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Full Length Research Paper

Effect of some alternative medicine and biological factors on *Candida albicans* in Saudi Arabia

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Yeast of the genus *Candida* has been recognized as important microorganisms responsible for *Nosocomial fungemia*. Six isolates of clinical *Candida albicans* isolated from patients and studied by electrophoretic karyotyping of chromosomal DNA by pulsed field gel electrophoresis. Six chromosal DNA profiles were more or less identical. Natural fungicide nystatin is more effective on *C. albicans* growth than the other synthesitic miconazole nitrate and clotrimazole. The minimum inhibitory concentration for 90% growth of *C. albicans* (MIC90) of nystatin is1500 unit/ml. The antimicrobial activities of alternative and traditional medicine in Saudi Arabia were evaluated against *C. albicans* growth. Natural acetic acid, plant extract, charcoal and Elshab are used as natural drug for *Candidiasis*. The percent of 36% Concentration of acetic acid in media causes maximum inhibition of *C. albicans* growth. There are direct relation between acetic acid concentrations and inhibition zones. Increase or decrease of pH inhibits the growth of *C. albicans*. The ideal pH for inhibiting *C. albicans* growth is pH 10. The combination of *Salvadora persica* extract (Meswak) and Elshab ($KAl(SO_4)_2$) in concentration of (28.5 + 12.75 mg/ml, respectively), have an antimicrobial effect on *C. albicans* growth (0.01 g/10ml of media). Charcoal in 20 mg/ml concentration or *Lawsonia alba* extract (Henna) in 250 mg/ml is the ideal concentration used for stop growth of *C. albicans*. The susceptibility of yeast to the previous alternative medicine shows that, it could be used in treating *candidiasis* resulting from different strains of *C. albicans* instead of commercial antibiotics used, without any adverse side effects. Also, it is an attempt to stop development of multidrug resistance organism. *Aspergillus fumigatus* performed a good tool as biocontrol for *C. albicans* which caused infections mainly *candidiasis*. Both are isolated as clinical isolates of *nosocomial* of infected patients.

Key words: *Candida albicans,* chromosomal DNA, *Lawsonia alba, Salvadora persica*, antibiotics, biocontrol.

INTRODUCTION

Candida species have become an important cause of *Nosocomial* infection, and the fourth most common organisms isolated from culture blood (Banerjee et al., 1991). *Candida* spp are among the most frequently isolated microorganisms in clinical microbiology laboratories and are becoming increasingly important, especially as causes of opportunistic and hospital-acquired infections. These organisms are normal inhabitants of human skin and mucous membranes (Joe et al., 2009). Dermal and mucosal human fungal infections have increased at an alarming rate at the last 30 years; it is mainly due to the growing population of immune-compromised individuals such as cancer patients receiving chemotherapy and patients submitted to organ trans- plantation, being treated with immunosuppressive drugs (Portillo et al., 2001; Schmourlo et al., 2005). The natural habitat of *Candida albicans* as common colonizers of skin and mucous membranes probably contributed in part to opportunities for *Candida* exposure to antibiotic that are used for different treatment indications (Powderly et al., 1999).

Resistance of microbes to antimicrobial agents is a worldwide concern (Joe et al., 2009). A number of recent studies have reported emerging antimicrobial resistance among *Nosocomial* isolates particularly *Candida* spp. The use of newer agent may help to avoid selection of resistant mutants.

Control of *C. albicans* was carried out by application of some alternative and traditional medicine (Gabriela et al., 2001). The use of plant extract or plant-derived chemicals

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to treat diseases, topical, subcutaneous and systemic, has stood the test of time (Oladunmoye, 2006). In recent years, there has been a gradual revival of interest in the use of medicinal plants in developing countries because herbal medicines have been reported safe and without any adverse side effect especially when compared with synthetic drugs (Iniaghe et al., 2009). Also, there has been little or no report of any form of microbial resistance during the use and administration of herbal medicines (Stephen et al., 2009). Also, acetic acid is an important factor affecting on the hydrogen ion concentration that has a role in metabolic activity of the microbes. Lactic and acetic acids are a chemical compounds extracted naturally which act as synthetic antiseptic chemical agents (Bangwart, 1989).

This study was aimed at *Nosocomial* infections with special interest in skin and mucous microorganism flora that could predispose patients to infectious *Candidiasis*. Also, it was aimed to determine the *in vitro* standard antibiotic susceptibility pattern of *C. albicans* isolated from patients. Then this study evaluated treatments of alternative medicine including acetic acid, Elshab (KAl $(SO_4)_2$, *Salvadora persica* extract, *lawsonia alba* extract and charcoal against *Candida* compared to standard antibiotic, in a trail to get a good first line drug for infection mainly *Candidiasis*.

MATERIALS AND METHODS

Tested organism

The Fungal surveillance cultures were obtained on admission and weekly thereafter cultures were collected and processed for more studies. Six isolates of Candida species from patients were saved. The isolates were submitted to electrophoretic karyotyping of chromosomal DNA by pulsed field gel electrophoresis technique modified by Doebbeling et al. (1993). Organisms were identified to the species level by using the API 20C system (Analytab Products, Plainview, N.Y.) and stored in distilled water at room temperature. For rapid identification of *Candida* to species level, DNA based identifications have been the most dominate techniques in recent years (White et al., 1998; Patel et al., 2000).

A novel method for DNA based identification was developed. A universal forward and reversed primer pair of U1 and U2 is used to amplify fungal 28S ribosomal DNA region, the PCR products were cleaved with a proprietary reagent kits (SNAP71) at both A and G nucleotides positions to obtain base specific fragments. A high 24 capillary multiplexed gel electrophoresis system with fluorescence detection was used to analyze the fragments with single base separation resolution. A sequencing pattern of PCR product was compared to a database for *Candida* species identification (Banerjee et al., 1991).

Comparative study on the effect of natural and synthetic antifungal on the growth of *C. albicans*

Nystatin (10×10^4 unit/ml) was obtained from Squib and Estrya farma sotica- Spanish, Miconazole nitrate (20×10^3 unit/gm) from Gansen farma sotica- Pelgica and Clotrimazole (10×10^3 unit/ml) was obtained from Pyer liver cozon- Germany. Half ml of yeast suspension (6×10^3 cell /ml) was inoculated onto solid Sabouraud dextrose agar. After 2 h the agar plates were cutten using 1 cm cork borer 0.1 ml of each Nystatin, Miconazole nitrate and Clotrima-zole were filled in wells separately. Addition of 0.1 ml sterile distilled water in a well was used as control. The plates incubated at 28°C for 24 - 72 h. The diameter of inhibition zones were measured for 72 h.

Determination of the minimal inhibitory concentration (MIC) of the growth of *C. albicans* by antifungal (Nystatin)

The minimal inhibitory concentration (MIC) was determined by the broth microdilution technique. Ten ml of Sabouraud dextrose broth was added to get different concentrations of Nystatin (250, 500, 1000, 1500, 2000 and 2500 unit/ml) then inoculated by 0.5 ml of *Candida* suspension to each tube. The tubes was incubated at 25°C for 48 h followed by centrifugation (3000 /min) for 15 min. The fresh and dry weights of the precipitated yeast cells were calculated as g/10 ml concentrations that would inhibit 90% growth of the tested isolate of MIC_{90} of Nystatin.

Effect of different concentration of acetic acid and pH on the growth of *C. albicans*

The standard agar well diffusion as described by Perez et al. (1990) was used. One ml of yeast suspension was added to Sabouraud dextrose agar media. After 2 h agar plates were cut using 1 cm cork borer followed by addition of 1 ml of different concentrations of acetic acid (1, 2, 5, 9, 18 and 36%) in the wells. Addition of 1 ml sterile distilled water in a well was used as control. The plates were incubated at 28°C for 5 days. The diameter of inhibition zones were measured every 24 h. Effect of different hydrogen ion concentrations on the growth of *C. albicans* was studied using the tubes containing Sabouraud dextrose broth. Buffered solutions 0.2 M KH_2PO_4 and 1.0 M citric acid for pH 2.8, 4.4, 6 and 7.6 while, 0.2 M boric acid and 0.2 M NaOH for pH 8.2 and 10 were used. The tubes were inoculated with 1 ml yeast suspension and incubated at 28°C for 5 days followed by centrifugation (3000 /min for 10 min). The fresh weight of the precipitated yeast cells was calculated as g / 10 ml.

Effect of *Salvadora persica* extract (Meswak) and Elshab ($KAl(SO_4)_2$ on the growth of *C. albicans*

S. persica extract and $KAl(SO_4)_2$ solution were prepared by boiling 19 and 8.5 g of *S. persica* stalks and $KAl(SO_4)_2$ separately in 500 ml distilled water for 10 min to get 38 and 17 mg/ml, respectively, followed by filtration and the extract was stored in refrigerator at 4°C. The following concentrations were prepared for *S. persica* extract and ($KAl(SO_4)_2$ solution (9.5, 19 and 28.5 mg/ml) and (4.25, 8.5 and 12.75 mg/ml), respectively, and added to Sabouraud liquid media.

Addition of 1 ml of yeast suspension and incubated at 25°C for 24 - 48 h followed by centrifugation (3000 /min) for 15 min. The fresh and dry weights of the precipitated yeast cells were calculated as g/ 10 ml. The same previous method was repeated with addition of combined *S. persica* stalks and $KAl(SO_4)_2$ in the following concentrations 9.5 + 4.25, 19 + 8.5 and 28.5 + 12.75 mg/ml , respectively.

Effect of some adsorptive factor charcoal and astringent material *lawsonia alba* extract (Henna) on the growth of *C. albicans*

Dried charcoal (adsorptive factor) was sterilized by using ultra

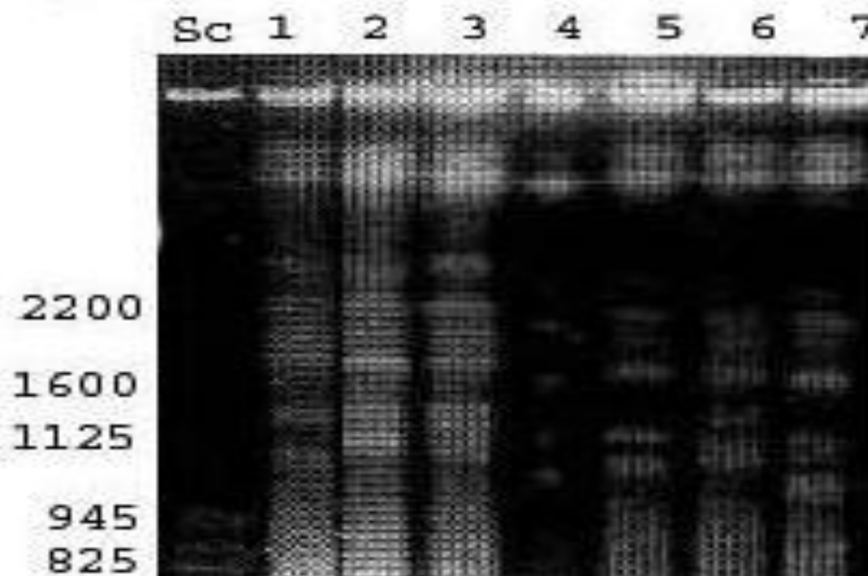

Figure 1. Electrophoretic karyotypes of *C. albicans* isolates isolated from nosocomial infections Sc = *Sccharomyces cerevisiae* chromosome DNA size standards (kb = kilobases), lanes from 1 to 7 except 5 show six isolates of nosocomial C*. albicans,* lane5 is control from the special microbiology lab Iowa, USA.

violet radiation and added to 50 ml of Sabouraud dextrose broth which inoculated with 1 ml yeast suspension to obtain the following concentrations of dried charcoal (0, 4, 10, 14 and 20 mg/ml) . The flasks were incubated at 25°C for 24 - 48 h followed by centrifugation (3000 /min) for 15 min. The fresh and dry weights of the precipitation were calculated as mg/ml. The method for preparation of *Lawsonia* extract was carried out according to Al- awadi and Al-Jedab, 2000. Twenty five gm of dried *Lawsoina* was added to 100 ml boiled distilled water and leave for 6 h and then filtered using seitz filter. Sabouraud dextrose broth was added by *Lawsoina* extract in conical flasks to obtain the following concentrations of *Lawsonia* extract (62.5, 125, 187.5 and 250 mg/ml) which inoculated with 1 ml yeast suspension. The flasks were incubated at 28°C for 24 - 48 h followed by centrifugation (3000 /min) for 15 min. The fresh and dry weights of the precipitated yeast cells were calculated as g/10 ml.

The effect of *Aspergillus fumigatus* as biocontrol of *C. albicans*

Clinical *A. fumigatus* was isolated and identified from nosocomial infections. Filtrate of *A. fumigatus* culture grown on Sabouraud dextrose for 5 days at 28°C was collected and sterilized by seitz filter. The filtrate was applied on *C. albicans* and the effect was indicated by measuring inhibition zone in cup plate method. Parallel and perpendicular streaking plate methods were applied on A. *fumigatus* and *C. albicans*. All assays were carried out in triplicates to calculate the mean results.

RESULTS AND DISCUSSION

Identification of tested organism

Differentiation among the isolates was achieved by visual comparison of electrophoretic karyotypes (Vasquez et al., 1993). Clinical six isolates from *Nosocomial* infections were considered as the same profile. All the bands in one isolate matched the bands in another, showing 2 bands over 2.2 kb, a 2.2 kb band and three bands between 2.2 and 1.0 kb. The bands were similar to the number of bands and respective molecular weights of control (Figure 1) were previously known and confirmed in this study. Figure 2 shows electropherogram of *C. albicans*. The U1-U2 primer pair is able to amplify all *Candida* isolates tested. The PCR product is approximately 270 pb. Software was also developed to search a database created with ATCC reference stains. The software is able to match and score the similarity of different species automatically.

This method appears highly accurate for micro-organism identification, likely because the information generated is based on the DNA sequence of regions previously shown to be of benefit for taxonomic cate gorization *Candida* species. In addition, high sample can be obtained through the use of a multiplexed capillary gel electrophoresis system allowing multiple samples to be simultaneously analyzed (Banerjee et al., 1991; Patel et al., 2000).

Comparative study on the effect of natural and synthetic antifungal on the growth of *C. albicans*

The widespread use of antibiotics has been responsible for the development of numerous problems including the emergence of multidrug resistant organisms. Antibiotics use is suggested to be major risk factor for development

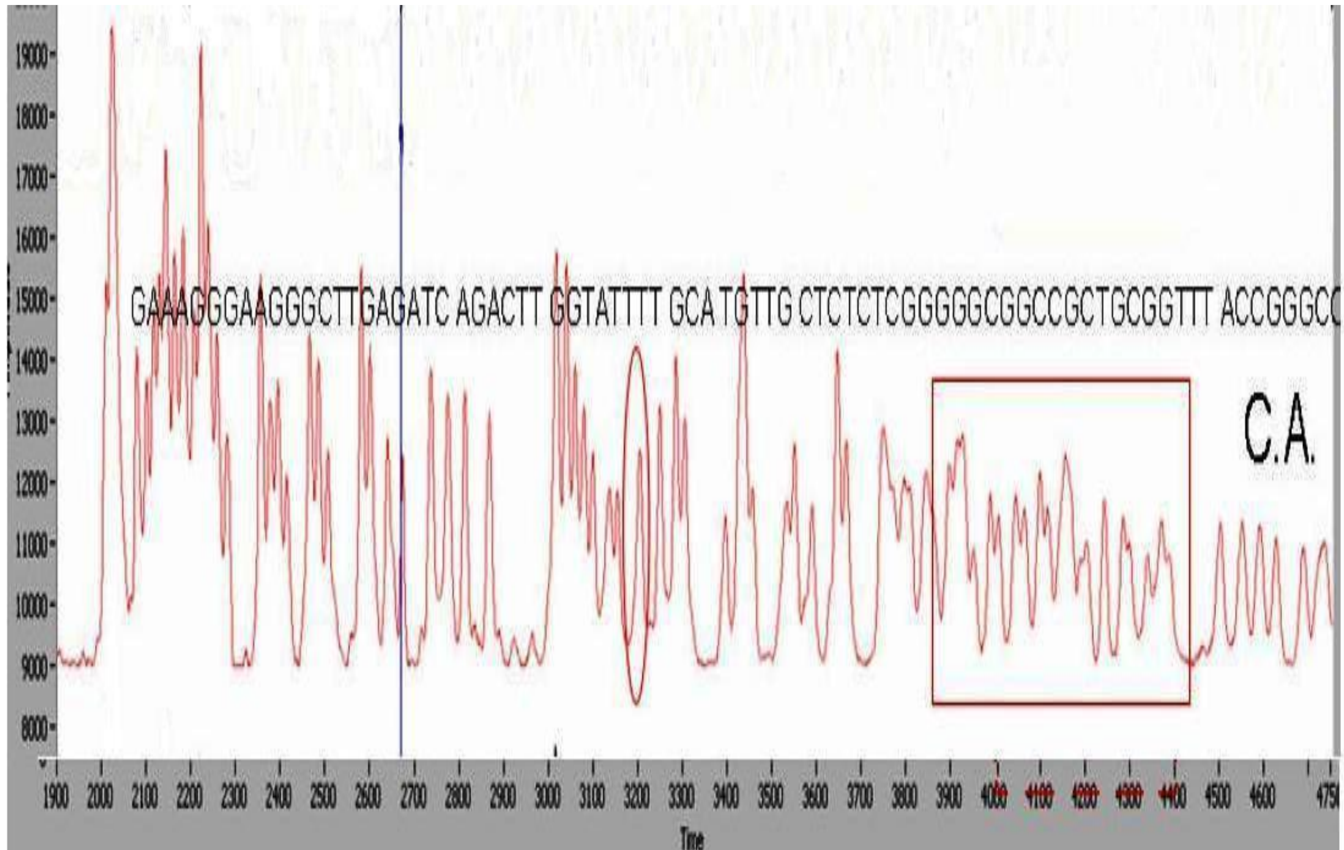

Figure 2. Electrophoregram of *C. albicans.*

Table 1. Comparative study on the effect of different antifungal (as recommended doses) on the growth of C. albicans (g/10 ml) grown on Sabouraud dextrose broth after 5 days incubation at 28˚C.

Antifungal	Inhibition zone (cm)
Nystatin	4.5
Miconazole nitrate	3.5
Clotrimazole	1.6
LSD at 0.05	0.6

of resistance (deMan et al., 2000). Antibiotics are the most important in the treatment of microbial infections and antibiotic resistance microbes become major problem of health for human and animals. The highest inhibitory effect on *C. albicans* was achieved with Nystatin followed by Miconazole nitrate while Clotrimazole has the lowest effect (Table 1). This result agrees with that obtained by Qadri et al. (1986) who stated that Nystatin fungicide has high inhibitory effect (99% inhibition) on 20 species of *Candida* compared with Miconazole nitrate, Clotrimazole and Amphotericin-B. Miconazole nitrate has a moderate effect on *C. albicans* due to the prevent induction of ergesterol in fungal cell and it can change the lipid components of plasma membrane, leading to fungal death (Qakley et al., 1999). Moreno et al. (1988) and Porretta et al. (1988) found that Imidazole fungicide have wide antifungal activity against *C. albicans.* Smith et al. (1990) investigated the fungicidal effect of Clotrimazole by its inhibitory effect on sterol biosyntheses. The obtained results reveal that natural fungicide Nystatin have the highest efficacy with low side effects compared with synthetic fungicide (Miconazole nitrate and Clotrimazole) with moderate efficacy and adverse side effect.

Determination of the minimal inhibitory concentration (MIC) of the growth of *C. albicans* by antifungal (Nystatin)

From Table 2, the MIC that would inhibit the growth of 90% of the tested bacterial isolates (MIC_{90}) of Nystatin for *C. albicans* was ≈ 1500 unit / ml. The highest efficacy of Nystatin against tested yeast could be due to the binding of the ploinic molecule of antifungal with ergesterol on the fungal membrane leading disturbance in cell osmosis and leakage out of potassium, magnesium, sugars and metabolic substances leading to fungal death (Debono and Gordee, 1994).

Table 2. Effect of different concentrations of Nystatin on *C. albicans* to obtain minimum inhibitory concentration (MIC).

Growth (g/10 ml)	Nystatin concentrations (unit/ml)							
	0 (control)	250	500	1000	1500	2000	2500	LSD at 0.05
Fresh weight	0.23	0.21	0.14	0.12	0.07	0.00	0.00	0.22
Dry weight	0.06	0.05	0.04	0.02	0.01	0.00	0.00	0.01

Table 3. Effect of different concentration of acetic acid on the growth of *C. albicans* by measuring inhibition zones (cm).

Acetic acid (%)	Inhibition zone (cm)		
	24 h	48 h	72 h
0	0	0	0
1	1.2	1.0	0.8
2	1.7	1.4	1.1
5	2.3	2.0	1.2
9	2.8	2.8	2.8
18	3.5	3.5	3.5
36	4.5	4.5	4.5
LSD at 0.05	0.5	0.2	0.3

Effect of different concentration of acetic acid and pH on the growth of C. *albicans*

Table 3 indicates that there are a direct relation between acetic acid concentrations and the diameters of inhibition zones reaching to the maximum inhibition at 36% concentration of acetic acid in media. On the other hand there are a reverse relationship between the inhibitory effect of acetic acid and incubation period. The maximum inhibition of acetic acid was recorded at the first 24 h; this may be referring to the fact that the pathogen is more sensitive at the beginning of growth. Turk and Porter (1978) reported that the microbes which reproduce fast at the log phase is more sensitive to antiseptics and antibiotics more than that at the stationary phase and this agree also with Carthy et al. (1985) who stated that the more sensitivity of pathogen against antifungal and antiseptics was at the log phase. *C. albicans* cells were inhibited at the log phase where the antibiotics are interfering with metabolic processes (Hammond and Klineger, 1974).

Bangwart, 1989 stated that the antifungal activity of acetic acid as organic acid affect on transport of necessary nutrient through cytoplasmic membrane which was destroyed due to increasing acidity. The toxic effect of acetic acid refers to penetration of weak acids inside the microbe stopping the nucleic acid and protein synthesis. The antifungal activity of acetic acid refers to the presence of potassium hydroxide which separated from apple acetic acid which prevents the absorption of water by microorganisms leading to stopping the growth (Matousek and Cambel, 2002).

Regarding the pH effect on the growth of *C. albicans*, the neutral acidic pH (5.6 - 6) lead to the maximum growth and with increasing the pH the growth decrease Figure 3. This result agreed with that obtained by Faergemann et al. (2000) who stated that the maximum growth of *C. albicans* was obtained at pH 6. Decreasing of pH leads to decrease the growth of normal flora of skin (Chikakan and Takhashi, 1995). Increasing pH leads to transformation *C. albicans* from Y form to M form and accordingly the cell energy will be directed towards threads formation with decreasing division process (Matousek and Cambel, 2002). Studying the effect of pH on *C. albicans* clarifies the presence of pathogen on the mucosal membrane and skin without causing disease, this refer that the skin surface is acidic due to secretion of organic acids by some natural flora as *Staphylococcus* and *Lactobacillus* (Brooks et al., 1991).

Effect of *S. persica* extract (Meswak) and Elshab $KAl(S0_4)_2$ on the activity of *C. albicans*

There is a significant inhibition of growth of *C. albicans* by decreasing of fresh and dry weights with increasing concentration of both. Applying of $KAl(S0_4)_2$ on *C. albicans* aggregated its cell, followed by precipitation process and this results due to the chemical properties of $KAl(S0_4)_2$ which used in precipitation process of soil microorganisms.

The negative charge of cells adsorbed on the positive charge of potassium and aluminum ions lead to neutralization process accompanied with inactivation of the enzymes and destruction of plasma membrane causing death of cells (Holmes et al., 1992). The inhibitory effect of *S. persica* extract refers to the presence of tannic acid which contains phenolic groups which have antimicrobial effect as antioxidant causing mutation for bacterial cells (Khan and Hadi, 1998). Antibacterial effect of aqueous and alcoholic extract of *S. persica* was tested by Al Bagieh and Almas (1997). *S. persica* fiber is non polluted fiber due to the presence of Sinnigrin perfumer which produced by the action of Myrosin enzyme on glycosidic compounds in mustard seeds. Combination of $KAl(S0_4)_2$ and *S. persica* extract recorded the maximum inhibition (0.01 mg/ml) on *C. albicans* and this refer to the synergistic effect for both the inhibitory compounds in *S. persica* extract and the precipitation phenomenon of $KAl(S0_4)_2$ (Table 4).

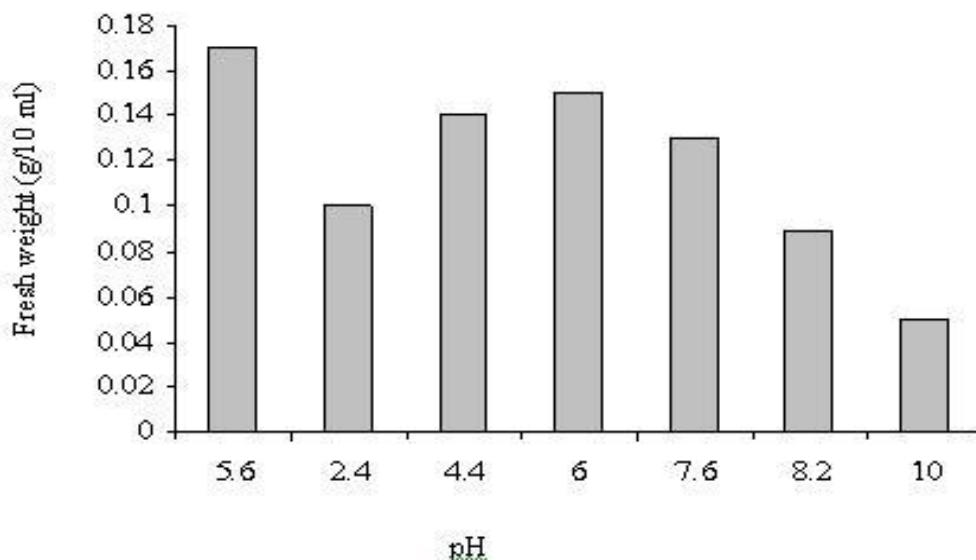

Figure 3. Effect of different hydrogen ion concentrations on the growth of C. albicans (g/10ml) grown on Sabouraud dextrose broth after 5 days incubation at 28˚C. LSD at 0.05 = 0.03.

Table 4. Effect of $KAl(SO_4)_2$ Elshab and *S. persica* extract (Meswak) on the growth of *C. albicans*(g/10 ml) grown on Sabouraud dextrose broth after 5 days incubation at 28˚C.

Growth (g/10ml)	**$KAl(SO_4)_2$ (mg/ml)**				***Salvadora persica* extract (mg/ml)**				**$KAl(SO_4)_2$ + *Salvadora persica* extract (mg/ml)**			
	0	**4.25**	**8.5**	**12.75**	**0**	**9.5**	**19**	**28.5**	**0**	**4.25+9.5**	**8.5+19**	**12.75+28.5**
Fresh weight	0.98	0.19	0.16	0.06	0.98	0.73	0.65	0.60	0.98	0.19	0.15	0.05
Dry weight	0.21	0.09	0.08	0.03	0.21	0.19	0.16	0.14	0.21	0.09	0.06	0.02
LSD at 0.05	0.62	0.08	0.05	0.03	0.62	0.50	0.35	0.30	0.62	0.02	0.09	0.01

Effect of some adsorptive factor (charcoal), astringent material *Lawsonia alba* extract (Henna) on the growth of *C. albicans*

Any increase in the adsorptive factor (charcoal) leads to decrease in the fresh and dry weights of yeast reaching to the minimum values (0.03, 0.02 respectively at 20 mg/ml charcoal) compared with non treated control (0.51 and 0.35, respectively) Figure 4. Charcoal has high adsorptive power on fungal cells.

The surface of activated charcoal has high adsorptive power due to the wide surface area and surface polarity. This binding may be as chemical contributive which result from the attractive force between the activated charcoal surface and the membrane of the yeast cell (Kazaz and Hashad, 1996).

Astringent material (Lawsonia extract) inhibited the growth of *C. albicans.* The lethal concentration was 250 mg/ml. *Lawsoina* extract contain astringent tannins which purified the skin surface from microbes (Azazy, 2002). The inhibitory effect of *Lawsoina* extract refers to the presence of *Lawsoina* which composed of naphtha-quinone and coumarin that dehydrates the water content of microbes. Hanna and Kassem (1985) reported that the antifungal effect of *Lawsoina* extract is due to the presence of 5 - 10% tannin and gallic acid with few of steroid.

The effect of *A. fumigatus* as biocontrol of *C. albicans*

The surfaces of the muscousal membranes are consequently support a diverse range of microorganisms that constitute the normal flora. These microorganisms interact with each other and with the immune system of the host. The consequence of these interactions in that, under normal conditions, the growth of individual microorganisms is regulated and infection is prevented. Infection is the results from either the acquisition of a virulent microorganism or uncontrolled growth of an existing organism because of lowered host resistance (Joe Lim Chung, 2009). The inhibition zone of *C. albicans* around the filterate of *A. fumigatus* after 24 h was well

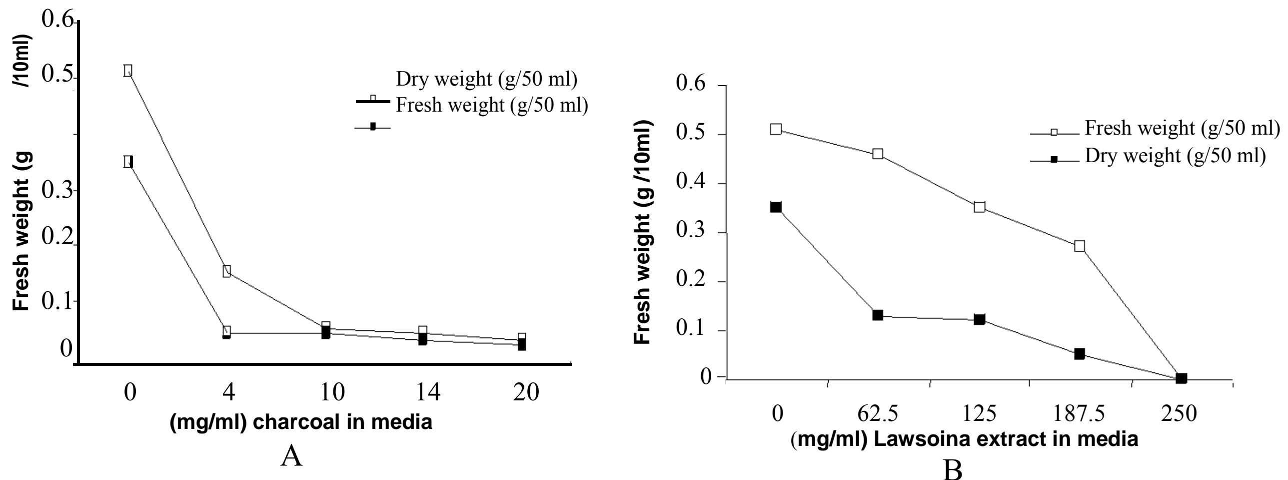

Figure 4. Effect of different concentrations of charcoal (A) ,LSD at 0.05 = 0.32 and L. alba extract (B), LSD at 0.05 = 0.40 on the growth of C. albicans(g/10 ml) grown on Sabouraud dextrose broth after 5 days incubation at 28˚C.

developed (3.5 cm). Amitani et al. (1995) separated 3 inhibitor factors from filterate of *A. fumigatus* ; the first is gliotoxins with low molecular weight while the others helvolic acid and fumigillin are high molecular weight > 2500, 35000, respectively. Also, Watanabe et al. (2008) isolated fungal toxins and antibiotics from *A. fumigatus* e.g. fumigillin, fumigantin and treptoquivaline. In this study, perpendicular streaking plate, a clear lysis area was established on the growth of *C. albicans* . Also, the growth of *A. fumigatus* spread on all surfaces of parallel streaking plate after 5 days incubation at 25°C. The lysis may be due to the high enzymatic system of *A. fumigatus* e.g. glutamate dehydraogenase, malate dehydrognase, glucose phosphate isomerase, phosphorgucomtase, hexokinase, esterase, peptidase, fructose, kinase, purine nucleoside, phosphorylase, phosphatase (Kamei et al., 2002; Tomee et al., 2002).

In conclusion, antibiotics are the most important in the treatment of microbial infection. Antibiotic resistance microbes become major problem of health for human animals. The results of the *in vitro* inhibitory activity of natural products against *C. albicans* are quite appreciable when compared by standard antibiotics used. The susceptibility of the tested organism shows that the total leaf extracts of Meswak stalks or Henna extract could be used as an alternative in treating *Candidiasis* resulting from different strains of *C. albicans*. The active ingredients of the plant would be isolated, chemically identified and purified for commercial uses. It is wisely to mention that the *in vitro* biocontrol effect of *A. fumigatus* is a good first line drug for infection mainly *Candidiasis* caused by *C. albicans.* Both are isolated of *Nosocomial* infections. If the traditional medicine can be developed as an anti-fungal agent, it will provide another powerful weapon against infectious disease. It may be a therapeutic option to use medicine, as alternative medicine in patients undergoing immune deficiency to reduce such infection.

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