showed various morphological changes. There were reduced numbers of *Candida* biofilm cells after treatment with thymol as compared to control. There were also shrinkage of cell membrane and leakage of intracellular material [Fig. 4 (C1 and C2)]. Microscopy revealed distorted cell shape as well as reduced hyphae formation in CT-04 after treatment with thymol as shown in Fig. 3(C1 and C2). Treated *Candida* biofilm cells had scattered aggregation. Shrinkage of the cells and permeabilization of cell membrane was also observed in CAJ-01 and CT-04 at sub-MIC of oils.

4.5. Eradication of pre-formed biofilms

Sessile MIC (SMIC) of test compound was considered as the concentration eradicating 80% of pre-formed biofilms. SMIC of *T. vulgaris* and thymol varied from 6.25- 100 µg/ml and 3.12-25 µg/ml respectively, against one or other *Candida* strains. The test agents (*T. vulgaris* and thymol) showed 2-4 folds increased in SMIC against the strains of *C. albicans* (CAJ-01, CAJ-12 (KGMU028 and *C. albicans* MTCC3017). Thymol showed no increase in SMIC compared to PMIC against CAJ-12 (KGMU028), CT-03 and CT-04. Whereas SMIC of *T. vulgaris* against CT-04 was found to be 100 µg/ml. SMICs of *T. vulgaris* and thymol were increased only 2-folds against *C. tropicalis* NRLLY12968 respectively (Table 3).

4.8. Kinetics of inhibition of sessile cells

The time dependent killing of CAJ-01, CAJ-12 (KGMU028), CT-04 and *C. tropicalis* NRRLY12968 by the *T. vulgaris*, thymol, fluconazole and amphotericin B is shown in Fig. 5. Treatment of pre-established biofilms with 2 × SMIC of test oils showed strong fungicidal effect on the strains of *C. albicans* and *C. tropicalis* biofilms. Within 24 h of treatment with *T. vulgaris*, $\log_{10}$ CFU count was reduced from 7.2 to 3.8 against the strains of CAJ-01. Similarly, CAJ-12 (KGMU028), CT-04 and *C. tropicalis* NRRLY12968 also showed significant reduction in viable count within 24 h of treatment of *T. vulgaris*. All the test

strains showed significant reduction in $\log_{10}$ CFU count within 12 h of treatment of thymol. However, amphotericin B and fluconazole could not produce killing effect even upto 48 h.

4.9. Synergistic interaction of essential oils with antifungal drugs in planktonic mode

The synergistic effect of *T. vulgaris* and thymol with fluconazole and amphotericin B were evaluated against CAJ-01, CAJ-12(KGMU028), *C. albicans* MTCC3017, CT-03, CT-04 and *C. tropicalis* NRLLY12968 strains as shown in Table 6a and 6b. *T. vulgaris* and thymol exhibited synergy with fluconazole and amphotericin B against all the tested strains. Thymol showed highest synergy with fluconazole (FICI values 0.156) against CAJ-01, *C. albicans* MTCC3017, CT-03 and CT-04. *T. vulgaris* also exhibited highest synergy with fluconazole (FICI values 0.140) against *C. tropicalis* NRLLY12968. MICs of fluconazole and amphotericin B were reduced upto 32-folds against the strains of *C. albicans* and *C. tropicalis* whereas reduction in *T. vulgaris* and thymol MICs were 8 to 16-folds against the test strains.

4.10. Synergistic interaction of essential oils with drugs in sessile mode

The synergistic effect of *T. vulgaris* and thymol with fluconazole and amphotericin B were evaluated against CAJ-01, CAJ-12(KGMU028), CT-04 and *C. tropicalis* NRLLY12968 sessile cells. Table 7a and 7b revealed the synergistic interaction of essential oils (*T. vulgaris* and thymol) with fluconazole against the above test strains. Thymol exhibited highest synergy with fluconazole against CAJ-01 (FICI values 0.187) and CT-04 (FICI values 0.125). Interestingly, SMICs of fluconazole with thymol were reduced upto 16-folds against all the test strains of *Candida* species. There was also reduction in SMICs of thymol upto 8-folds against the test strains of *C. albicans* (CAJ-01 and CAJ-12(KGMU028)). SMIC of thymol was reduced up to 16-folds against the strains of *C. tropicalis* (CT-04 and *C. tropicalis* NRLLY12968).

5. Discussion

Variation in biofilm forming ability under *in vitro* condition is commonly observed among *Candida* species. However, the ability to form biofim *in vivo* condition may not be directly correlated with *in vitro* ability. Different factors are known to influence the biofilm formation under *in vivo* as well as *in vitro* condition [22]. Such variations have also been previously reported [15, 21]. However *in vitro* biofilm study is important to evaluate the relative characteristics of cell growth in planktonic and sessile mode and provide a platform to asses an antibiofilm activity of bioactive compounds. Biofilm formation by *C. albicans* and *C.*

tropicalis has been documented by many researchers [4, 22, 23, 24]. *In vitro* strong biofilm formation by *C. albicans* and *C. tropicalis* has formed the basis of selection of these strains in the test system for antibiofilm screening. Role of biofilm in virulence and pathogenicity of *Candida* is well documented which provides several advantages to the organisms such as enhance resistance level and protection against host defence system [22].

The strains of *C. albicans* were also previously studied for their antifungal susceptibility profile and found resistant to common antifungal drugs [15]. The MIC values of fluconazole, amphotericin B and ketoconazole against *C. albicans* showed variation from 4 to 32 µg/ml except CAJ-12 (KGMU028) strain where it was ranged from 2 to 1024 µg/ml. Relatively higher MIC values of above antifungal drugs was recorded against *C. tropicalis*. Interestingly, all the test strains of *C. albicans* and *C. tropicalis* showed high level of MIC (1024 µg/ml) against itraconazole. Similar level of variation in MIC values of antifungal drugs was also recorded in *Candida* species by other researchers [21, 25, 26].

Azoles are fungistatic rather than fungicidal so the treatment provides the opportunity for acquired resistance to develop in the presence of these drugs [27]. Amphotericin B has been used as the drug of choice when acquired drug resistance emerges to azoles. However, resistance to amphotericin B has been attributed to absence of ergosterol in the cell membrane, activation of antioxidant mechanisms and decrease in mitochondrial activity [28]. Antifungal activities of plant essential oils and active constituents and their mode of action are documented [29, 30]. The antifungal activity of essential oil is attributed due to the presence of functional groups such as phenols, aldehydes, ketones, alcohols, esters, hydrocarbons [31, 32].

Considering the problem of drug resistance to conventional antimicrobials in pathogenic strains of fungi, *T. vulgaris* and thymol were screened for their efficacy against the drug resistant strains of *Candida* species. Anti-candidal activity of *T. vulgaris* and thymol against drug resistant strains of *C. albicans* and *C. tropicalis* demonstrated promising anti-candidal activity of thymol as compared to thyme oil as shown by their MIC values. Similar activity of *T. vulgaris* and thymol were also reported by many other researchers [33, 34].

Furthermore, *T. vulgaris* constitutes high percentage of phenolic compound such as thymol. Thus, it is speculated that the fungicidal and/or fungistatic activity of *T. vulgaris* can be attributed to its main component, thymol whereas role of other compounds might be contributing in nature.

Inhibition of biofilm and eradication of pre-formed pathogenic biofilm by anti-infective agents are considered as an effective approach to combat biofilm associated infections. Therefore, *T. vulgaris* essential oil and thymol was evaluated for biofilm inhibition at sub-MICs. Different concentrations including sub-MICs of *T. vulgaris* and thymol tested in our previous study showed no cellular toxicity to red blood cells [35].

Varying level of attenuation of *C. albicans* and *C. tropicalis* biofilms in the presence of *T. vulgaris* and thymol indicated that these agents inhibited biofilm either by preventing adherence or subsequent biofilm development.

Further to assess the structural changes in the biofilm development, light and scanning electron microscopy were conducted on glass coverslip surface. Thymol treated cells exhibited disorganization of *C. albicans* and *C. tropicalis* biofilm cells. There were also reduced number of *C. albicans* and *C. tropicalis* biofilm cells with the increase in concentration of thymol. Light microscopy examination also revealed reduced hyphae formation in CT-04 with the increase in concentration of thymol compared to control (untreated biofilms).

Further, SEM examination of *C. albicans* and *C. tropicalis* biofilm cells revealed the dense cell architecture with huge amount of matrix in the control sets. Whereas treated cells exhibited unorganised biofilm cells at sub-MICs of oil. Similar changes in cell morphology of *C. albicans* in the presence of thymol were also reported [34, 36]. SEM images of *C. albicans* and *C. tropicalis* biofilm cells also revealed distorted cell shape, permeabilization of cell membrane and contraction of cell wall that may caused leakage of intracellular material at sub-inhibitory concentrations of thymol.

SEM analysis clearly demonstrated the mode of action of thymol in yeast which showed interaction with cell envelop and intracellular targets as evident from disruption of the cell membranes. Many authors have suggested the similar mechanism of disruption of cell membrane integrity [21, 37, 38].

In the present study, *T. vulgaris* and thymol showed the potential to eradicate the sessile cells of *C. albicans* as well as *C. tropicalis*. The sessile MICs of antifungal drug against the test strains exhibited several folds increase in MICs as compared to PMIC. SMIC of fluconazole and amphotericin B was raised upto 1000-folds in the test strains whereas sessile MIC of *T. vulgaris* and thymol was raised only 2-4 folds against the test strains. Planktonic cells shed from the biofilm surface may get killed by conventional antimicrobial drug therapy however they fail to eradicate sessile cells that are embedded within the EPS matrix [22].

Furthermore, EPS production is considered as an important virulence factor of *Candida* species. It is also responsible for persistence, colonization and firm adherence of pathogen in the host tissues. It is reported that metabolically inactive non dividing persister cells within biofilms may be present. These persister cells are tolerant to a number of antimicrobial drugs despite the fact that they are genetically identical to the rest of the microbial population. It is believed that these cells are responsible for recurring of biofilms on treatment with antimicrobial drugs [22, 39, 40].

Furthermore, efficacy of *T. vulgaris* and thymol were investigated in terms of the time dependent killing of established *C. albicans* and *C. tropicalis* biofilms. Interestingly, test oil and its active compound showed good fungicidal activity against the test isolates. In contrast, antifungal drugs were showing least activity. The present data indicates that test oils exhibited fungicidal activity rather than fungistatic which is very important to combat with recalcitrant infections.

The present study highlights the synergistic interaction between the test essential oil and active compounds with antifungal drugs against the test strains. In our study, interaction of *T. vulgaris* and thymol with fluconazole and amphotericin B is clearly indicated in FICI index against planktonic and sessile mode. Antifungal drugs activity is greatly increased with thymol against the *C. albicans* and *C. tropicalis* strains. The FICI index also revealed the synergy of thyme oil with fluconazole or amphotericin B in planktonic mode. Furthermore, *T. vulgaris* and thymol has anti-candidal activity alone as well as in combination with drugs. Thyme oil and its major component thymol, also showed significant synergy with fluconazole in sessile mode. However, thymol showed more synergy with fluconazole against *C. albicans* and *C. tropicalis* strains in sessile mode. These findings are encouraging and could be exploited in combination therapy as also suggested by other authors [21, 35, 41, 42].

The two antifungal drugs were selected based on their different mode of action and their associated side effects or toxicity. Unfortunately, these drugs may not be used alone to combat fungal infections caused by drug resistant strains of *Candida* species which may require higher doses application resulting in increasing adverse side effects [43]. To overcome such problems, combination therapy is advantageous over monotherapy as it can exhibit more effective way of killing or attenuating pathogenic organisms. Such synergistic/combinational interaction might results in enhanced efficacy of drugs, decreased chances of resistance emergence as well as reducing dose related toxicity [20, 21].

6. Conclusions

The findings of the present study highlight the promising role of *T. vulgaris* and thymol as alternative agents in the treatment of biofilm associated with *C. albicans* and *C. tropicalis* infections. Further, their synergistic interaction with antifungal drugs could be exploited against infection caused by the drug resistant *Candida* species.

Disclosure of interest

The authors declare that they have no conflict of interest.

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