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Original Article

Evaluation of antifungal activity and cytotoxicity of *Thymus vulgaris* essential oil

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ABSTRACT

Objective: To evaluate the antimicrobial properties of *Thymus vulgaris* against seven pathogenic fungi strains and its cytotoxic effect on a human breast cancer cell line (MDA-MB-231) **Methods:** The susceptibility test was carried out in terms of by determining minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) using micro-dilution methods and additional end point by using MTS chromogenic assay. Cytotoxicity activity was assessed using MTT assay, the viability and the IC50 were used to evaluate this test. **Results:** All tested fungal strains were sensitive to the (*Thymus vulgaris* essential oil) TEO and this inhibitory effect is dose-dependent. A remarkable ($p<0.001$) inhibitory effect at concentrations 2.5-10mg/ml for all tested organisms comparable with control was found. The MIC_{50} values ranged between 0.5 to 5mg/ml. The MFC values varied from 2.5–10 mg/mL and were equal to MIC values for majority of the tested organisms. As far as the antifungal activity is concern, out of the seven strains tested, *Candida kefyr* exhibited the highest sensitivity with minimal inhibition growth 2.5mg/ml whereas, *Aspergillus niger* exhibited the lowest inhibition ≥10mg/ml. *Thymus vulgaris* oil inhibited proliferation of MDA-MD-231 cancer cells in a time- and dose dependent manner. IC_{50} value of the complex was found to be time dependent. At 24h treatment, the IC_{50} values ranging between 108-115µg/ml, while at 48h treatment the values were between 71-78µg/ml against cancer cell. **Conclusion:** *Thymus vulgaris* essential oil exhibited significant antifungal and cytotoxic effects on living cells. Therefore, it can be used as alternative antifungal natural substances and also play a significant role in discovery of the new drugs.

KEY WORDS: Antifungal, cytotoxicity, Thymus, MTT assay, MIC

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INTRODUCTION

Mycotic infections caused by various pathogenic and opportunistic strains are on the rise in different parts of the world and are a common diseases in developing countries. During the last decade, fungal infections, mainly those caused by opportunistic microorganisms, have been a problem of growing clinical importance.[1-3] The number of infections caused by new, reemerging, or drug resistant pathogens is growing daily, and the increased proportion of hospitalized patients with immunodeficiency has resulted in an increase of severe and invasive infections.[4] In the literature, the incidence of opportunistic infections in hospital environments or nosocomial infections is related to fungi belonging to the genera *Candida, Aspergillus, Rhizopus, Penicillium, Fusarium* and *Cryptococcus.*[5, 6] *Candida* and *Aspergillus* species accounted for the majority of infections.

Essential oils and their phytoconstituents have shown promising antifungal activity *in vitro* and *in vivo*, where they have been extensively studied against *Candida* spp., *Trichophyton* spp. and A*spergillus* spp.[7-11] The development of protective products of natural origin as alternatives to synthetic fungicides is currently in the spotlight.

Plant-derived drugs remain an important resource, especially in developing countries, to combat serious diseases. In years, a large number of essential oils (especially those of some species of *Thymus*) and their phenolic components have been investigated for their antimicrobial properties against certain bacteria, [12,13] and fungi.[14, 15]

Thymus vulgaris (commonly known as Basil) is a pleasant smelling perennial herb belonging to family Lamiaceae. It grows in several regions of the world.[16] *Thymus* spp. contain a number of chemicals including thymol, γ-terpinene, ρ-cymene, linalool, myrcene, α-pinene, carvacrol and α –thujene.[17] It also contains the eugenol which is a powerful antibacterial agent.[18] Traditionally basil has been used in the treatment of headache, cough, diarrhea, constipation, warts, worms and kidney malfunction.[19] In addition, it is also used as antiseptic, carminative, antimicrobial and for its anti-oxidative properties.[20] The purpose of this study was to evaluate the *in vitro* antifungal activity of the *Thymus vulgaris* essential oil (TEO) against different pathogenic fungal strains and its cytotoxicity effects on breast cancer cell line (MDA-MB-231).

MATERIALS AND METHODS

Extraction of volatile oil by steam distillation

The dried leaves of *Thymus vulgaris* were collected from a local market in Riyadh, Saudi Arabia. The leaves were coarsely powdered and used for analysis. For volatile oil extraction, thirty grams of coarsely powdered leaves were placed in a 1liter round-bottom flask and 750 mL of water was added. The flask was attached with Clevenger apparatus and heated using a heating mantle to boil the water. The volatile oil, along with the water vapor, was passed through a condenser and the oil was accumulated in a graduated side arm of the Clevenger apparatus. Distillation continued until there was no difference in successive readings of the oil volume. The oil was drained out, dried over anhydrous sodium sulfate, filtered through 0.22-µM filter paper, and kept at 4°C in sealed in amber colored vials. The phytochemical analysis was carried out using standard screening tests.[21]

Antifungal activity

Preparation of fungal suspensions

The *Thymus vulgaris* essential oil (TEO) was tested against seven pathogenic fungal strains, including *Candida albicans* (ATCC 10231), *Candida glabrata* (ATCC 15126), *Candida kefyr* (ATCC 66028) and *Candida parapsilosis* (ATCC 22019), *Aspergillus flavus, Aspergillus niger* and *Fussarium* sp. The clinical fungal strains were obtained from the Microbiology Department in Prince Sultan Military Medical City, Riyadh, Saudi Arabia. Microdilution susceptibility testing was performed according to the NCCLSM-27A criteria.[22] *Candida* spp. were cultivated on 4% Sabouraud Dextrose Agar (SDA, Oxoid) for 24h at 35˚C. The suspension inoculum was carried out in MicroScan inoculum water (Siemens Healthcare Diagnostics Inc. USA) from a colony alone. This suspension, after shaking in vortex by 15s, was adjusted to 0.5 of McFarland scale, resulting in a concentration of $1x10^6$ CFU/mL, diluted, 1:10 in RPMI 1640 medium with GlutaMAX™ supplement (Gibco, Life Technologies, New York, NY, USA). Filamentous fungi, susceptibility testing was performed in RPMI–2% glucose medium as described by Espinel-Ingroff *et al.* [23] To induce conidium formation, filamentous fungi were grown on SDA slants of agar at 27°C until they were judged to have formed maximal numbers of conidia or sporangia. Then, each fungal culture was covered with 1 ml of sterile saline containing 0.1% Tween 80, and the spores were washed off by gently probing the colonies with the tip of a pipette. Finally, the suspension was vortexed for 10 s to break up clumps of cells. This was then filtered through four layers of sterile gauze. The conidia or sporangia were counted by using a hemocytometer, adjusted to a density of 10^6CFU/ml. Stock inoculum suspensions were further diluted in RPMI 1640 medium to achieve two-fold final concentrations 5 x 10^4 CFU/ml each of fungus species was exposed to a double dilution of oil extract. *Determination of Minimum Inhibitory Concentration (MIC)* The MIC values were determined for the fungi strains which were sensitive to the essential oil in macro-dilution assay on solid media. The broth micro-dilution bioassay was used to determine the MIC of *T. vulgaris* essential oil (TEO). For this, 96-well flatt bottom plates were used. The 96-well plates were prepared by dispensing 90µL of RPMI 1640 into each well. A 100µL from the stock emulsion of essential oil was added into the first wells. Then, 100µL from their serial dilutions were transferred into consecutives wells, excluding the last ones. In each well of the column, aliquots of 10µL of the inoculum were dispensed for each strain tested. In parallel, the last well contained 90µL of broth inoculated with 10µL fungal inoculums to confirm the cell viability (viability control). A positive control was carried out in the same way with standard antifungal using Amphotericin B (Sigma Aldrich®). Also, a sterility control was performed to verify whether the medium used in antifungal essay was contaminated before test procedures. To determine MIC values, the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyldiphenyl)-2-(4-sulfophenyl)-2H tetrazolium inner salt (MTS) a water-soluble salt was performed as described by the supplier (Promega Corporation). After 24 h of incubation with TEO, 20 µl of a solution containing MTS and phenazine methosulfate (PMS) was added to each well (333 mg of MTS per ml and 25 mM PMS final concentrations). Incubations were continued for another 4h at 35°C, and the plates were read at 490 nm. The endpoints were read with a Hidex Sense micro plate reader (HIDEX Oy, Turku, FINLAND). According to CLSI criteria 100% growth inhibition is defined as clear wells. Therefore, the OD readings at MIC value were similar to the negative control without *Candida* also defined as minimum fungicidal concentration (MFC). The lowest concentration that inhibited 50% of visual growth was recorded and interpreted as the MIC_{50}.

Determination of Minimum Fungicidal Concentration (MFC)

After determining the MIC, the inhibitory and two following higher concentrations as well as the positive controls were sub-cultured on Sabouraud dextrose (Difco®) agar plates in triplicate. After 24h of incubation at 35 ± 2°C, the readings of MFCs were carried out based on the growth controls. The minimum fungicidal concentration was regarded as the lowest extract concentration that hindered visible growth of the subculture (did not yield any fungal growth on the solid medium used).

Cytotoxicity studies

Cell Culture

Human breast cancer cells MDA-MB-231 (obtained from breast mammary glands) [24] were kindly supplied by the cancer research facilities, King Saud Bin Abdulaziz Medical City, Riyadh, Saudi Arabia and had been originally procured from the American Type Culture Collection (ATCC), USA. The cells were maintained in DMEM medium supplemented with 10% FBS (PAA Laboratories, Germany) 2mM L-glutamine, 50µg/mL of penicillin-G, and 50µg/mL of streptomycin sulfate. The culture was maintained as described earlier.[25]

Determination of the cytotoxic property of essential oil from T. vulgaris adopting MTT assay: To evaluate the cytotoxic property of *T. vulgaris* essential oil, MTT colorimetric assay was performed.[26] The extracts were dissolved in DMSO (dimethyl sulfoxide) (Sigma Chemical Co., St. Louis, MO, USA). The cells were seeded in 96-well plates at a density of 5×10^4 cells/well and treated with TEO at concentrations ranging 0-200 µg/ml, and incubated at 37°C, for 24 h and 48 h. At the end of the exposure period, the cells were subjected to assessment of viability using the MTT assay. The IC_{50} concentration was determined as the dose that would be required to kill 50% of the cells.

Morphological assessment of cell death

a). Phase contrast microscope

The morphological features of cell were observed using phase contrast microscopic method. In brief, the MDA-MB-231 cells were cultured and treated with IC_{50} concentration of TEO and incubated for 24 h and 48 h. At the completion of the treatment period, cells were harvested and wash with PBS. The morphological changes of the cells were assessed using phase contrast microscope (Olympus, Tokyo, Japan) and images were captured using a scientific camera (Olympus D73, Tokyo, Japan) at 200x magnification.

b). DAPI staining 4',6-diamidino-2-phenylindole

Cultured and treated washed cells were fixed in 3.7% formaldehyde and stained with 1 µg/ml DAPI for 15 minutes in a glass slide in dark conditions. The stained cells were viewed with a fluorescent microscope at 340-380 nm wave length and 400x magnification (Olympus, Tokyo, Japan) and pictures were taken as described above. The percentage of normal, apoptotic and necrotic cells were calculated.

c). PI staining

Cellular changes of TEO treated cells were observed using Propidium iodide (PI) staining. The cultured MDA-MB-231 cells were treated with TEO and incubated for 24h and 48h. At the end of the treatment period, cells were harvested and washed with PBS. Next, the cells were stained with PI florescent stain and observed through a fluorescent microscope using green filter (Olympus, Tokyo, Japan) and images were taken as described previously.

Statistical Analysis

Data were statistically analyzed by ANOVA using the Graph Pad software package (Graph Pad Instat®, version 3.05, USA). MICs and MFCs of different Candida isolates were compared through analysis of variance followed by comparison between geometric means using the Tukey-

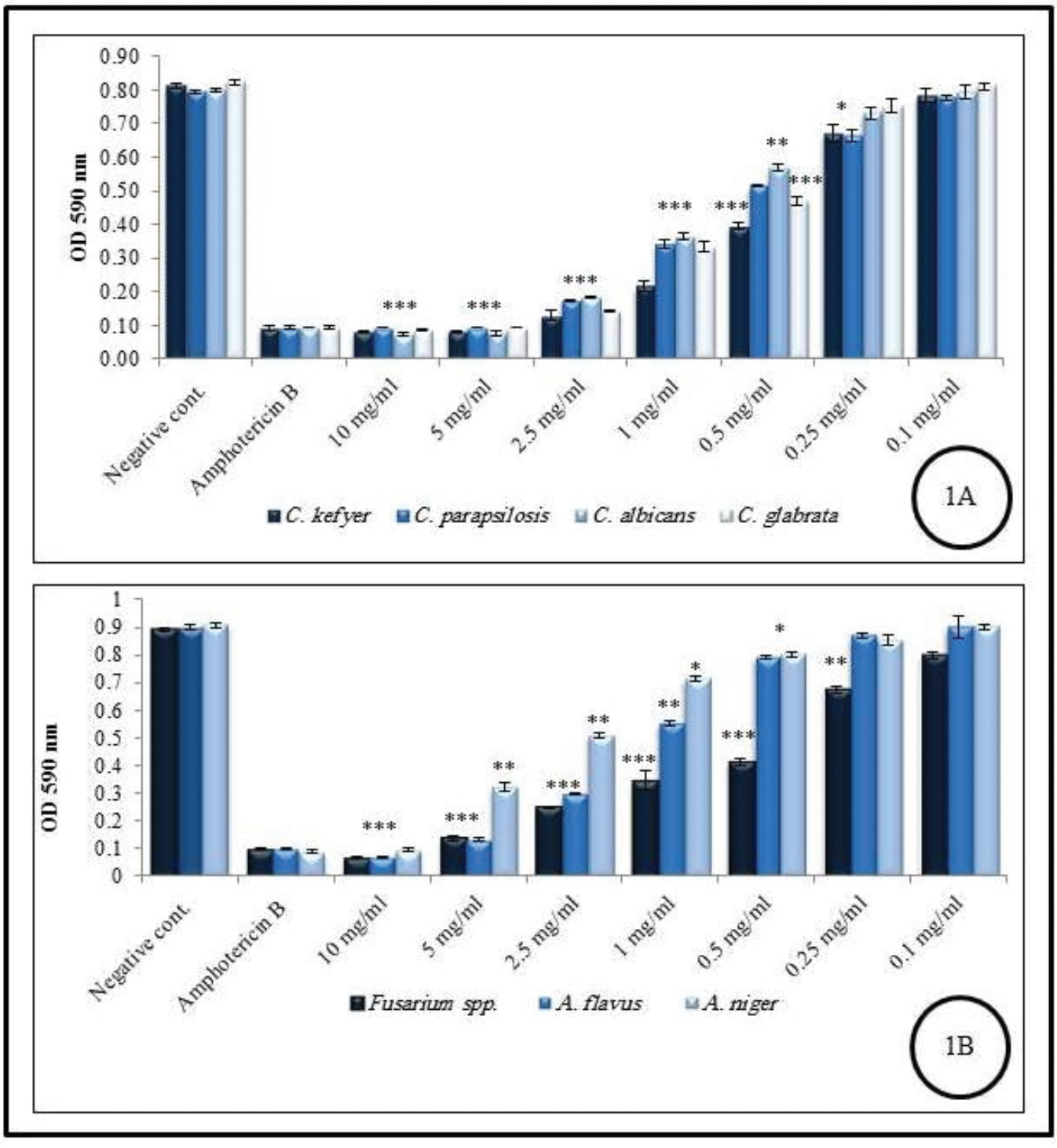

Figure-1:1A). Antifungal activity of TEO against different clinical fungi strains, *Fusarium* sp. *A.flavus*, and *A.niger*; (1B). Antifungal activity of TEO against *C. albicans, C. glabrata, C. kefyr* and *C. parapsilosis*. Data points represent the means of triplicate replications and error bars represent the standard deviations from the means (SEM ±), n=3. *P<0.05, **P<0.01 and ***P<0.001 indicates results that are significantly different to the untreated control (no TEO in the inoculated well) using Tukey test at 5% probability level.

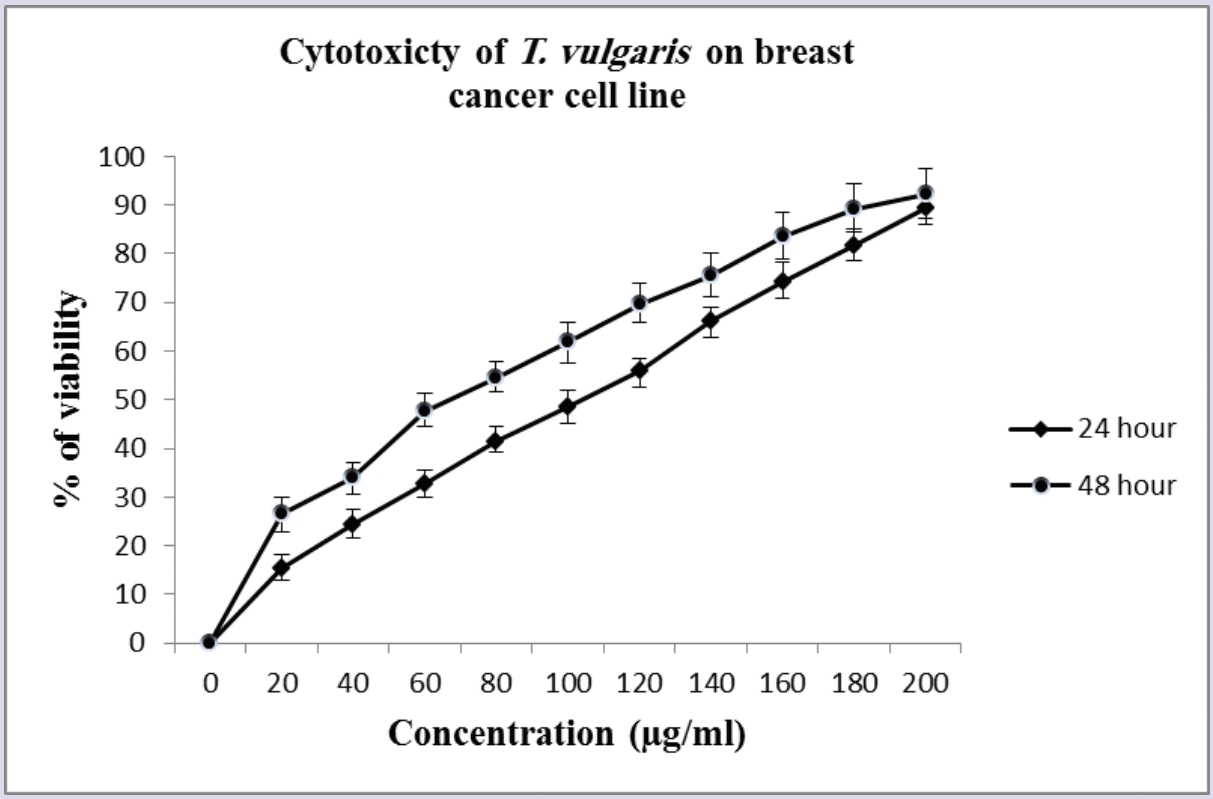

Figure-2: Cytotoxic effect of TEO on MDA-MB-231 breast cancer cell line at 24h and 48h treatment.

Kramer test, at the significance level $p < 0.05$.

RESULTS

Effect of TEO on fungal strains

Antifungal activity of *T. vulgaris* essential oils (TEO) was assessed quantitatively by micro-dilution methods and additional end point using MTS chromogenic assay. The susceptibility test for the TEO was carried out in terms of minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The results revealed that TEO exhibited strong antifungal properties against all tested strains in the concentrations range from 0.5 to 10 mg/ml. Investigation of the influence of TEO on the growth of seven fungi species, showed that this inhibitory effect is dose-dependent at increasing concentrations of the extract in the culture media, reduction in growth was obvious (Figure. 1A, 1B). Our results show a strong significant correlation between the TEO concentrations and growth inhibition ($P<0.001$). The corresponding inhibition growth and MICs are listed in Table 1. The values of MIC and MFC ranged between 2.5 and 10 mg/ml. On the other hand, TEO exhibited the most potent action against *C. kefyr* ($MIC_{50;}$ 0.5 mg/ml) followed by *C. glabrata* (≥0.5 mg/ml), and *C. albicans, C parapsilosis* ($MIC_{50;}$ 1 mg/ml) each whereas, exhibited the lowest potent action against *A. niger* ($MIC_{50,}$ 5mg/ml) followed by *A. flavus* ($MIC_{50,}$ 2.5mg/ml).

Cytotoxicity studies

Effect on breast cancer cells as revealed in MTT Assay

MTT assay determines the integrity of mitochondria and reflects the viability. The IC_{50} values obtained by plotting the cell viability profile against the concentration of extract. The results of MTT assay showed that the TEO inhibited proliferation of MDA-MD-231 cancer cells in time- and dose dependent manner. As shown in the figure 2, the IC_{50} value of the complex was higher for the 24 h treatment groups, i.e., in the range of 108 - 115µg/ml, whereas for the 48 h treatment groups the IC_{50} value fall in the range of 71 - 78µg/ml.

Morphological assessment of cell death

The cancer cells were treated with TEO, at IC_{50} concentration for 24 h and 48 h, MDA-MB-231 responded with different morphological changes such as DNA fragmentation, chromatin condensation and marginalization, membrane blebbing and cell shrinkage (Figures 3A, D, G). At 24 h treatment, around 29% apoptosis and 18% necrosis were observed (Figures 3B, E, H), whereas at 48h treatment, the rate of apoptosis further increased to 37% and necrosis were decreased to 9% (Figures 3C, F, I).

DISCUSSION

Invasive fungal infections cause substantial morbidity and mortality. These are a common health care issue, resulting in a high cost for healthcare. With increasing number of incidents and the spectrum of invasive fungal infections, antimicrobial drug resistance has become a major consid-

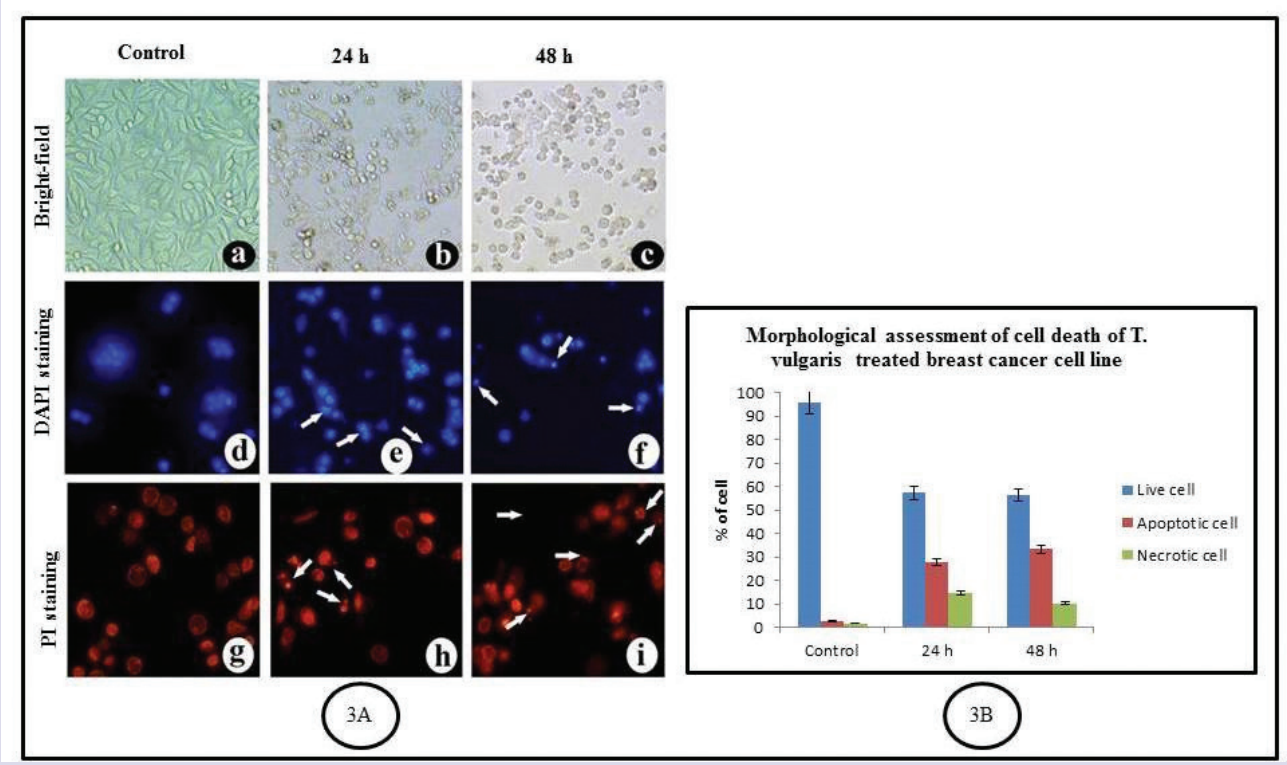

Figure 3: (3A). MDA-MB-231 breast cancer cells treated with TEO for 24 h and 48 h and morphological changes of cell were assessed (a) bright field (BF) -untreated. (b) BF- 24h treatment (c) BF- 48h treatment (d) DAPI-Untreated. (e) DAPI-24 h treatment (f). DAPI-48h treatment (g) PI-Untreated. (h) PI-24h treatment (i) PI- 48h treatment. Arrows indicate the apoptotic cells. (3B). Percentage of normal, apoptotic and necrotic cells.

Table 1: Minimum inhibitory concentration (MIC, mg/mL) and minimum fungicidal concentration (MFC, mg/mL) of TEO against fungi. MIC_{50} the concentration inhibited 50% of the fungal growth. ***$P < 0.001$ highly significant different, **$P < 0.01$.significant different compared with control group.

Tested Strains	MIC_{50}	MIC	MFC	P value
Fusarium spp	1	10	10	***$P<0.001$
Aspergillus flavus	2.5	10	10	***$P<0.001$
Aspergillus niger	5	10	10	**$P<0.01$
Candida albicans ATCC 10231	1	5-≤10	10	***$P<0.001$
Candida glabrata ATCC 15126	≥0.5	5-≤10	5-≤10	***$P<0.001$
Candida parapsilosis ATCC 22019	1	5-≤10	5-≤10	***$P<0.001$
Candida kefyr ATCC 66028	0.5	2.5	2.5	***$P<0.001$

eration in the management of patients. Consequently the need for new drugs and new cost-effective therapies with better efficacy has become important. The usefulness of plant extracts for antimicrobial therapy and/or other diseases have been found to be promising remedies since ancient time in Chinese medicine, Ayurveda, Arabic, and Unani medicine.[27] In these modern days, a substantial number of drugs are developed from plants which are active against a number of diseases.[28] In this study, antifungal activity of *T. vulgaris* essential oil was tested against *Candida albicans*, *Candida glabrata, Candida parapsilosis, Candida kefyr, Fusarium sp, Aspergillus flavus* and *Aspergillus niger*. The present study demonstrated that the *T. vulgaris* essential oil was overall effective against all tested organisms. MIC_{50} and MIC_{100} values ranged between 0.5 - 5mg/ml and 2.5 -10mg/ml, respectively. The current study shows higher anti-fungal activity in comparison with previous published work by Mekonnen *et al.* [29], which reported that the minimum inhibitory concentration values of *Thymus schimperi* EO were <15.75mg/mL for most of the bacteria and fungi used in the study. After determination of the MIC, the fungicidal effect of the product was investigated. The

MFC values of the essential oil varied 2.5–10 mg/mL (Table 1), where the MFCs of the TEO were obtained in this study was equal to MIC values for majority of the tested organisms. These findings prompted by previous studies which demonstrated antifungal activity of *Thymus* ssp. and their phenolic components (thymol and *p*-cymene) against the species of yeasts and filamentous fungi isolates.[14, 15, 30] A similar study reported that both thymol and the essential oil of *T. vulgaris*, whose main components are p-cymene (36.5%) and thymol (33.0%), showed strong fungicidal and/or fungistatic activities against *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., *Trichoderma* spp., *Mucor* spp. and *Rhizopus* spp. [15] In a separate study [31] the antifungal activity of *Thymus vulgares* EO and its constitutuant thymol were investigated against *Rhizopus oryzae*, it was reported that the MIC of TEO and thymol varied 128-512µg/ml, but the MFC of EO and thymol varied 512-1024µg/ml respectively. Giordani *et al.* [32] carried out a study on the antifungal potential of essential oils of various chemotypes of *T. vulgaris* against *Candida albicans*. The essential oil of the thymol chemotype of *T. vulgaris* was the most potent, with a MIC 80% of 0.016µL/mL, where the efficacy was mainly due to the high level of thymol (63.2%). In another study on the essential oil of *T. spathulifolius* (whose thymol content is 36.5%) the growth of *Trichophyton sp*., *Fusarium sp*, *Penicillium sp*., *Rhizopus spp*., *Alternaria spp*. and *Aspergillus spp*., was inhibited with MICs varying between 31 and 250µg/mL.[33] Nevertheless, the range of antimicrobial activities varied depending on the species, subspecies, or varieties of the microorganisms. In fact, essential oils of some plants belonging to the same taxa but collected from different localities showed different levels of antimicrobial activities.[32]

Plants have been used traditionally for the remedy of several diseases. They may act as anti-inflammatory, antimicrobial and anticancer agents. This alternate way of treatment encourage the researchers to explore further herbs and plants to be used against several chronic diseases including that of cancer.[34] The *Thymus* oil killed the breast cancer cells by inducing apoptosis.

CONCLUSION

This study highlighted the antifungal and cytotoxic effect of *Thymus vulgaris* essential oil. The TEO showed excellent antifungal and anticancer activity indicating that it could be a potential candidate to develop new drugs and disinfectants to control infective fungal agents, and also for the treatment of cancer. Further studies are needed to identify the chemically bioactive compounds present in the extract which are responsible for the antifungal activity as observed in this study. Specific studies, *in vivo*, are recommended to determine the efficacy of TEO in the treatment of fungal infections and other diseases in laboratory mice.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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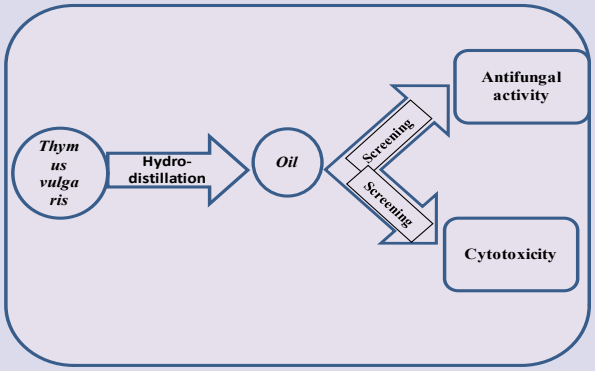
PICTORIAL ABSTRACT



SUMMARY

- *Thymus vulgaris essential oil (TEO) exhibited significant antifungal and cytotoxic effects.*
- *All tested fungal strains were sensitive to the TEO and effect is dose-dependent.*
- *A remarkable* ($p<0.001$) *inhibitory effect were found at concentrations 2.5-10mg/ml for all tested organisms.*
- *Candida kefyr exhibited the highest sensitivity with minimal inhibition growth 2.5mg/ml whereas, Aspergillus niger exhibited the lowest inhibition ≥10mg/ml.*
- *TEO inhibited proliferation of MDA-MD-231 cancer cells in a time and dose dependent manner.*
- *Thymus vulgaris essential oil can be used as play a significant role in discovery of the antifungal and anticancer drug.*

ABOUT AUTHOR

Dr Abdulrahman Al Asmari leads a research team in Research Center of Prince Sultan Military Medical City (PSMMC). His research involves the pharmacological effect and phytochemical studies on the traditional medicinal plants. This research leads to a number of publications in a variety of peer reviewed journals