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Antifungal activity of Essential Oils of *Moringa Oleifera* and *Punica Granatum* Seeds against Candidal Vaginitis in Mice Model

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Abstract: *Moringa oleifera* (*M. oleifera)* and *Punica granatum L (P. granatum L)* are two of important plants found in different parts of Al-Baha region, Saudi Arabia, the seeds essential oils of the two plants have broad spectrum antimicrobial activity against a lot of pathogens. *Candida albicans (C.albicans*) is a common cause of mucosal infectious disease in women in the form of candida vaginitis. Marked resistance by the microorganisms to conventional antifungal drugs has been documented, so the study designed to evaluate the effect of *M. oleifera and P. granatum* seeds essential oils on infections caused by *C.albicans* (candidal vaginitis) using mice model. The essential oils were extracted with n-hexane and soxhlet extractor and tested its activities. Agar diffusion method, minimum inhibitory concentrations (MIC) and Minimal fungicidal concentration (MFC) were performed in vitro and histological diagnosis using H & E and PAS stains. The M. *oleifera* E.O showed high ability to inhibited candidal growth compared to the *P. granatum L* E.O with 18.3 ±1.5mm inhibition zone and MIC & MFC were recorded 0.0312 mg/ml and 0.0625 mg/ml respectively. The *P. granatum L* E.O gave 12.7 ±1.5 mm inhibition zone and MIC & MFC were recorded 0.0625mg/ml and 0.125 mg/ ml respectively. The lost of squamous epithelium cells without hyphae appeared in histological examination of *M. oleifera* treated group. Clear ability to recover vaginal lumen from *C. albicans* infection showed in histological sections of *P. granatum* E.O compared to infected non treated group. The results of the present study indicated that seeds E. Os of *M oleifera* and *P. granatum* exhibited strong antifungal activity against candidal vaginitis in mice.
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Key wards: Antifungal activity, Essential Oils, *Moringa Oleifera, Punica Granatum L,* Mice**.**

1. Introduction

Therapeutic agents of plants origin have been subjected to a huge number of pharmacological investigation **(Eilert et al. 1981).** The family of Moringaceae contains more than 12 species and only one Moringa genus It commonly occur in the Red Sea area, Saudi Arabia, and the Indian subcontinent**.** The most economically valuable species are *M. oleifera* **(Bhoomika et al. 2007).**

Natural products essential oils are produced by aromatic plants extracted where the trees were cultivated, by seeds boiling with water and oils collecting from the water surface**) Lalas and Tsaknis 2002).** Oils acting against *Candida* by interact with the mitochondrial membrane leading to cidal effects **(Rajkowska et al. 2016) or** inhibiting ergosterol synthesis altering cell wall morphology **(Adil et al., 2014 Samber et al., 2015, Cardoso et al., 2016).**

In humans and animals, *Candida* species are one of the most prominent agents of fungal infections and *Candida albicans* is the most prevalent agent of candidal infections among Candida species. Cutaneous and mucosal candidiasis is more common than other clinical Candida infections. The most common lesion types are vaginal candidiasis, thrush, and onychomycosis **) Fichtenbaum et al., 2000, Dignani et al., 2009, Tavernier et al., 2015 and Zhang et al 2015).**

Many studies have documented that Candida species are a wide range resistant to many of antifungals, therefore replacement treatments are necessary for eliminating drug resistant infections and the undesirable side effects of synthetic and chemical drugs **(Dignani et al., 2009, Tavernier et al., 2015 and Zhang et al 2015).**

Pomegranate (*Punica granatum L.*) is a broad spectrum antimicrobial effective herbal extract with to the ability to modulate the immune response **(Harris 2002, Budzyńska et al., 2011).** Due to the increased resistance to routine antifungal agents increases search about new remedy for treatments of *Candida* infections. and the herbal medicines using appeared as new solution. The purpose of the present work was to evaluate the antifungal effects of seeds E. Os of *M oleifera* and *P. granatum* were tested against *Candida albicans*.

2. Materials and Methods

Extraction of the antifungal agent from plant

Plant samples collection

Plant samples of *M. oleifera, and P.granatum L* were obtained from Al-Baha Region, Saudi Arabia and identified at Department of Biology, Faculty of Science, Al-Baha University.

Essential oil extraction

Approximately 60g of finely ground powder of *M. oleifera*, *Punicagranatum L* seeds were drayed and put in a thimble, then extracted using Soxhlet extractor for 8 h using 250 ml of hexane as solvents. Solvent was removed and the obtained plant oil was dissolved in 10% dimethyl sulfoxide (DMSO) W/W. The extracts were saved in amber bottle and kept in the refrigerator at 4°C **(Ashraf et al., 2011)**.

In vitro extracted essential oil antifungal activity

Very small inoculums of candidal colony was picked up with a sterile inoculating loop and was suspended in test conical flasks containing 30 ml of Sabouraud Dextrose broth medium and all flasks were incubated at 37^{o}C and 120 rpm for 48 hr. in incubator shaker.

Agar-well diffusion method

The antifungal activity of active material in E.O of *M.aoleifera, and P. granatum L* against *Candia albicans* was evaluated by using agar well diffusion method. The surface in agar plate is inoculated by spreading a 100 µl of standardized inoculum (0.5x106 CFU/ml) of the *Candia albicans* inoculum over the entire agar surface. Then, 7 mm diameter hole with is punched aseptically with a sterile cork borer or a tip, and a volume (100 µL) of the extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The Essential oils diffuses in the agar medium and inhibits the growth of the microbial strain tested after 48 hr. of incubation at 37°C. The zone of inhibition was measured and expressed in millimeters (mm). Antifungal activity was recorded if the zone of inhibition was greater than 7 mm **(Balouiri *et al.*, 2016).**

Determination of minimum inhibitory concentrations (MIC)

The minimal inhibitory concentrations of the extracts against *C. albicans* were determined by the modified broth micro dilution test according to CLSI method in 96 microtiter plates **(Rex et al., 1997)**. Red phenol was used as a color indicator; it appeared red in a basic or neutral medium and yellow in an acidic medium. The yeasts growth was indicated by the color.

Minimal fungicidal concentration (MFC)

The minimum fungicidal concentrations (MFCs) were determined in wells which showing no visible growth in microtiter plates coming before MIC wells in order.

The antifungal activity of Essential oil extracted *in vivo*

Fifty female BALB/C Mice (6 weeks old) weighing 20–25 gm were obtained from the excremental animal store. Mice were 5 kept in stainless steel cages under controlled temperature (22±2°C), humidity was at 55±10%, and 12/12 hrs. cycle of light and dark with an access to food and drinking water ad libitum. The experimental procedures were carried out in accordance with the international guidelines for the care and the use of the animals in laboratory.

Induction of vaginal candidiasis in mice model

All animal were randomly arranged into5 groups:

Group (1) Control non infected treated with physiological solution.

Group (2) infected non treated.

Group (3) *M. oleifera*: treated with *Moringaoleifera* essential oils.

Group (4) *P.granatum L*: treated with *P. granatum L* essential oils.

All mice were infected with *C. albicans* as previously described and modified by **(Pietrella et al., 2011)** excepted mice in Control Positive group. Mice were maintained under pseudoestrus condition by subcutaneous injection of 0.2 mg of estradiol valeratein 100 µl of sesame oil 6 days prior to infection and weekly until the completion of the study. Mice were infected twice at a 24 h interval with 10 µl of 109 cell/ml of *C. albicans*. Cell suspensions were administered from a mechanical pipette into the vaginal lumen, close to the cervix. To favour vaginal contact and adsorption of fungal cells, mice were held head down for 1 min following inoculation. Mice were then allowed to recover for 24-48 h, during which the *Candida* infection was established. The intravaginal treatment with physiological solution, *M. oleifera* E.O *and P.granatum L* E.O (500 µg/mouse) was repeated every two days until day +21.

Microbiological evaluation of progression of the vaginal candidiasis infection

At day 3 the whole vaginal cavity mucosa was swabbed using a cotton pad. The end of the cotton pad was then cut off and placed in a tube containing 5 ml sterile saline. After mixing on a Vortex mixer to release *Candida* cells from the swab into the saline, serial 100-fold dilutions of the cell suspension were incubated on *Candida* Sabouraud dextrose agar plate at 37°C for 24 hr. The CFU (colony forming units) of *Candida* colonies was counted **(Takakura *et al.*, 2003).**

Light microscope study (LM).

Mice were sacrificed by cervical dislocation. Vagina were excised and immediately fixed in 10%

neutral buffered formalin. Fixed samples were embedded in paraffin wax then sectioned (5.m). Lung specimen were stained with hematoxylin-eosin (H & E) and Periodic acid–Schiff (PAS) stains, examined randomly with no knowledge of the group. Histopathological lesions were evaluated based on the degree of tissue damage and absence or presence of candiadiasis.

Statistical analysis

The values of agar well diffusion assay was expressed as mean±SD. The data obtained from the Microbiological evaluation of *C. albicans* cells number in the vagina before and after treatment were analyzed by Student's t test, SPSS version 17.

3. Results

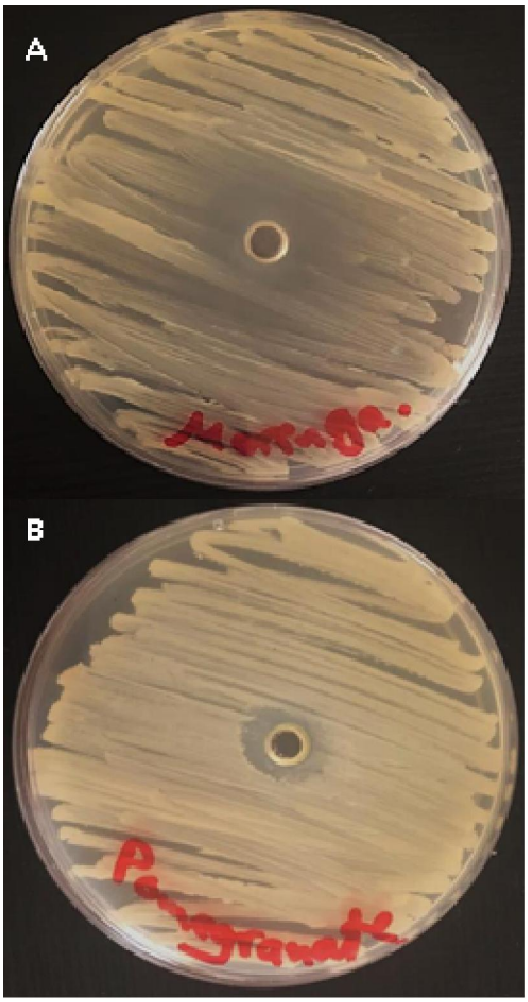

Figure 1: The antifungal activity of essential oils of (A) *M. oleifera* (B) *P.granatum L* against *Candida albicans* using agar well diffusion assay.

The activities of essential oils against *Candida albicans* were obtained by inhibition zones development and calculation. The M. *oleifera* E.O showed high ability to inhibited candidal growth compared to the P. *granatum* E.O with 18.3 ±1.5mm inhibition zone **(Table 1, Fig.1)** and MIC & MFC were recorded 0.0312 mg/ml and 0.0625 mg/ml respectively. The *P. granatum* E.O gave 12.7 ±1.5 mm inhibition zone and MIC & MFC were recorded 0.0625mg/ml and 0.125 mg/ ml respectively **(Table 2., Fig.2,3)**.

Table 1. The antifungal activity of natural extracts against *Candida albicans* using agar well diffusion assay

Essential oil	*Zone of inhibition (mm)±SD
M. oleifera	18.3±1.5
P. granatum L	12.7±1.5

Table 2. Determination of minimum inhibitory concentrations (MIC) and Minimal fungicidal concentration (MFC)

Essential oil	MIC (mg/ml)	MFC (mg/ml)
M. oleifera	0.0312	0.0625
P. granatum L	0.0625	0.125

Figure 2: MIC and MFC for *M.oleifera* E.O on *C. albicans.*

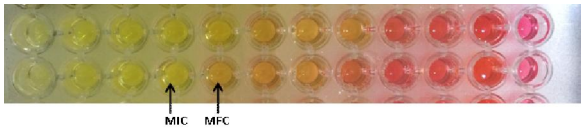

Figure 3: MIC and MFC for *Punicagranatum L* E.O on *C. albicans*

Microbiological **as**sessment of *C. albicans* cells number in vagina before and after treatment with essential oils showed that the relative abundance of the *C.albicans* was found to be significantly influenced by vaginal treatment with the two used essential oils ($P<0.001$). The loading of *C.albicans* was decreased by using *Moringa oleifera and Punica granatum* from 6.01 to 1.9 CFU/ml and from 6.20 to 2.21 CFU/ml respectively (Table 3).

Table 3: Microbiological evaluation of *C. albicans* cells number in vagina before and after treatment with Essential Oils.

Treated animal groups	Log_{10} CFU/ml of *C. albicans* cells		T test (treated groups VS Control negative group)
	Before treatment	After treatment	
Control positive	0	0	
Control negative	6.23	6.64	
Moringaoleifera	6.01	1.9	0.000*
Punicagranatum L	6.20	2.12	0.000*

*: Highly significant results compared to Control negative group P<0.001

Histological findings

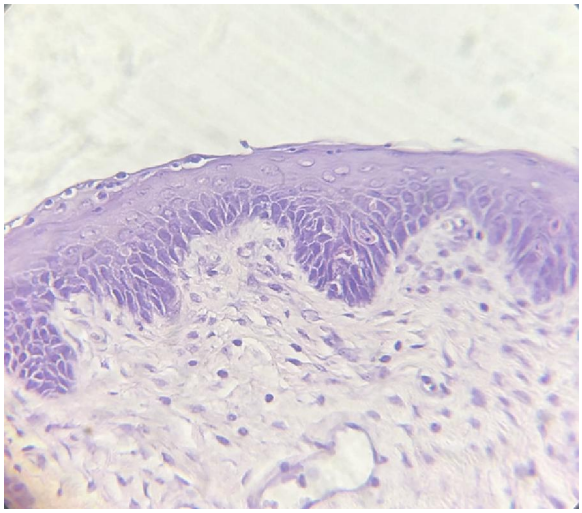

Figure 4**:** Sagittal section of vagina of control positive group stained with PAS stain at magnification of 400 X.: no *C. albicans* cells in normal appearance of epithelial layer**.**

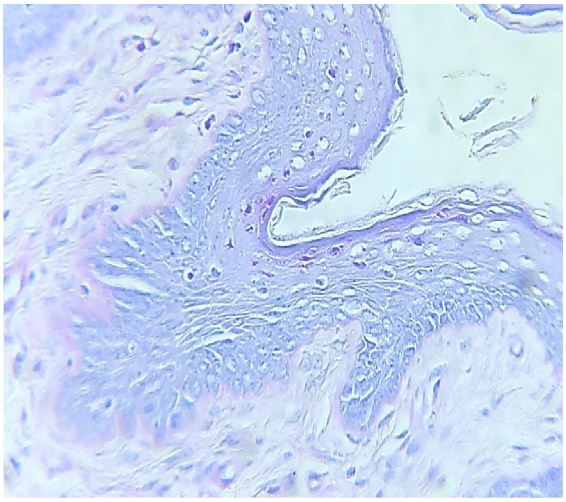

Figure 5**:** Sagittal section of vagina of *Moringa oleifera* group stained with PAS stain at magnification of 400 X: semi-normal appearance of vaginal epithelium and no presence of *C.albicans* yeast or hyphae.

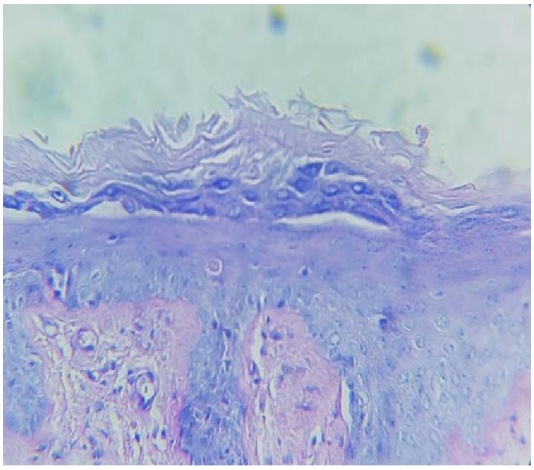

Figure 6**:** Sagittal section of vagina of *Punica granatum L* group stained with PAS stain at magnification of 400 X: huge loss of stratified squamous epithelium in vagina lumen no candida albicans yeast or hyphae appeared in sections

Vagina of control positive group showed absence of *C. albicans*, epithelial cells layer in normal appearance Figure 4. Also, histological examination with routine HE staning revealed normal appearance of stratified squamous epithelium and normal lamina propria. Meanwhile**,** *Moringa oleifera* group is showing absence of *Candida albicans* yeast or hyphae Figure 5. with resolution of pyogranulomatous inflammation and normal histological appearance of both covering mucosa and lamina propria of vagina and *P. granatum* E.O is showing absence of *Candida albicans* yeast or hyphae with PAS stain Figure 6. meanwhile, histological examination with HE staining revealed macrophagic infiltration in the lamina propria with loss of superficial epithelial lining of vagina.

4. Discussion

It is known that *Candida* species are the most common fungal causes of deep-seated and disseminated infections in immunocompromised human hosts, and are associated with high morbidity and mortality in this population **(Rossoni et al., 2018).** Therefore, many researchers seeking new alternatives therapies due to the high number of infections whose agents are resistant to conventional treatments. One of

the economical alternatives and safe is the use of the product of plant origin like Essential oils having antifungal activity **(Carvalhinho et al., 2012 and Szweda et al., 2015 and Gomes et al., 2016).**

Due to marked resistance to conventional antifungal drugs, the objective of the present study was to evaluate the antifungal activity of *M. oleifera and P.granatum L* seeds essential oils against *C. albicans*. This study successfully extracted E.O. using a Soxhlet extractor with hexan as a solvent. This extraction of E.O. showed a high effect *in vitro* and *in vivo*. M. *oleifera* E.O has high ability to inhibited candidal growth compared to the P. *granatum* E.O with 18.3 ±1.5mm and12.7 ±1.5 mm inhibition zone These findings were in accordance of **(Suarez *et al* 2003)**. Again, these activities are much higher as MIC & MFC were recorded 0.0312 mg/ml and 0.0625 mg/ml and0.0625mg/ml and 0.125 mg/ ml respectively in M. *oleifera* and P. *granatum* **(Dahham et al., 2010).** In addition, the extract causes serious damage of the cellular structure the *C. albicans* yeasts, preventing fungal growth and development and invasion of tissue **(Anibal et al., 2013).**

Histological examination of the present study documented the anti-candidiasis effects of *M. oleifera and P. granatum L* seeds essential oils that improved recovery from vaginitis infection in an experimental mice model. where either losses of squamous epithelium cells or *Candida albicans* yeast or hyphae do not appeared in treated groups with either *M. oleifera* or *P. granatum* E. Os. compared to infected non treated groups proving that the antifungal activity corresponded with previous reports of **(Rajkowska et al. 2016)** and **(Nazzaro et al. 2017).** In spite of still presence of macrophagic infiltration in the lamina propria of vagina of *P. granatum* E.Os. group indicate incomplete resolution of chronic inflammatory infiltrate, compared to *M. oleifera* or group that alleviated inflammatory exudates in the lamina propria and normal appearance of superficial mucosa**.** The results of the present study indicated that seeds E.Os of *M oleifera* and *P. granatum* exhibited strong antifungal activity against candidal vaginitis in mice.

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