Research Journal of Medical Sciences 9 (1): 1-7, 2015
ISSN: 1815-9346

Molecular Characterization and Inhibition by Natural Agents of Multidrug Resistant Candida Strains Causing Vaginal Candidiasis

[1]Talaat I. EL-Sayed, [2]Dina Atef, [1]Mahmoud Amer, [1]Ahmed Mahdy and [3]Gamal Enan
[1]Department of Botany, Faculty of Science, Benha University, Benha, Egypt
[2]Department of Clinical Pathology, Faculty of Medicine,
[3]Department of Botany and Microbiology, Faculty of Science, Zagazig University, Zagazig, Egypt

Abstract: The prevalence percentage of vaginal candidiasis appeared to be 49% within Egyptian pregnant women suffering from vaginal pain and vaginal discharge. The 61.2% of the 49 patients were multigravidae and almost in the age range 20-30 years. Based on phenotypic and biochemical characteristics, the infective 49 isolates causing vaginal candidiasis could be classified and identified to *C. albicans* group (32 isolates), *C. tropicalis* group (12 isolates) and *C. dubliniensis* group (5 isolates). Three strains of them viz. *C. albicans* ZUH_1, *C. trobicalis* ZUH_{13} and *C. dubliniensis* ZUH_9 were resistant to 6 antifungal agents used and the antifungal resistance ability in each of them was genetically encoded and linked to one plasmid in each of them, almost in the range 600-800 bp. Of many natural agents used as antifungals, aqueous extracts of alum and clove inhibited vigorously the antifungal resistant strains: *C. albicans* ZUH_1, *C. trobicalis* ZUH_{13} and *C. dubliniensis* ZUH_9; no colony forming units were detected onto Sabouraud agar after 5 days of incubation from samples treated with either alum or clove extracts.

Key words: Candidiasis, *Candida* sp., antifungal resistance, identification, genetic linkage, aqueous extract of alum and clove

INTRODUCTION

Candidiasis is a fungal disease caused by *Candida* sp. which is predominantly common in immuno compromised individuals and also a known causative agent of vaginal candidiasis in pregnant women (Chengo, 2012). Vaginal candidiasis is common and frequent disease among women during their child bearing age; women with this age group face at least one episode of vaginal candidiasis in their lifetime (Aslam *et al*., 2008). Number of gravida and stage of trimester have been associated with development of vaginal candidiasis. Studies showed that women in their 3rd trimester and multigravidae women had the highest rate of candidiasis occurrence (Aslam *et al*., 2008). Pregnant women are more susceptible to many diseases including candidiasis due to immuno suppression and hormonal imbalance; progesterone and estrogen are the known hormones that are elevated during pregnancy. Consequently, studies on prevalence of vaginal candidiasis among pregnant women are necessary and must be continued.

Several new antifungal agents have become available for the treatment of vaginal candidiasis (Tobin, 1995). The available therapeutic agents for treatment of vaginal candidiasis offers few options and include compounds of polyenes and azoles. However, newly resistant strains of *Candida* sp. were isolated from pregnant women suffering from vaginal candidiasis (Liu *et al*., 2005). This makes a necessity to continue research to understand the genetic linkage of the antifungal resistance ability and to find out natural extracts effective in inhibition of *Candida* sp. Alum (aluminium potassium sulphate) has recommended as category I active ingredient in mouthwashes by FDA (Olmez *et al*., 1998). Also cloves (*Zyzygium aromaticum*) extract was reported to inhibit bacterial and fungal pathogens (Alta'ee *et al*., 2014). Those two natural agents were used with other natural agents in this study as an antimycotic agent.

The present research was undertaken to study the prevalence of candidiasis among pregnant Egyptian women, study the sensitivity of causal pathogens of candidiasis to some antifungal agents with selection and studying the resistant strains and to study the inhibition of the isolated multidrug resistant Candida strains by aqueous extracts of potassium alum and cloves.

MATERIALS AND METHODS

Isolation of *Candida* sp.: A population consisting of 100 pregnant women that were admitted to Zagazig University

Corresponding Author: Gamal Enan, Department of Botany and Microbiology, Faculty of Science, Zagazig University, Zagazig, Egypt

Hospital, Zagazig, Egypt were choosed as all of them were suffering from vaginal pain and vaginal discharge. Samples of vaginal fluids were taken by cotton swabs and were streaked onto Sabouraud agar (Hi-media). After incubation at 37°C for 3-5 days, single colonies appeared were picked up, purified on the same medium and were then streaked on slope cultures of Sabouraud agar.

Identification of Candida isolates: Fourty nine isolates were isolated on Sabouraud agar. They were subjected to identification studies. They were examined under light microscope to show the budding cells with or without pseudohyphae, blastospores and germ tubes (Milne and Mitchell, 1998). Also Gram reaction and growth of isolates on Bismuth sulfite Glucose Glycine Yeast (BIGGY) agar was carried out to differentiate among yeast species (Ingham *et al*., 2012). In addition, biochemical tests and carbohydrate fermentation profiles were studied using Hi-Candida™ API identification kit (KB006 Hi-Candida™ identification kit, India).

Antifungal susceptibility test: This test was carried out by well diffusion assay using six different antifungals including fluconazole, clotrimazole, itraconazole, ket oconazole, amphotericin B and nystatin (Pfaller *et al*., 2010). Results were taken according to National Committee for Clinical Laboratory Standard (NCCLS, 2004).

Induction of mutation in the identified Candida strains and plasmid profile: Three Candida strains: *C. albicans* ZUH_1, *C. tropicalis* ZUH_{13}, *C. dupliniensis* ZUH_9 were showed to be resistant to antifungals used. Elimination of the antifungals resistance ability was studied using elevated temperature. The above three Candida strains were grown at 40°C and colonies appeared were grown at 41 and 42°C. The colonies grown were picked up and assayed again for their sensitivity to antifungals used (Enan *et al*., 1996). Plasmids were extracted from the wild strains and their mutants and agarose gel electrophoresis was carried out as described previously (Sambrook *et al*., 1989). The 25 μL of taking dye were added to 100 μL of plasmid DNA preparation. The sample was loaded on 0.7% agarose gel in Tris-EDTA buffer pH 8.2 and electrophoresis was conducted at 100 Volt for 1-2 h. The gel was stained in EtBr (0.5 g mL^{-1}) for 15 min., destained in 1 mM $MgSO_4$ for 30 min, photographed by gel documentation system.

Preparation of aqueous extracts and inhibition of the experimental Candida strains: Alum material (aluminium potassium sulfate) was provided from Alexandria Company, Egypt. Two concentrations of alum were prepared (3 and 5%) w/v. Clove plants used in this study were obtained from local market at Zagazig City, Egypt, 2013, immersed in distilled water and left for 24 h. This content was mixed by the blender and filtered to remove the large un-homogenized particles to get clear aqueous extract. Two concentrations (3 and 5%) were prepared and kept in refrigerator (Ismaiel *et al*., 2014). The Maximum Inhibitory Concentrations (MIC) of aqueous extract of alum and clove were determined by critical dilution assays using the experimental *Candida* sp. as an indicator organism (Ismaiel *et al*., 2014). The lowest concentrations of both aqueous extracts that inhibited visible growth were the MIC.

Qualitative inhibition of both alum and clove aqueous extracts was studied by the agar well diffusion assays (Ismaiel *et al*., 2014; Enan *et al*., 2014a, b). Quantitative inhibition of *Candida* sp. *in vitro* by aqueous extracts of both alum and clove was studied (Ismaiel *et al*., 2014; Enan *et al*., 2014a, b). A series of test tubes; each containing 10 mL Sabouraud broth (Hi-media) were prepared, inoculated with almost $4\text{-}4.5\times10^3$ CFU mL^{-1} of the tested *Candida* sp. (*C. albicans* ZUH_1, *C. trobicalis* ZUH_{13}, *C. dubliniensis* ZUH_9). The inoculated and treated tubes were incubated at 37°C for 3-5 days and then colony forming units were determined.

RESULTS

One hundred pregnant women suffering from vaginal pain and vaginal discharge in general, admitted to Zagazig University Hospitals, Zagazig, Egypt were subjected to microbiological analysis based on requests of physicians to find out the existence of vaginal candidiasis which was detected in only 49 patients of them as their cultures of their vaginal fluids showed fungal growth by prevalence percentage of vaginal candidiasis around 49% within patients tested. Other patients appeared to be infected by other microbial or protozoan infections. The 49 cases were diagnosed by physicians as vaginal candidiasis based on culture results and other symptoms such as itching, irritation, painful micturation, dyspareunia and vaginal soreness. Out of the 49 vaginal candidiasis cases, 5; 29; 15 cases were in the age range (year) 16-20; 21-30; 31-40, respectively. Also 19 cases; 30 cases of the 49 cases tested were primigravidae; multigravidae by occurrence percentage of 38.8 and 61.2%, respectively.

It was necessary to identify the 49 fungal isolates causing vaginal candidiasis. All the 49 isolates were

examined under microscope. All of them showed budding cells and pseudohyphae but only 37 isolates formed germ tubes. A key for differentiation of the experimental Candida isolates was designed and showed in Fig. 1. The experimental isolates could be differentiated into three groups. Group 1 include 32 isolates which formed brownish-black colonies on BIGGY agar media and formed germ tubes and could be follow *C. albicans*. Group 2 include 5 isolates which grew on BIGGY agar media and formed light brown colonies and those could be classified as belonging to *C. dubliniensis*. Group 3 include 12 isolates, all of them did not form germ tubes and could be classified as *C. tropicalis*.

The biochemical identification of the 49 Candida isolates was carried out using API identification kits (KB006 Hi-Candida™ identification kit, India). Results are given in Fig. 2. API identification confirmed that the groups 1, 2, 3 of Candida isolates which included 32, 5, 12 isolates could be classified and identified as belonging to *C. albicans*, *C. dubliniensis*, *C. tropicalis*, respectively. The prevalence percentages of *C. albicans*, *C. tropicalis*, *C. dubliniensis* were 65.3, 24.5 and 10.2%, respectively within 49 isolates identified herein (Table 1).

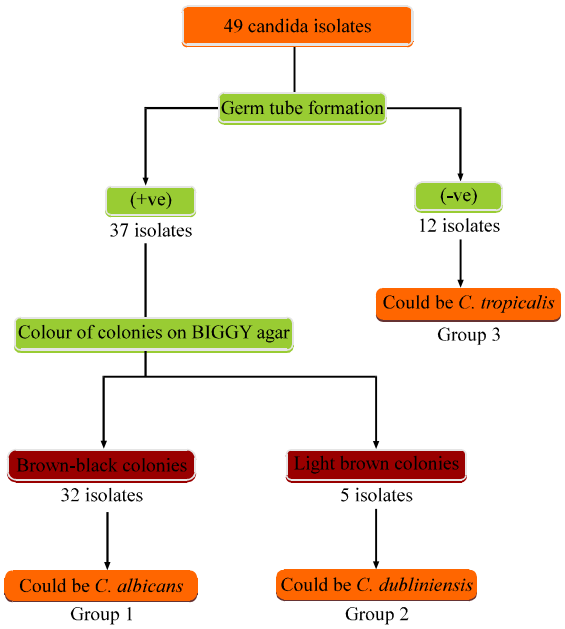

Fig. 1: Key for preliminary differentiation of the 49 Candida isolates obtained

The antifungal resistance profiles of the 49 Candida strains were studied. Only three strains: *C. albicans* ZUH_1, *C. trobicalis* ZUH_{13} and *C. dubliniensis* ZUH_9 were multidrug resistant as they were resistant to the 6 antifungals used. To understand the genetic linkage of the antifungal resistance ability within their strains, mutation of them were obtained by their growing and subculturing at 42°C. The mutants obtained rendered sensitive to almost 50% of antifungal drugs used. Plasmid profiles of wild strains and their mutants were obtained in Fig. 3. It was obvious that the plasmid profiles of *C. albicans* ZUH_1, *C. tropicalis* ZUH_{13} and *C. dubliniensis* ZUH_9 wild strains are different from that showed for their mutants. One plasmid in each Candida mutant strain tested of a molecular size almost 600-800 bp was deficient. This clearly indicated that the antifungal resistance ability in the wild strains of Candida is linked to gene(s) located in this deficient plasmid but not due to other factors such as modification of cell wall or specific site(s) receptors.

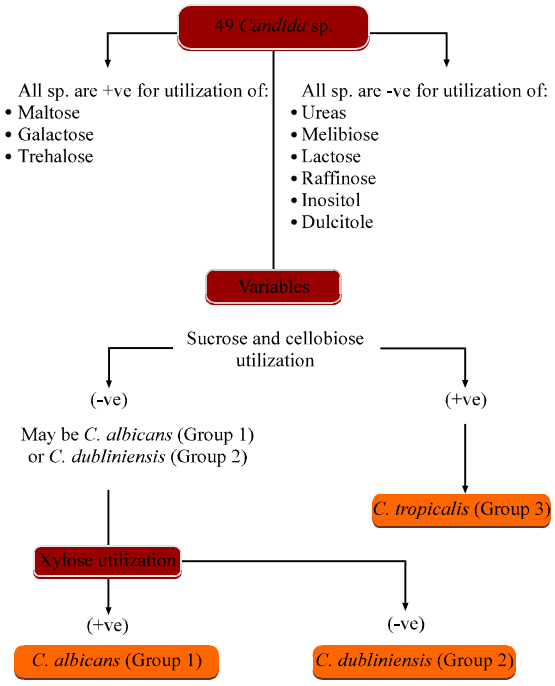

Fig. 2: Key for differentiation and biochemical characterization of the 49 Candida isolates as showed by API-Candida kits

Table 1: Prevalence of *Candida* species in both pregnant women (subject study) and total *Candida* species obtained

Candida species	No. of *Candida* species obtained (%)	Percentage within total *Candida* species (%)	Percentage of *Candida* species with total samples (%)
Candida albicans	32	65.3	32
Candida tropicalis	12	24.5	12
Candida dubliniensis	5	10.2	5
Total number	49	100.0	49

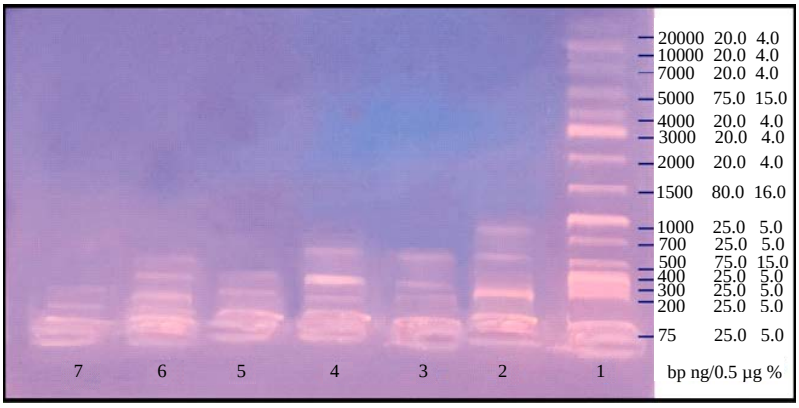

Fig. 3: Agarose gel electrophoresis of chromosomal DNA isolated from both wild strains and their mutants obtained after curing by elevated temperatures. Lane 1: standard DNA; Lane 2 and 3: *Candida albicans* ZUH_1 before and after curing, respectively; Lane 4 and 5: *Candida tropicalis* ZUH_{13} before and after curing; Lane 6 and 7: *Candida dubliniensis* ZUH_9 before and after curing

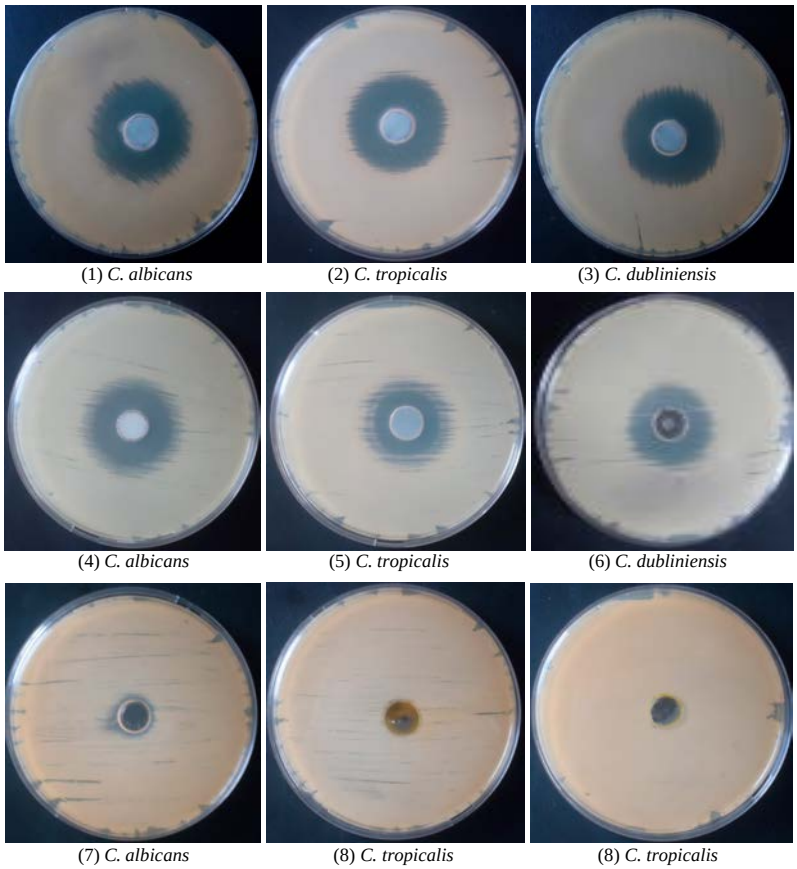

Fig. 4: Inhibition of *Candida* species by agar well diffusion assay by different essential oils and alum. The 1, 2, 3: clove oil; 4, 5, 6: alum; 7, 8, 9: one example of an essential oils (olive oil) without effect

It was necessary to inhibit such antifungal resistant Candida strains. Natural agents such as potassium alum and clove were used. The MIC values for aqueous alum solution; clove extract were 1.5 and 2%, respectively. Consequently, 3 and 5% w/v of both of them were used. Figure 4 showed the inhibition of the three multidrug

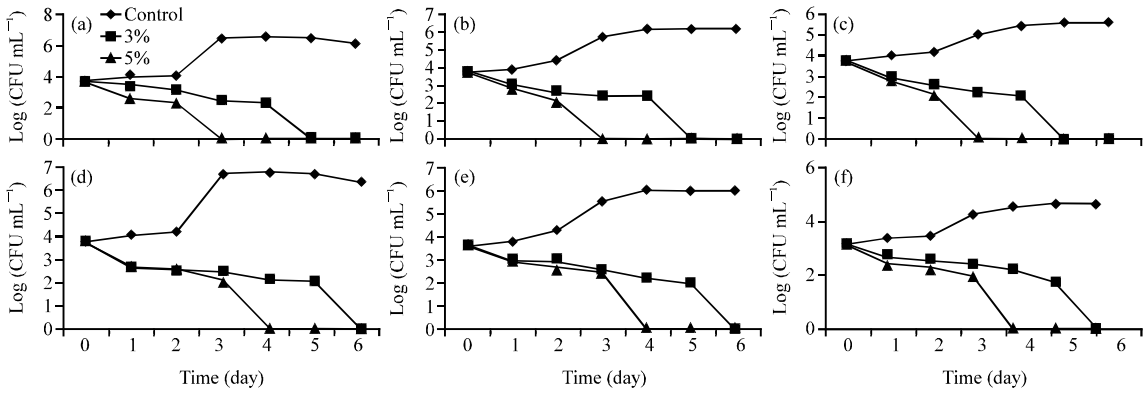

Fig. 5: Inhibition of Candida strains by natural agents: a-c) growth (CFU/mL) of *C. albicans* ZUH_1; *C. tropicalis* ZUH_{13} and *C. dubliniensis* ZUH_9 treated with clove extract; d-f) growth (CFU/mL) of *C. albicans* ZUH1; *C. tropicalis* ZUH_{13}; *C. dublin-iensis* ZUH_9 treated with aqueous alum extract

resistant Candida strains used by 5% w/v aqueous extract of either alum or clove. Diameter of inhibition zone ranged from 18-30 mm.

The quantitative inhibition of multidrug resistant Candida strains by aqueous extract (3 and 5% w/v) of either alum or clove is given in Fig. 5. Viable cell populations in control experiments of the three multidrug resistant Candida strains tested increased from almost 4.6×10^3 CFU mL^{-1} to almost 10^6 CFU mL^{-1} within 4 days. However, in treated and inoculated samples growth of multidrug resistant Candida strains tested (CFU/mL) declined rapidly almost two log cycles within 4 days and no growth of the three multidrug resistant Candida strains tested was detected in the 5th day of incubation of samples treated with 3% w/v of all treatments. No growth of all three multidrug resistant Candida strains tested after almost 4 days from samples treated with 5% w/v aqueous extract of either alum or clove.

DISCUSSION

The high prevalence of vaginal candidiasis among pregnant women makes an interest to study the behaviour of its causal pathogens and their susceptibility to antifungals. This is necessary to design an effective treatment programmes. The prevalence percentage of candidiasis in pregnant women suffering from vaginal pain and/or vaginal discharge appeared on this study was 49%. Previous published results by Kamara *et al.* (2000) and Njunda *et al.* (2012) showed that the percentage of incidences of candidiasis within pregnant women were of about 30.7 and 60%, respectively. The prevalence of vaginal candidiasis among pregnant women is a variable property due to many reasons such as personal hygiene, diagnostic facilities, dietary habits, levels of reproductive hormones, prolonged use of antibiotics which kill the good and beneficial bacteria which are not fixed criteria in all women.

It was showed previously that the age of the patient possessed certain relation with spreading of vaginal candidiasis (Oyewole *et al.*, 2013). Higher percentage of vaginal candidiasis prevalence was showed in this study in the age group 21-30 years and this is similar to previous published results in this respect (Willacy and Jackson, 2011). This is because the hormonal mileau of the vagina during pregnancy in this age can affect Candida colonization and serve as risk factors for this phenomenon and thus women of childbearing age groups are more vulnerable to vaginal candidiasis. The higher progesterone secretion in the age group 20-30 has suppressive effects on the anti-Candida activity of neutrophiles and formation of yeast blastospores are increased by this hormone (Fidel Jr., 2005; Isibor *et al.*, 2011).

Among 49 pregnant women which were found to be positive with vaginal candidiasis, 30 cases of them (61.2%) were found to be multigravidae (women that have experience of more than one pregnancy) while 19 cases (38.8%) were found to be primigravidae (women with first pregnancy). This high prevalence among the multigravidae may be due to long sexual history and use of contraceptives and antibiotics were considered as important risk factors associated with vaginal candidiasis (Neerja *et al.*, 2006). It was shown herein that the pregnant women in the third trimester have the highest occurrence of Candida infection (59.2%); this is in conform to previous published results which showed that the hormonal factors in the third trimester could enhance vaginal candidiasis (Aslam *et al.*, 2008).

The 49 Candida isolates obtained in this study were preliminary, characterized by their growth on Sabouraud

agar, Gram staining and appearance of pseudohyphe and blastospres under light microscope. Previous results characterized Candida isolates by similar taxonomic criteria. Species differentiation was carried out by growth of isolates on BIGGY agar (Hi-media), germ tube formation and API identification kits (KB006 Hi-Candida™ identification kit, India). Out of the 49 Candida isolates, 32 isolates (65.3%) were identified as *C. albicans*. This was followed by non-albicans species which were *C. tropicalis*, 12 isolates (24.5%) and *C. dubliniensis*, 5 isolates (10.2%). *C. albicans* was the predominant species because it was reported to be the more adhesive species than other non-albicans species and hence can attach epithelial and smooth tissue by a more capability than other non albicans species (Milne and Mitchell, 1998; Grigoriou *et al*., 2006).

Three Candida strains *C. albicans* ZUH_1, *C. trobicalis* ZUH_{13} and *C. dubliniensis* ZUH were resistant to the six antifungal drugs used and the antifungal resistance ability was reported previously for similar *Candida* species. Reasons of resistance was due modification of specific site(s) receptors or due genetic reasons (Akins, 2005). To understand the genetic linkage of the antifungal resistance ability, mutants of the antifungal resistant strains (ZUH_1, ZUH_{13}, ZUH_9) were obtained by growth of those strains at elevated temperature in a way similar to previous published work in this respect. Plasmid profiles of the wild strains (antifungal resistant strains) and their mutants were carried out. About three plasmids were showed in wild strains with varying base pairs and one plasmid of almost 800, 600 and 500 bp was deficient or diffused in plasmid profiles of mutants of ZUH_1, ZUH_{13}, ZUH_9 strains, respectively indicating that those deficient plasmids were the genetic determinants of the antifungal resistance ability. This is in conform with previous results in this respect which confirmed the existence of plasmids in Candida isolates and such plasmids were the responsible for genetic linkage of the antifungal resistance ability (Vandeputte *et al*., 2012).

Due to selection of antifungal resistant strains of *Candida* sp. causing vaginal candidiasis and based on the increasing side effects of chemical antifungals, novel antifungal therapies with fewer side effects on humans like different essential oils or natural chemical agents like potassium alum were used in this study for inhibition of *in vitro* growth of the antifungal resistant *Candida* species tested. From different natural essential oils used, aqueous extract (3 and 5% w/v) of cloves showed significant inhibition of the antifungal resistant Candida strains tested (ZUH_1, ZUH_{13}, ZUH_9). Also, aqueous extract of alum inhibited these strains significantly.

Growth of Candida strains tested recorded zero within 6 days of incubation in all treatments. Potassium alum was known to decrease the free vaginal fluids which lowers vaginal water activity and this prevents proliferation of vaginal candidiasis by increasing bound water around potassium alum and limiting metabolic activity of *Candida* species in such environment (Alta'ee *et al*., 2014). The antifungal activity of clove was reported to be due to phenolic compounds such as hydroxibenzoic acids, gallic acids and eugenole which is the main bioactive compound. It was reported that eugenole and other phenolic compounds of clove can denature proteins and react with cell membrane phospholipids changing their permeability and inhibiting pathogenic bacterial and fungal organisms (Flayeh, 2010; Mohammad, 2013; Enan *et al*., 2014a, b). Further resaerch will be necessary to study the effect of both clove oil and aqueous extracts on candidiasis *in vivo*.

CONCLUSION

Three multidrug resistant Candida strains were isolated and characterized from women infected with vaginal candidiasis. The genetic linkage was plasmid encoded in each of them. They were inhibited vigorously by 5% aqueous extract of both potassium alum and clove.

REFERENCES

Akins, R.A., 2005. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. Med. Mycol., 43: 285-318.

Alta'ee, A., I.A. Bnyan and N.H. Kadhum, 2014. Antibacterial activity of aluminum potassium sulfate and Syzygium aromaticum extract against pathogenic microorganisms. J. Natioral Sci. Res., 4: 11-14.

Aslam, M., R. Hafeez, S. Ijaz and M. Tahir, 2008. Vulvovaginal Candidiasis in pregnancy. Biomedica, 24: 54-56.

Chengo, N.M., 2012. Isolation, identification and susceptibility profile of Candida species to antifungal agents in pregnant women in Thika District Hospital, Kenya. Ph.D. Thesis, Kenyatta University in Nairobi, Kenya.

Enan, G., A.R. Al-Mohammadi, G. El-Didamony, M.E.F.A. Haliem and A. Zakaria, 2014a. Antimicrobial activity of *Enterococcus faecium* NM2 isolated from urine: Purification, characterization and bactericidal action of enterocin NM2. Asian J. Applied Sci., 7: 621-634.

Enan, G., M.E.F.A. Haliem and E. Tartour, 2014b. Evaluation of the antimicrobial activity, starter capacity and technological properties of some probiotic bacteria isolated from Egyptian pickles. Life Sci. J., 11: 967-985.

Enan, G., M. Uytendaele, A.A. El-Essawy and J. Debevere, 1996. *Lactobacillus plantanum* UG1: Characterization, production and bactericidal actin of plantaricin UG1. Int. J. Food Microbiol., 30: 189-215.

Fidel, Jr. P.L., 2005. Immunity in vaginal candidiasis. Curr. Opin. Infect. Dis., 18: 107-111.

Flayeh, R.A.M., 2010. Antifungal activity of some plant extract and alum on yeast isolated from mouth lesions. Euphrates J. Agric. Sci., 2: 1-4.

Grigoriou, O., S. Baka, E. Makrakis, D. Hassiakos, G. Kapparos and E. Kouskouni, 2006. Prevalence of clinical Vaginal candidiasis in a university hospital and possible risk factors. Eur. J. Obstet. Gynecol. Reprod. Bio., 126: 121-125.

Ingham, C.J., S. Boonstra, S. Levels, M. de Lange, J.F. Meis and P.M. Schneeberger, 2012. Rapid susceptibility testing and microcolony analysis of *Candida* sp. cultured and imaged on porous aluminum oxide. PLoS One, Vol. 1. 10.1371/journal. pone.0033818.

Isibor, J.O., S.O. Samuel, C.I. Nwaham, I.N. Amanre and O. Igbinovia *et al*., 2011. Prevalence of Bacterial and *Candida albicans* Infection Amongst Women Attending Irrua Specialist Teaching Hospital, Irrua, Nigeria. Afr. J. Microbiol. Res., 5: 3126-3130.

Ismaiel, A.A.R., A.E.S. Ali and G. Enan, 2014. Incidence of *Listeria* in Egyptian meat and dairy samples. Food Sci. Biotechnol., 23: 179-185.

Kamara, P., T. Hylton-Kong, A. Brathwaite, G.R. del Rosario and S. Kristensen *et al*., 2000. Vaginal infections in pregnant women in Jamaica: Prevalence and risk factors. Int. J. STD AIDS, 11: 516-520.

Liu, T.T., R.E. Lee, K.S. Barker, R.E. Lee, L. Wei, R. Homayouni and P.D. Rogers, 2005. Genome-wide expression profiling of the response to azole, polyene, echinocandin and pyrimidine antifungal agents in *Candida albicans*. Antimicrob. Agents Chemother., 49: 2226-2236.

Milne, L.J.R. and T.G. Mitchell, 1998. Mackie and McCartney Practical Medical Microbiology. 14th Edn., Churchill Livingstone Publication, New York, USA., pp: 537-541.

Mohammad, H.H., 2013. *In vitro* antibacterial activity of propolis, alum, miswak, green and black tea, cloves extracts against porphyromonas gingivalis isolated from periodontitis patients in Hilla City, Iraq. Am. J. Phytomed. Clin. Therap., 1: 140-148.

NCCLS., 2004. Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts: Approved Guideline. National Committee for Clinical Laboratory Standards (NCCLS), Wayne, PA., USA., ISBN-13: 9781562385323, Pages: 23.

Neerja, J., A. Aruna and G. Paramjeet, 2006. Significance of Candida culture in women with vulvo-vaginal symptoms. J. Obstet. Gynecol. India, 56: 139-141.

Njunda, A.L., D.S. Nsagha, J.C.N. Assob, H.L. Kamga and P. Teyim, 2012. *In vitro* antifungal susceptibility patterns of Candida albicans from HIV and AIDS patients attending the Nylon Health District Hospital in Douala, Cameroon. J. Public Africa, Vol. 3. 10.4081/jphia.2012.e2.

Olmez, A., H. Can, H. Ayhan and H. Okur, 1998. Effect of an alum-containing mouthrinse in children for plaque and salivary levels of selected oral microflora. J. Clin. Pediat. Dent., 22: 335-341.

Oyewole, O.A., I.N. Okoliegbe, S. Alkhalil and P. Isah, 2013. Prevalence of vaginal candidiasis among pregnant women attending Fedral University of Technology, Minna, Nigria, Bossa Clinic. Res. J. Pharmaceut. Biol. Chem. Sci., 4: 113-120.

Pfaller, M.A., D.J. Diekema, D.L. Gibbs, V.A. Newell and D. Ellis *et al*., 2010. Results from the artemis disk global antifungal surveillance study, 1997 to 2007: A 10.5-Year Analysis of susceptibilities of Candida Species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. J. Clin. Microbiol., 48: 1366-1377.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Habour Laboratory Press, New York, USA., ISBN-13: 978-0879693091, Pages: 1659.

Tobin, M.J., 1995. Vulvovaginal candidiasis: Topical vs. Oral therapy. Am. Family Phys., 51: 1715-1720.

Vandeputte, P., S. Ferrari and A.T. Coste, 2012. Antifungal resistance and new strategies to control fungal infections. Int. J. Microbiol. 10.1155/2012/713687.

Willacy, H. And C. Jackson, 2011. Vaginal and vulval candidiasis. http://www.patient.co.uk/doctor/vaginal-and-vulval-candidiasis.