Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)
ISSN: 2349-2759
Available online at www.jipbs.com

Research article

Biological and physiological responses of *Candida albicans* to some natural products and some heavy metals

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Key words: Candida albicans, Nigella sativa, Syzygium aromaticum, Mentha biperatta.

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Vol. 6 (4), 42-52, Oct-Dec, 2019.

Abstract

The factors responsible for *Candida albicans* pathogenesis are still not fully understood and increasing resistance to commonly used antifungal agents necessitates the search for new formulations. The effect of heavy metals on the growth of *Candida albicans* (ATCC 10231) was tested, where Se, CO and Zn caused 100%, 72.65 and 17.4% dry weight inhibition, respectively. The inhibitory effect of different natural extracts on *Candida albicans such as* ethanolic extract of propolis, methanolic extract of Miswak (*Salvadora persica*), methanolic extract of Black seed (*Nigella sativa*), Musk oil (*Moschus moschiferus*), essential oil of clove bud (*Syzygium aromaticum* and Peppermint oil (*Mentha biperatta*) on *Candida albicans* was evaluated. The antifungal agent fluconazole was used as a positive control. Combination between 50ppm of selenium and 50ppm of fluconazole increased the activity of fluconazole against *Candida albicans*. *Moschus moschiferus* showed more potent inhibitory effect on the growth of *Candida albicans*. Addition of 50ppm selenium to *Moschus moschiferus,* showed highly increase of its antifungal potency against *Candida albicans*. Total protein and lipid content of *Candida albicans* decreased greatly in presence *Moschus moschiferus,* selenium or both together in the growth media, while, carbohydrate content increased slightly in the presence of selenium but sharply decreased in the presence of *Moschus moschiferus* alone and in the combination between *Moschus moschiferus* and selenium.100ppm of cobalt prevented formation of germ tube while equivalent 1ml of *Moschus moschiferus* 100ml media had seemed to destroy the cells of *Candida albicans*, which can make these formulations an effective and safe alternative source for elimination of *Candida albicans*.

Introduction

Candida albicans causes a wide variety of disorders (Candidiasis), which include thrush, candidal enteritis, vulvovaginitis and urinary tract candidiasis, mucocutaneous candidiasis, and invasive candidiasis. The importance of *C. albicans* in causing human diseases requires that the organism be identified from clinical specimens early enough. Because germ tubes are developed quickly, they are used as a rapid presumptive diagnostic identification of *C. albicans*, usually within 90 minutes. Moreover, Germ tube formation is believed to contribute to pathogenicity of *C. albicans*. Two important factors that affect the pathogenicity of *C. albicans* are the ability to switch between yeast growth and filamentous growth, i.e. the hyphal switch**,** and the ability to form biofilms, which enables *C. albicans* to adhere to the surface of substrates. In biofilms, cells show an increased resistance to the immune system and to antifungal drugs [1-2].

Aromatic plants have been widely used in folk medicine. It is known that most of their properties are due to their volatile oils. Essential oils from many plants are known to possess antifungal activity [3], but only limited information is available about their activity toward human fungal pathogens. Volatile vapours emanating from musk, phulwari, jasmine, nagchampa and bela caused approximately 100% inhibition in spore germination *Candida albicans* [4]. Moghim H. [5] indicated that *Nigella sativa* extract is effective against *Candida albicans*, and can be used to develop antifungal medicinal herbs. *Syzygium aromaticum* essential oil due to its anti-biofilm activity can be efficiently used in the prevention of the tested abiotic surfaces colonization by *Candida sp.* [6]. Apart from plants, certain metals have also shown to possess antimicrobial properties. Metal ions may penetrate into pathogens, inactivating their enzymes and thus killing the pathogens [7]. Azole drugs and their derivatives continue to dominate as the antifungal agents of choice against *Candida*-related infections, as either topical applications or oral drugs. Even though they are widely acclaimed for their efficacy, these drugs are known to have side effects [8-9]. Fluconazole, commonly used to treat various *Candida albicans* infections, is fungistatic in nature and there are reports of emerging resistance among clinical isolates of *C. albicans* [10].

Therefore, there is a need to isolate new antifungal agents, mainly from plant extracts, with the goal of discovering new chemical structures without the above disadvantages [11]. Many plant extracts and essential oils have biological activity both *in vitro* and *in vivo*, which has justified research on traditional medicine focused on the characterization of their antimicrobial activity [12]. The antimicrobial activity shown by plant oils is mainly due to a number of phenolic and terpenoid compounds, which have antibacterial or antifungal activity [13]. In addition, it is expected that plant compounds with target sites other than those currently used by antimicrobials will be active against drug-resistant microbial pathogens. Yet, the information available regarding plants (particularly medicinal plants) that are active against this microorganism has, until recently, not resulted in effective formulations for human use. For this reason, the present study assessed six natural products for their effect against *C. albicans* by standard disc diffusion and using the most effective one in combination with some heavy metals and studying the influence of these heavy metals on the efficiency of these natural products as a trial for finding high efficiency natural antifungal products to avoid the side effects of the chemical drugs.

Materials and methods

Microorganisms

Candida albicans used for this study was obtained from the culture collection of the Microbiology Department, Faculty of Medicine, Mansoura University, Egypt.

Plant materials

Propolis samples were collected from Temey El-Amdied, Dakahlia Governorate in April and May, Thepropolis samples of Apismellifera L. were handly collected by using a plastic propolis trap and were kept under desiccation in the dark up to their processing. Buds of *Syzygium aromaticum* and fresh seeds of *Nigella sativa* L were collected from the local market of Mansoura, Dakahlia Governorate, Egypt. Essential oil of Musk and dried root of *Salvadora persica* were kindly provided from "the International Commission on Scientific Signs in the Qur'an and the Sunnah" (KSA). Musk essential oil was kept at 4°C.

Methods

Preparation of propolis extract

An aliquot of crude propolis (7 g) was dissolved in 80 % ethanol by shaking at 50°C for 3days protected from light. The resulting aqueous ethanol extract was filtered three times through paper filter and concentrated at 50°C. The resin obtained was dissolved in absolute ethanol to a final concentration of 3 mg/mL. This final solution was used, itself for the antifungal assays, to be non toxic application.

Preparation methanol extracts (MeOH) of *Salvadora persica*

The powdered stems (100 gm) were extracted with methanol (because methanol gives high dissolution with high resolution during isolation) using a soxhlet extractor for 10 h or until the solvent turned pure and colorless [14]. The solvent was then removed using a rotary vacuum evaporator at 40 °C to give the concentrated extract, which was frozen and freeze-dried until use.

Preparation ethanol extracts (MeOH) of *Syzygium aromaticum*

For extraction, the freshly collected bud of *Syzygium aromaticum* was washed with tap water followed by sterile distilled water. The material was dried in an oven at 50°C for 48 hrs followed by grinding in to a fine powder. Solvent, ethanol (95%) was used for the phytochemical extraction of buds. For extraction with ethanol, 25 g of powdered plant material was dissolved in enough sterilized ethanol to make 100ml of ethanol extract (25% w/v). The mixture was kept undisturbed at room temperature for 24 hrs in a sterile flask covered with aluminum foil to avoid evaporation and subjected to filtration through sterilized Whatman no.1 filter paper. After filtration, the extract was evaporated in water bath at 50ºC until 25 ml extract was left in the container. Ethanolic extracts thus obtained were immediately evaluated for antifungal activity.

Extraction of the essential oil *of Nigella sativa*

25 g seeds were crushed and extracted with petroleum ether for 4 h in a Soxhlet apparatus. After extraction, the solvents were removed by rotary vacuum and dried in a vacuum oven at 30ºC. The essential oil dissolved in dimethylsulfoxide (DMSO).

Effect of some metal ions on *Candida albicans* grown on solid media

To see the effect of metal ion on the antimicrobial activities of the plant extracts against *Candida albicans*, the plate diffusion method as described by the National Committee of Clinical Laboratory standard guidelines. In this method, the culture media {malt extract medium (MYGP)} was prepared. Malt extract medium (MYGP) consists of (g/L : malt extract, 3; yeast extract, 3; glucose, 10; peptone, 5; agar, 15 and distilled water, 1000 ml). The Petri plates and prepared culture media were then autoclaved. The media were carefully poured in the Petri plates and allowed to get solidified. After the media solidified, seeded with 1.0 ml culture (2 x10^6 spore/ml) of *Candida albicans* under aseptic conditions, (before inoculated, the yeast suspension was measured at

650 nm, and adjusted to be the wanted concentration which corresponds the number of cells). Wells were made in the plates with the help of sterile porer (5mm). Aliquots (50 µl) of different concentrations (25, 50, 100, 250, 500, and 1000 ppm) of tested metal ions ($COCl_2$-.$6H_2O$, $ZnSO_4.7H_2O$ and Na_2SeO_3) were then loaded into the wells and plates were incubated at 30°C for 72 hr. At the end of the incubation period, the inhibition zones were measured. The growth or activity were observed after the incubation and was determined by measuring the diameter of zone of inhibition in mm.

Antifungal assay

The inhibitory effect of different natural extracts on *Candida albicans* were evaluated by using plate diffusion method as described by the National Committee of Clinical Laboratory standard guidelines. The concentration of ethanolic extract of propolis and methanolic extract of *Salvadora persica* (50 ppm, 100 ppm, 250 ppm, 500 ppm, 1000 ppm, 2000 ppm, 3000 ppm and 5000 ppm) were used. An aliquot amount of *Moschus moschiferus* and *Syzygium aromaticum* extract (5, 10, 25, 50, 100 µl) were used. An aliquot amount of the essential oil of Nigella sativa (5, 10, 15, 20, 25,30 µl). Each concentration was poured into a well of inoculated plates with *Candida albicans* and incubated at 25°C for 48 h.Ethanol, methanol and dimethylsulfoxide (DMSO) were used as a negative control which was introduced into a well instead of plant extract. The antifungal agent fluconazole was used as a positive control. The inhibition zones were measured (mm). The antifungal activity was expressed in term of the diameter of inhibition zone surrounding the well.

Effect of 50 ppmselenium in combination with *Moschus moschiferus* and antifungal drug on growth of *Candida albicans*

MYGP agar medium was prepared, sterilized and distributed in sterile plates. After solidification, 0.5 ml culture ($2x10^6$ spore/ml) of Candida albicans was spreaded on the agar plate under aseptic conditions. 50 ppm selenium with *Moschus moschiferus* (100µl) or fluconazole (50 ppm) were applied into the well, left one hr at 5°C to allow diffusion, then incubated at 30°C for 72 hr. At the end of the incubation period, the inhibition zones were measured. The antifungal activity was expressed in term of the diameter of inhibition zone surrounding the well.

The effect of *Moschus moschiferus* in combination with selenium on total proteins, lipids and carbohydrates content of *Candida albicans*

This experiment was carried out in 250ml Erlenmeyer flasks (50 ml Malt extract broth in each flask). The flasks were autoclaved at 120°C for 15 min. After sterilization the flasks were supplemented with (Na_2SeO_3) by the addition of the appropriate volume from the sterile stock solution to the medium to get final concentration of (50 ppm). An aliquot amount of *Moschus moschiferus* (100µl) was added. Triplicate sets of 250 ml Erlenmeyer flasks each containing 50 ml were used for each treatment. Aliquot of 1.0 ml culture (2×10^6 spore/ml) of Candida albicans was used as an inoculum. After incubation at 28°C for 3 days at 120 rpm, total proteins, lipids and Carbohydrates were determined.

Determination of carbohydrate

Carbohydrates determination was carried out using the anthrone technique as described by Umbreit *et al*. [15].

Determination of proteins

The cellular Protein content was determined by the method of Lowry OH *et al*. [16] using bovine serum albumin as a standard protein.

Determination of lipids

Total lipid content was determined by the phosphovanillin method [17].

Effect of *Moschus moschiferus* on the formation of *Candida albicans* germ tube

The formation of germ tube by *Candida albicans* as influenced by natural oil and metal was carried out as described by Elmer WK [18]. The yeast was grown on sabouraud agar medium. A small portion of a pure colony ($2x10^6$ spore/ml) was inoculation in to 3 sterile test tubes containing 0.5ml of human blood serum. The first tube used as control. The second tube contains tube contains musk solution (100µl of *Moschus moschiferus*/50 ml serum). The resulting mixtures were incubated aerobically at 37°C for 2 hrs. After 2 hrs, a drop of the yeast-serum mixture was placed on a clean microscope slide, covered with a cover slip and examined microscopically, using scanning (JEOL-JSM-5500 LV) and electron microscope (TEM-100cx, electron microscope, okenShojico, Ltd., Japan). The appearance of small filaments projecting from the cell surface confirmed formation of germ tubes.

Statistical analysis

Variance analysis of data was done using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 1% and 5% level by using [19]. New multiple range tests as described by Snedecor GW *et al*. [20]. Means followed by the same letter are not significantly different at $P \leq 0.01$.

Results

Effect of some heavy metals on the growth of *Candida albicans* on solid media

The efficacy of heavy metals on the growth of Candida albicans were assayed on Malt extract (MYGP) agar medium using the standardized disc diffusion method. In general, selenium and cobalt had the highest antimicrobial activity but zinc had the lowest effect on Candida albicans (Table 1).

Effect of some heavy metals on *Candida albicans* grown on liquid media

The influence of different metals on the cellular dry weight of the same isolate is shown in table 2 that revealed the same effects as the toxicity indexes of Se, CO and Zn are 100%, 72.65 and 17.4%. Table 2 also revealed that the relative potency of selenium was the highest (7.26, 5.46 and 7.01) at 24, 48 and 72 hrs respectively. The same results achieved in table 2 by using dry weight growth criterion, the relative potency of selenium values were 5.16, 8.49 and 5.72 at 24, 48 and 72 hrs respectively.

Effect of traditional folk medicinal natural products on growth of different isolates of *Candida albicans*

The data represented in table (2, 3 and 4) revealed that all of the tested natural extracts exhibited antifungal activity against *Candida albicans*, the growth inhibition varied among plant extracts. That the most effective natural extracts was *Moschus moschiferus* oil, followed by the *Syzygium aromaticum* ethanolic extract, methanolic extract of *Salvadora persica*, ethanolic extract of propolis but the essential oil of *Nigella sativa* has weak antifungal activity on *Candida albicans*.

Table 1. Antifungal activity of some heavy metals (Se, Co and Zn) on Candida albicans grown solid media. ± SE (Standard Error) of the mean. Means followed by the same letter in each column are not significantly different by Duncan's multiple range test (p=0.01) indicated by different letters are significantly different (P < 0.01) according to Duncan's test.

Conc. (ppm)	Inhibition zone diameter (mm)		
	Selenium	Cobalt	Zinc
25	5±0.08 l	3±0.09 lmn	0 L
50	16 ±0.52 hig	6 ±0.08 k	5±0.08 hi
100	20±0.99 def	14 ±0.96 hi	7 ±0.19 gh
250	21 ±1.52 cde	15 ±0.03 h	9 ±0.26 fg
500	27 ±1.95 b	22 ±0.66 de	16 ±0.24 cd
1000	35 ±1.04 a	29 ±0.91 a	24 ±0.64 a

Table 2. Antifungal activity of ethanolic extract of propolis, and methanolic extract of *Salvadora persica* on growth of *Candida albicans*.

Concentration	Inhibition zone diameter (mm)	
	Folk medicinal natural products	
	Propolis	*Salvadora persica*
50 ppm	2 ±0.03 jkl	0.0 n
100 ppm	4 ±0.02 hij	0.0 n
250 ppm	5 ±0.03 ghi	12±0.05 j
500 ppm	7 ±0.06 fg	12±0.22 j
1000 ppm	10 ±0.08 de	13±0.18ij
2000 ppm	15 ±0.16 c	16±0.08g
3000 ppm	22 ±0.99 b	25±0.98 d
5000 ppm	30 ±0.54 a	32±2.04 b
LSD 1% =	1.88	1.48

Means followed by the same letter in each column are not significantly different by Duncan's multiple range test (p=0.01) indicated by different letters are significantly different (P < 0.01) according to Duncan's test.

Table 3. Antifungal activity of different concentration of *Nigella sativa* essential oil extract on growth of *Candida albicans*.

Concentration (µl)	Inhibition zone diameter (mm)
5	4±0.25 m
10	8±0.18 jk
15	9±0.26 ij
20	12±0.09
25	14±0.13 de
30	18±0.34 b
LSD 1% = 1.36	

Table 4. Antifungal activity of ethanolic extract of *Syzygium aromaticum* and *Moschus moschiferus*on growth of *Candida albicans*.

Concentration (µl)	Inhibition zone diameter (mm)	
	Folk medicinal natural products	
	Syzygium aromaticum	*Muschus moschiferus*
5	9 ±0.05 mn	10±0.36gh
10	14 ±0.06 jkl	19±0.34ig
25	19 ±0.14 ij	30±1.09e
50	23 ±0.25 hi	39±2.4 d
100	35±0.28 gh	52±2.04 d
LSD 1% = 2.02.48		

Effect of different concentrations of antifungal drug (Fluconazole) on growth of *Candida albicans*

The data represented in table 5 revealed that fluconazole showed an inhibitory action on the growth of *Candida albicans*. The growth of *Candida albicans* decreased with increasing of concentrations antifungal drug. Fluconazole has strongest effect on the growth of *Candida albicans* at of 1000ppm.

Table 5. Antiungal activity of different concentrations of antifungal drug (Fluconazole) on growth of *Candida albicans*. ±: SE (Standard Error) of the mean.

Concentration(ppm)	Inhibition zone diameter(mm)
50	4±0.13 o
100	9±0.32 n
250	15±0.09 L
500	23 ±1.2 ij
1000	29±0.88fg
LSD 1%= 2.18	

Effect of 50 ppm selenium in combination with *Moschus moschiferus* and antifungal drug antifungal drug on growth of *Candida albicans*

Obviously, to obtain more accurate evidence about combination we must use another high concentration of selenium like 50 ppm selenium with some natural products and antifungal drug.

Table 6 showed that the addition of 50ppm selenium increased the antifungal potency of Fluconazole, and *Moschus moschiferus* against *Candida albicans*.

Table 6. Combined effect of 50 ppm selenium with *Moschus moschiferus* and antifungal drug on growth of *Candida albicans*. ±: SE of the mean.

Conc. (ppm)	Inhibition zone diameter (mm)
50 ppm Fluconazole	4±0.28 cd
50 ppm Fluconazole + 50 ppm selenium	26 ±1.56a
50 µl *Moschus moschiferus*	3±0.02e
50 µl *Moschus moschiferus* + 50 ppm selenium	38±1.24a
50 ppm selenium	16±0.14b
LSD 1%	2.0

Effect of combination of selenium with *Moschus moschiferus* on total proteins, lipids and carbohydrates of *Candida albicans*

The present study was carried out to analyze the antifungal activity of *Moschus moschiferus* with effect of someµmetal ion. The data obtained in table 7 showed that total proteins content in *Candida albicans* was decreased sharply in the presence of combination between selenium and *Moschus moschiferus*, while the *Moschus moschiferus* alone exhibited approximately the same effect on total protein then the selenium alone showed less effect on total protein. On the other hand, 50ppm selenium showed a low effect on total lipids in *Candida albicans* if compared with the other values. Whereas, *Moschus moschiferus* in combination with selenium

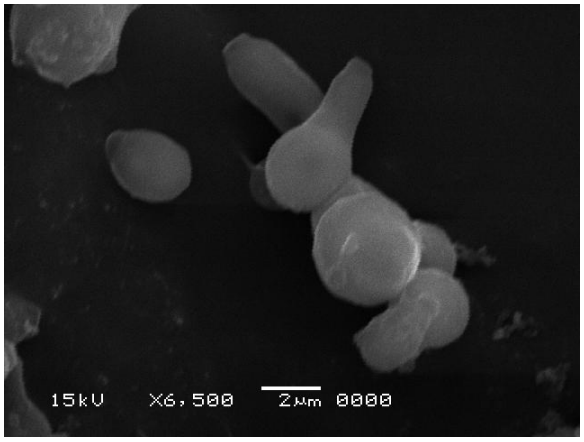
represented a high decrease in total lipids content than alone on *Candida albicans*. Total carbohydrates being highly reduced when *Candida albicans* grown in the presence of combination between selenium and *Moschus moschiferus*, and also decreased in presence of *Moschus moschiferus* but more reduction occurred with *Moschus moschiferus* during the combination with selenium than its presence or its effect alone. Total carbohydrates of the tested organism being increased slightly with selenium.

Effect of *Moschus moschiferus* on the formation of *Candida albicans* germ tube

The data represented in figure 1 Showed that all cells in the first tube (control) formed germ tube, but in the second tube which contains *Moschus moschiferus* solution (100µl of musk oil/50 ml media) the cells were destroyed. So no formation of germ tube had been occurred as shown under scanning electron microscope. Figure 2 showed damaging of the plasma membrane resulted in the inhibition of growth or death of the cell due to the leakage of cellular constituents.

Table 7. Total proteins, Lipids and Carbohydrates content of Candida albicans grown in absence or in the presence of selenium, Moschus moschiferus and selenium combined with Moschus moschiferus. Data are expressed as mg/gm cells dry weight. ±: SE of the mean.

Cellular content	Treatments				
	Control	100µl *Moschus moschiferus*	50 ppm Selenium	50 ppm Se + 100µl *Moschus moschiferus*	LSD 1%
Protein	158 a	72 c	85 b	70 c	2.74
Carbohydrates	170 b	158 c	204 a	82 d	2.74
Lipids	65 a	37 c	b53	25 d	2.73



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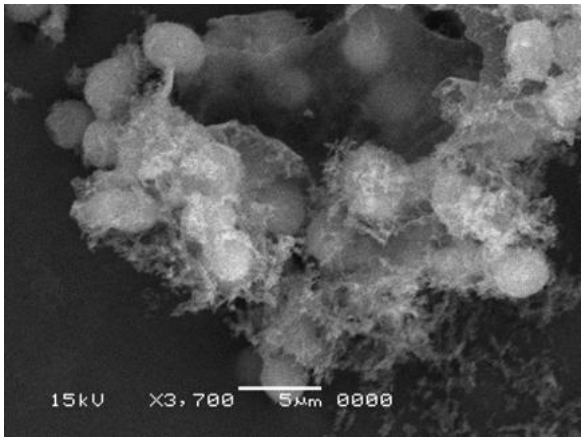
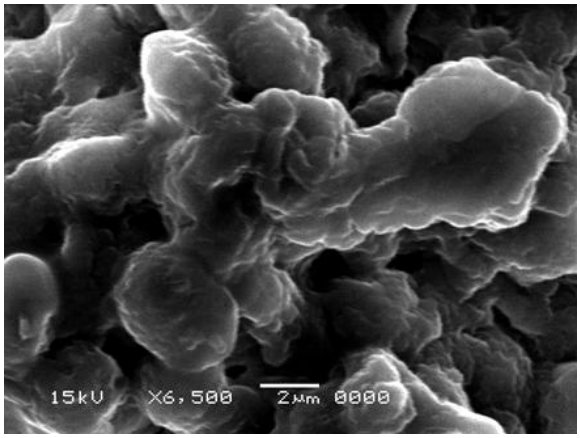
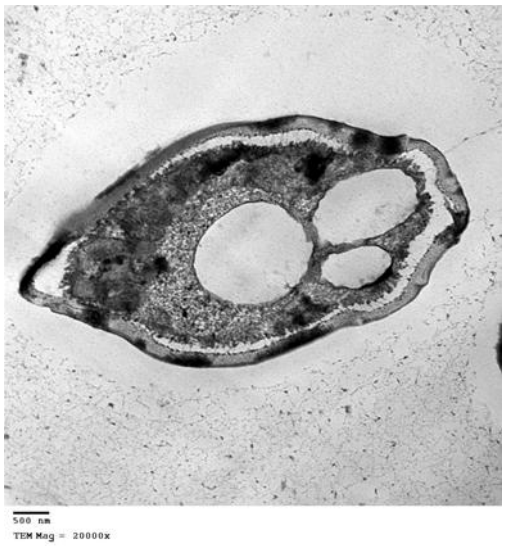


b1



b2

Figure 1. Effect of *Moschus moschiferus* oil on the formation of germ tube in *Candida albicans* under scanning electron microscope. (a) Control (*Candida albicans* alone) and (b1, b2) *Moschus moschiferus* oil effect on formation of germ tube in *Candida albicans.*



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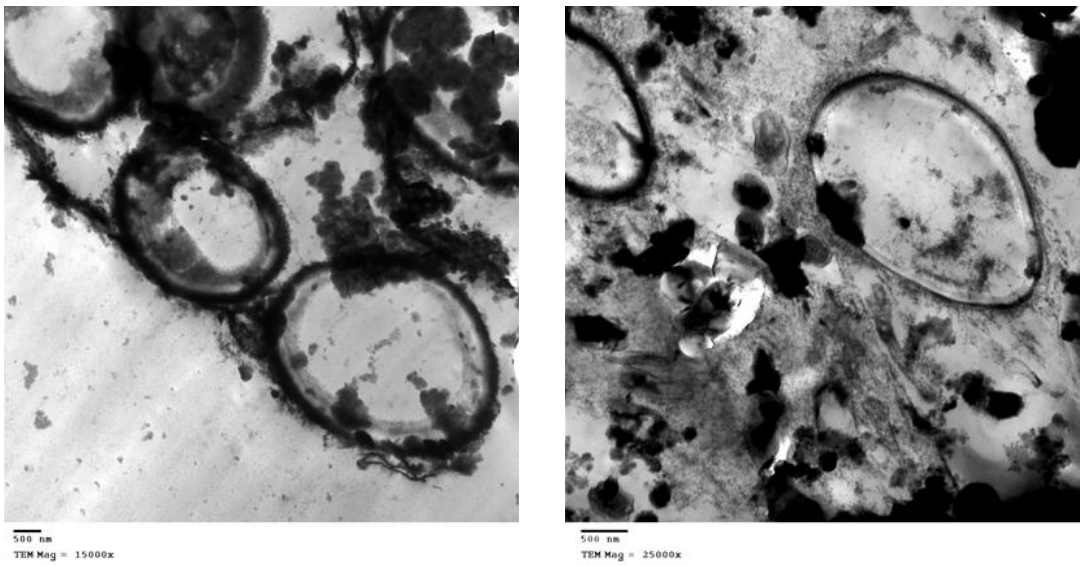

b

Figure 2. Effect of *Moschus moschiferus* oil on the cell of *Candida albicans* under electron microscope (a) Control (*Candida albicans* alone) and (b) *Moschus moschiferus* oil effect on the cell of *Candida albicans.*

Discussion

The increasing failures of chemotherapeutics and antibiotics exhibited by pathogenic microbial infection have led to screening of several medicinal plants for potential antimicrobial activity [21]. Medicinal plants possess potent medicinal value that is due to the presence of variety of phytochemical constituents in the plants tissue which cast a definite physiological action on the human body. Very few of these chemicals are toxic also [22]. Plant oils used as cooking and flavoring agents are increasingly claimed to have broad spectrum antimicrobial activity. Selected oils have been suggested to have potent antimicrobial activity against skin infections, insect bites, chicken pox, colds, flu, measles, sinus congestion, asthma, bronchitis, pneumonia, tuberculosis and cholera, probably due to their phenolic, alcoholic and terpenoid constituents [8-9]. However, azole antifungal agents and their derivatives continue to dominate as the drugs of choice for treating *Candida* infections as either topical applications or oral drugs [23-24]. The present study was under taken with the prime objective of assessing the antifungal properties of selected natural products against *C. albicans*. Fluconazole, commonly used against *Candida* infections, was chosen as the control in the study. Our results clearly demonstrate that the most effective natural extracts was *Moschus moschiferus* oil, followed by the *Syzygium aromaticum* ethanolic extract, methanolic extract of *Salvadora persica*, ethanolic extract of propolis but the

essential oil of *Nigella sativa* has weak antifungal activity on *Candida albicans*. The antifungal activity of propolis has been specifically evaluated against *Candida* [25-28]. The two propolis samples used significantly inhibited the *C. albicans* strains tested, showing a rapid (between 30 seconds and 15 minutes), dose-dependent cytocidal activity and an inhibitory effect on yeast-mycelial conversion (Y-M) at a concentration of 0.22 mg/ml. Moreover, the hyphal length was reduced even at lower propolis concentration. Propolis also caused a dose- and time-dependent inhibition of phospholipase activity. No clear effect was shown on adherence to buccal epithelial cells and surface structure hydrophobicity, but damage to the plasma membrane structure was demonstrated with the Propidium Iodide test [29]. Propolis, a natural resinous product of honeybees, is known to possess antimicrobial properties [30]. [31] Reported that all propolis extracts evaluated are capable of inhibiting the development of *Candida* spp. and the MIC for *C. albicans* ranged from 197 µg mL-1 to 441 µg mL-1. However, they show significant differences in the concentration of polyphenols present and in antifungal activity. Possible modes of action of essential oil constituents (phenolic and terpenes) have been reported in different reviews [32]. However, the mechanisms have not been completely elucidated. [33] Mentioned that the effect of phenolic compounds is concentration dependent. At low concentrations, phenols affect enzyme activity, especially of those enzymes associated with energy production; at greater concentrations, they cause protein denaturation. The effect of phenolic antioxidants on microbial growth and toxin production could be the result of the ability of phenolic compounds to alter microbial cell permeability, permitting the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in their structure and functionality [34]. [35] reported that strong antimicrobial activity could be correlated with essential oils containing a high percentage of monoterpenes, eugenol, cinnamic aldehyde and thymol. [36] suggested that the antimicrobial activity of the essential oils of herbs and spices or their constituents such as thymol, carvacrol, eugenol, etc., could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds. Eugenol, the major phenolic components of *Syzygium aromaticum* essential oil, the antifungal antibiotics amphotericin B, itraconazole, fluconazole and ketoconazole are more antifungal than the essential oils. Perhaps, the mode of extraction of the oils may have contributed to the active agents being suboptimal in the extracts [37]. The effect of euganol on adherent cells and subsequent biofilm formation was dependent on the initial adherence time and the concentration of this compound, and that euganol can inhibit filamentous growth of C. albicans [38]. Also, eugenol was found to disrupt the cell wall of *C. albicans* through altering in ultra structure and cell surface morphology of of *C. albicans* [39]. The antimicrobial sensitivity of the volatile oil *of Syzigium aromaticum* (*Syzygium aromaticum*) against some Gram-negative bacteria (*Escherichia coli* ATCC 35218, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella paratyphi*, *Citrobacter* spp. and *Enterobacter cloacae*), a Gram-positive bacterium (*Staphylococcus aureus* ATCC25923), and a fungus (*Candida albicans*) showed a broad spectrum of activity. The minimum inhibitory concentration (MIC) was determined for each organism as 2.4, 1.6, 0.27, 0.016, 0.23, 1.63, 0.73 and 0.067 mg/ml for *S. aureus* ATTC 25923, *E. cloacae*, *S. paratyphi*, *K. pneumoniae*, *E. coli* ATTC 35218, *E. coli*, *Citrobacter* spp. and *C. albicans*, respectively. Antioxidant screening of *Syzygium aromaticum* oil with 2,2-diphenylpicryl-hydrazyl radical (DPPH) was positive, indicating the presence of free radical scavenging molecules which can be attributed to the presence of eugenol, a phenolic compound [40]. *Nigella sativa* seed composition includes nutritional components such as carbohydrates (glucose, xylose, rhamnose, and arabinose), vitamins as thiamine, riboflavin, pyridoxine, niacin and folic acid, mineral elements (calcium, iron and potassium), proteins, alkaloids (nigellidine, nigellimine and nigellicine), 36%-38% fixed oil and 0.4%-2.5% essential oil [41]. The oil of *Nigella sativa* was also effective against multi-drug resistant strains of *Staphylococcus aureus* [42]. The antifungal spectrum included *Aspergillus niger, Aspergillus flavus, Candida albicans, Microsporum gypseum* and *Rhizoctonia solani* [43]. The antimicrobial activity of *N. sativa* was further established against several species of pathogenic bacteria and yeast [44-45]. Ethanolic and methanolic extracts *Nigella sativa* (L.) seeds exhibited dose-dependent activity. Ciprofloxacin exhibited a synergistic effect with ethyl acetate extract giving zone of inhibition of 18.0mm against S. aureus. When 0.1% Cu was mixed with Ethyl acetate extract it proved 72.0% increase in the activity as it decreased the Minimum inhibitory concentration up to 1.25mg/ml from 4.5mg/ml. No growth was found in the Nutrient agar plate which was mixed with crude oil of *Nigella sativa* (L.) seeds [46]. The results indicated that. *N. sativa* and was good antifungal agent against oral species of *Candida* isolated from individuals wearing complete dentures, hence, there is a possible usefulness as therapeutic agent [47]. The extracts of *Salvadora persica* posses various biological properties, including significant antibacterial [48], antifungal [49]. The antimicrobial and cleaning effects of *Salvadora persica* may be attributed to various chemicals contained in its extracts, such as sodium chloride and potassium chloride, as well as salvadourea and salvadorine, saponins, tannins vitamin C, silica, and resin [50], in addition to cyanogenicor lignan glycosides [52], alkaloids, terpenoids, and oleic, linoleic, and stearic acids [51]. It has been demonstrated by [53] that

Salvadora persica is more efficient antimicrobial agent against *Staphylococcus aureus* and *C. Albicans* tested as oral pathogen than tooth paste. The main objective of this work was to establish the new treatment for invasive *C. albicans* by using natural product with low toxicity for human. The results showed that the presence of metals, in particular selenium, in the growth media inhibited the growth of *C. albicans*, however the presence of zinc in the growth media displayed low effect. Similar observations have been reported by [54] who reported that selenium containing compounds were 30- to 75 fold more cytotoxic than other compounds against *C. albicans* and dermatophytes. In previous studies cobalt and nickel ions showed a highly significant inhibition on the growth of *C. albicans* and Candida tropicalis [55-56]. Copper ion was found best elicitor among all the metal ions selected in the study to enhance the activity of the plant extract against all the bacterial pathogens, followed by Iron and Zinc [46]. The results of the present work showed that fluconazole caused a high inhibitory action against *C. albicans* at low concentration (100 ppm). Similar observations were reported by [57]. Most of the aromatic volatile compounds are known to interfere with cell metabolism, although exact mechanism of inhibition by various active components of volatile compounds is not known. The literature showed that they act as regulators of intermediary metabolism of activation (or blocking) of an enzyme reaction removing or neutralizing an inhibitor, influencing nutrient uptake from the medium, acting as a depressor or otherwise effecting enzyme synthesis at a nuclear or ribosomal level, changing membrane structure, or substituting a limiting factor in intermediary metabolism [58]. In the present study the musk (*Moschus moschiferus*) was effective against *C. albicans*. The results are similar to some extent to that have been reported by [59]. Musk, which is a mixture of musk ambrete, musk ketone, 13-Lonone, pappy oil, sandal oil, and benZY1 benzoate in alcohol, is the only perfume tested based on the derivatives of secretion from perputial follicles of the male musk deer. All other perfumes used in this study are based on derivatives of plant oils only. The presence of sporostatic activity in the plant-based perfume oils may be due to their components such as phenols, aliphatic acids, aldehydes, terpenes and alcohols [60]. In the present study the incorporation of 50ppm selenium with 50ppm fluconazole highly increased their potency against *C. albicans*. The effect of incorporation of metal ion with medical drugs also has been studied and showed an increase of the antifungal potency of some medical drugs. [61] Reported that addition of zinc to antifungal drug "nystatin" increased its activity in treatment of some *C. albicans* infections. Also, [62] showed that mixing of sodium with econazole increased its activity in treatment of candidiasis in skin. The present results showed that the addition of selenium to the natural products *Moschus moschiferus* increased their activity against *C. albicans*. Increasing in the activity of Musk when they were incorporated with selenium may be attributed to the toxic effect of this metal. In contrast, the addition of this metal to some plant extracts appeared to decrease their potency against all the isolates of *C. albicans*, this may be attributed to blocking of the active sites in the active compounds of these plant extracts. The effect of antifungal drugs are divided into inhibitors of sterol biosynthesis such as (terbinafine, miconazole, ketoconazole, clotrirmazole, econazole, fluconazole, itraconazole & amorolfine) and inhibitors of nucleic acid or protein biosynthesis such as (5- fluorocytosine, rifampicin & chlortetracycline) as reported by [63]. In the same manner our results showed that the biosynthesis of lipids and proteins *C. albicans* were highly affected by the presence of selenium and *Moschus moschiferus* and their combination together. Carbohydrates highly affected by presence of *Moschus moschiferus*. Environmental stress including changes in temperature, water content, osmolarity, pH, oxidation, nutritional starvation and chemical compounds. Under severe stress conditions, cellular macromolecules such as proteins, nucleic acid and membranes are seriously damaged and lead to growth inhibition or cell death. To survive these stresses and to avoid potentially lethal damage the cells adapt a variety of stress proteins, accumulation of compactable solutes etc. Proline is an important amino acid which is also known as a stress substrate. It is believed to have multiple functions as it stabilizes proteins and membranes and scavenge reactive oxygen species. It has been reported that proline level increases in the blood serum when the body has an infection. *Candida* species are reported to germinate in high proline medium and the *Candida spp*. change from yeast phase to mycelial phase, the virulence phase [64]. The presence of *Moschus moschiferus*and metal ionprevent the formation of germ tube while equivalent 100µl of *Moschus moschiferus*/50ml media had a destructive effect on *C. albicans*. The importance of germination of *C. albicans* as a critical factor in candidal vaginitis is highlighted by the recent availability of a highly efficacious imidazole, ketoconazole. This agent is not only fungistatic, interfering with candidal cell membrane ergosterol synthesis, but is highly active at very low concentrations in inhibiting *Candida blastoconidial* germination [65]. Volatile vapours emanating from musk caused approximately 100% inhibition in spore germination of *C. albicans* [59]. [66] Indicated that phenolic compounds could denature the enzymes responsible for spore germination or interfere with the amino acids involved in germination. Once the phenolic compounds have crossed the cellular membrane, interactions with membrane enzymes and proteins would cause an opposite flow of protons, affecting cellular activity. [32] Reported that the exact cause effect relation for the mode of action of phenolic compounds, such as thymol, eugenol and carvacrol, has not been determined,

although it seems that they may inactivate essential enzymes, react with the cell membrane or disturb genetic material functionality.

Conclusion

This work highlights the effectiveness of *Moschus moschiferus* or antifungal drugs in combination with low concentrations of selenium against pathogenic *Candida* strains. The growth of *C. albicans* was decreased with increasing concentrations of cobalt and selenium. Combination between selenium and fluconazole increased the activity of fluconazole against *C. albicans*. Additionally *Moschus moschiferus* oil showed more potent inhibitory effect on the growth of *C. albicans*, and the addition of metal ion such as selenium to this *Moschus moschiferus* significantly increased their antifungal efficiency against *C. albicans*. This study denotes the potentiality of this natural product as a source as antifungal drugs and support its use in folk medicine for the treatment of fungal infections. Using the previous plant extract materials alone or with some metal ions as alternative treatment against *C. albicans* can be efficient and safe way as antifungal drug, specially *C. albicans* showed resistance to the common drugs.

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