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Antifungal Activity of Petroleum Ether and Ethanol Extracts of Moringa Oleifera Seeds

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ABSTRACT---- ***The activity of petroleum ether and ethanol extracts of Moringa olifera seeds have antifungal activity on different fungi including dermatophyte, non-dermatophyte molds and yeasts, which isolated from human skin and nail fungal infections. From 70 samples collected from skin and nail infection; 20 dermatophyte isolates, 21 non dermatophyte moulds and 25 yeast were isolated. While macromorphological and micromorphological as well as cultivation on differential media identified all isolated molds (derematophyte and non dermatophye molds), cultivation on corn meal media and chromogenic Candida agar could identified isolated yeasts. EME is having more antifungal activity than PME on all tested fungi.***

Keywords--- Moringa oleifera (MO) petroleum ether extract (Pe) ethanol extract (Eme) phytochemical dermatophyt non dermatophytes yeast

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1. INTRODUCTION

The products derived from several herbs and plants, being a source of multifunctional curing agents. About 70-80 % of the world's population relies on herbal and plants medicine to prevent and cure diseases specially in development countries (Ekor 2014). On the other hand about 25 % of the synthesized drugs are manufactured from medicinal plants (Pan et al., 2012). On recent years interests have been generated in the development of plant extracts to control fungal infections. Various plants extracts are found to have antifungal activities with no side effects on human and animals (Tabassum and Vidyasagar 2013). From Those plants which have antifungal effects is Moringa Moringa oliolifera. Moringa olifera is one of the most widely distributed and naturalized species of the monogeneric family Moringaceae (Ramachandran et al 1980). The plant is known for its nutritional and medicinal value. It contains some phytochemicals which make it is good source of antioxidant and antimicrobial substances (Kayode and Afolayan 2015). Concerning antifungal effect it was found that its extracts can affect different fungi causing skin fungal infections including yeasts and dermatophytes (Patel et al., 2010 ; Chuang et al., 2007).

2. AIM OF THE WORK

The present study is focused on the activity of Moringa olifera seeds on different fungi including dermatophyte, non-dermatophyte molds and yeasts, which isolated from human skin and nail fungal infections.

3. MATERIALS AND METHODS

Materials

The plants used in the present investigation were *Moringa oleifera Lam.* The seeds of *Moringa oleifera* were obtained from El-kanater El-khiria Horticulture Research Station, Ministry of Agriculture and El-Orman Garden, respectively.

1. Preparation of plant extracts

30g of collected powdered form of leaves and seeds extracts weighed and extracted with 90% of methanol, 80% of petroleum ether, 70% of ethanol and aqueous (distilled water), made up to 250ml and purified by soxhlet apparatus with their respective boiling temperature, allow to evaporate and collected in small labelled vials (Balajee el al. 2004).

2. Phytochemical analysis

Tannins

0.5 g of the moringa oil was mixed with 20ml water. The mixture was boiled and filtered. Then few drops of 0.1% ferric chloride were added. The development of the brownish green or blue-black color confirms the presence of tannins.

Saponins

2 g of moringa oil was mixed and boiled with 20ml of the water and then filtered. 10ml of this filtrate was further mixed with 5 ml of distilled water and was shaken vigorously for stable persistent froth. The formation of the froth confirms the presence of the saponins.

Flavonoids

The known quantity of the oil was taken with the ammonia solution and the concentrated sulfuric acid was added and was allowed to develop the color. Development of yellow color indicated the presence of the flavonoids.

Alkaloids

A 0.5 g of extract was diluted with 10 ml of acid alcohol, boiled and filtered. Two milliliter of diluted ammonia was added to 5 ml of the filtrate. Five milliliter of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Meryer's reagent was added to one portion and Draggendorffs reagent to the other. The formation of a cream (with Meryer's reagent) or reddish brown precipitate (with Draggendorffs reagent) was taken as positive for the presence of alkaloid (Trease and Evans, 1983).

3. Isolation and Identification of fungi from human

Samples were collected from patient attending to outpatient clinic of Dermatology Zagazig University and private Laboratory for mycological examination. All patients were suspected by dermatologist to have dermatomycosis and have positive direct KOH. From 70 samples collected from skin and nail infection; 20 dermatophyte isolates, 21 non dermatophyte moulds and 25 yeast were isolated.Concerning dermatophyte which isolated only from skin infections they were identified by macromorphological and micromorphological characters as well as cultures on rice grains, BCP medium and MHB medium into: Microsporum canis, Trichophyton violaceum, Trichophyton mentagrophytes and Trichophyton rubrum. M. canis and T.violaceum were isolated as the most predominant cause of Tinea capitis the result which coincide with previous works done by El Fangary et al (2011) and Soliman et al (2013). From Tinea corporis and tinea pedis T. rubrum, T.mentagrophytes , M. canis and T. violaceum were the most dermaophytes isolated the results which in agreement with El Fangary et al., (2011) and Taha et al., (2013).

In the present work 21 non dermatophytes moulds (42%) were isolated from cases of Onychomycosis, the identification of non dermatophytes moulds isolated from Onychomycosis were done according macromorphological and micromorphological characters revealed the isolation of Aspergillus niger (12%), Aspergillus nidulans 14%, Aspergillus flavus (10 %), Fusarium oxysporum (4%) and one Scytalidium dimidiatum 2%..The high incidence of non dermatophytes molds were Aspergillus species the result which was also observed by El Khafagy et al. (2002) and Taha (2011). Yeast isolates which obtained from Onychomycosis were identified by macroscopic on CCA and microscopic on corn meal agar besides and macromorphological on SDA as well as micromorphological after Gram stain.

4. Agar well diffusion susceptibility test determination of MIC (Gergova et al., 2005).

Petridishes were prepared with Müller Hinton for yeast and with potato dextrose for dermatophyte and non dermatophyte moulds. Five welles (6mm in diamater were then cut for agar). 100 Mu of ethanolic Moringa oleifera L. extract in concentration of (100%, 75%, 50%, 25%). 0% it was used as control where added into the wells after incubation for 3-5 days for yeasts and 5:8 days for non dermatophyte and 8 :10 days for dermatophyte dishes were examined for zones of growth inhibition the result were reported.

Agar dilution method (Irith et al., 2008)

For MIC determination ethanol extract of Moringa oleifera L. A serial dilution was done with in (100%, 75%, 50% and 25%). One volume of the solution was added to 100 vol. of Müller Hinton media for yeast and PDA for other moulds. mixed thoroughly and the mixture poured in the sterilized petri dishes after solidification one loop of the tests strain of fungi was inoculated in the surface of the medium. Control was done by inoculated tests strains on media free from the extract. The plates were incubated for 48 hours for yeast, 7 days for non dermatophyte moulds and 15 days for

dermatophyte. After confirming that sufficiency growth was obtained on the growth control plate end point were read. The lowest drug concentration of the plate showing no colony formation was recorded as MIC.

5. Transmission electron microscope:

In this experiment, transmission electron microscope was applied to detect the effect of *Moringa oleifera* seed extract in the conidia cell wall and mycelia of *A. flavus* at Petroleum ether extract and ethanol extract compared with control (without *Moringa oleifera* seed extract). A small portion of the fungal mat was fixed at room temperature in 2% (v/v) glutaraldehyde mixed with potassium in 2% (w/v) osmium tetraoxide buffered in 0.005 M sodium cacodylate at pH 6.5 for 40 min. After fixation, the material was washed overnight in the appropriate buffer, dehydrated at room temperature in acetone, and embedded overnight at 65°C in low viscosity epoxy resin **(Spuur, 1969)**. At these conditions, the material was polymerized; ultrathin sections were cut by glass knives of an ULKD ultramicrotome. Sections were collected each on stabilized copper grids, stained with lead citrate and examined in a GOL 100 CX electron microscope. This method was carried out according to the instructions of **Ellis and Griffiths (1974)** and was applied in T.E.M. unit in Faculty of Agriculture, Cairo University.

4. RESULTS AND DISCUSSION

Preliminary phytochemical analysis of petroleum extract (A) and ethanol extract (B)

The presence of Alkaloids, Flavonoids, Saponins and tannins were observed in (A, B) extracts in Table (1). Alkaloids are present in the ethanol extract (B) and absent in Petroleum ether extract (A). Flavonoids are present in (A) and (B). Saponins are present in (B) and absent in (A). Tannins are present in (A) and (B).

These results reavealed that ethanol extract contain phytochemical charaxteristics more than the other extracts .

Table (1) : preliminary phytochemical analysis of petroleum extract (A) and ethanol extract (B)

Tests	A	B
Alkaloids	-	+
Flavonoids	+	+
Saponins	-	+
Tannins	+	+

A = Petroleum ether extract, B = Ethanol extract
(+) = presence of compound, (-) = absence of compound.

Antifungal Activity

In the present work antifungal activity of Moringa oleifera seeds was done using ethanol extract as (EME) as well as petroleum ether extract. Susceptality test for Moringa olifera seeds extracts against dermatophytes (M. canis, T.mentagrophytes and T.rubrum). non dermatophytes moulds (A. niger, A. nidulens A. flavus, and F. oxysporum) as well as yeast (C. albicans. C.purpalers, C. Kirusei and R. mucilagenan) were done by well diffusion method comparing with itraconazole disc.

Table (2): Inhibition zone of EME, PME and itraconzole on different fungi/mm

Fungi	EME	PME	itraconzole
Dermatophytes			
M. canis	28	18	30
T. mentagrophytes	35	10	28
T. rubrum	30	12	25
Non dermatophyte moulds			
Aspergillus niger	20	8	22
Aspergillus nidulans	17	7	18
Aspergillus flavus	15	5	20
Fusarium oxysporum	20	10	15
Yeast			
Candida albicans	18-30	0-10	28
Candida parapsilosis	30	10	25
Candida krusei,	10	10	24
Rhodotorula mucilaginosa	35	15	30

The results reversed moderate to been effect of EMG against NDM and yeast comparing to itraconzole while inhibition varies from 15-22 mm as the other hand yeast inhibition zone done by EME reversed effective against C. albicans (18-30 mm) C. parapsilosis (30 mm) and 35mm inhibition zone against R. muciloginosa. C. krusei only showed been inhibition zone (10mm)

For all yeast PME showed been effective rang from 10-15 mm. Control disk of itraconzole showed been 24-30 mm for all yeasts (table 2).

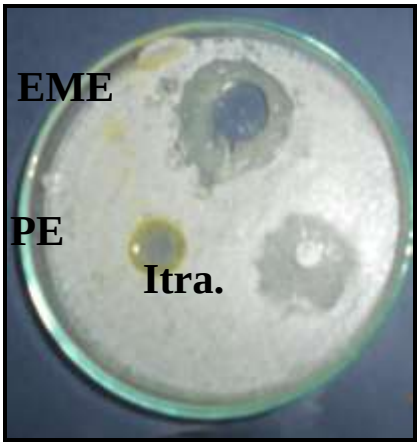


Figure 1: Antifungal effect of EME and PE compared to Itraconazole on candida albicans

MIC of ethanolic extract of moringa oleifera against dermatophyte reversed strongest antifungal activity for T. mentagrophytes 100% and T. rubrum 25 %. While Mic for M.canis moderate activity showed been 50 %. Ethanolic extract of moringa oleifera against non dermatophyte mould showed been inhibition where MIC ranging from 50-75 %.

On the other hand MIC of ethanolic extract of moringa oleifera against yeast were variant with increase inhibition against C. albicans, C. parapsilosis and R. mucilaginosa 25% and moderate inhibition effect by C. krusei where MIC was 50%.

Table (3): MIC of ethanol extract of Moringa oleifera L. against dermatophyte

Concentration	M. canis	T. mentagrophytes	T. rubrum
75%	-	-	-
50%	-	-	-
25%	+	-	-
10%	+	-	+

+ indicates growth of dermatophyte - indicates inhibition of growth

Table (4): MIC of ethanol extract of Moringa oleifera L. against Non dermatophyte moulds

Concentration	A. niger	A. nidulans	A. flavus	F. oxysporum
75%	-	-	-	-
50%	-	+	+	-
25%	+	+	+	+
10%	+	+	+	+

Table (5): MIC of ethanol extract of Moringa oleifera L. against yeast

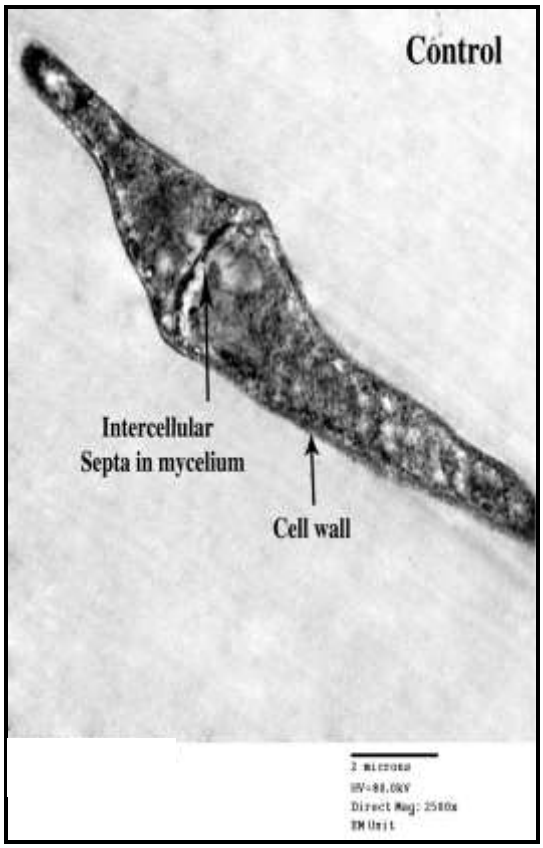
Concentration	C. albicans	C. parapsilosis	C. krusei	R. muciloginosa
75%	-	-	-	-
50%	-	-	-	-
25%	-	-	+	-
10%	+	+	+	+
5%	+	-	-	+

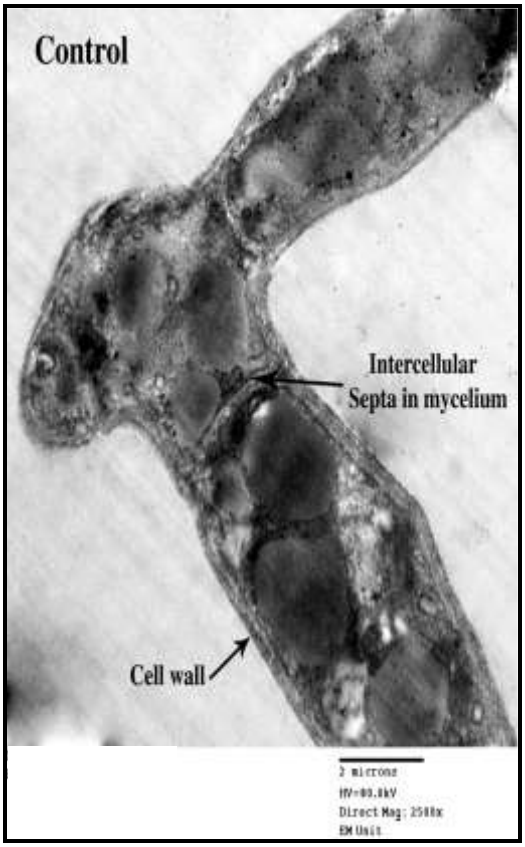
The current work concerning the antifungal activity of EMO on dermatophyte, non-dermatophyte molds and yeasts are in agreement with Riad (2014).

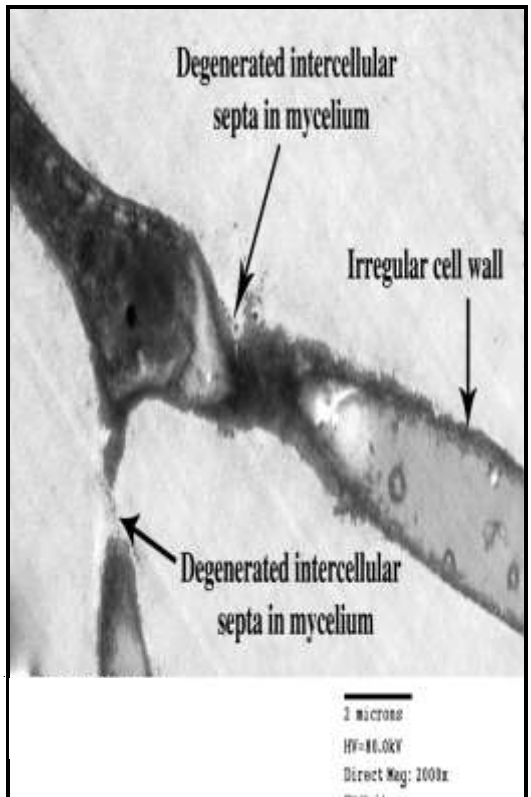
Effect of Moringa oleifera seed extract on the cell wall of Aspergillus flavus by transmission electron microscope:

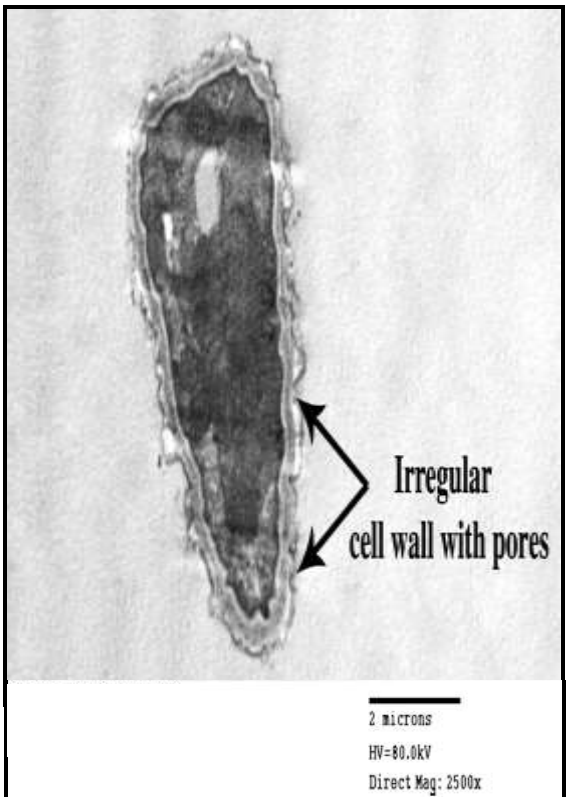
From the table (2), Inhibition zone of EME, PME on different fungi we found that the antifungal activity of *Aspergillus flavus* in PME gave 5 mm which is lowest effect on different fungi and EME gave 15 mm, thus we had done a comparison between the effect of EME, PME on the cell wall of *Aspergillus flavus* by transmission electron microscope.

Examining *A. flavus* morphology in the control sample indicated normal structure for mycelia and conidia of the fungus (Fig. 2 A). Petroleum ether extract of *Moringa oleifera* seed had affects on cell wall; forming some external protrusion, in both conidia and mycelium, degenerated intercellular septa in mycelium with torned cell wall in conidia and cause irregular cell wall (Fig. 2 B). *A. flavus* treated with ethanol extract of *Moringa oleifera* highly affected the fungus morphology and led to irregular cell shape with destroyed cell wall and shrinkage of cell cavity (Fig. 2 C).









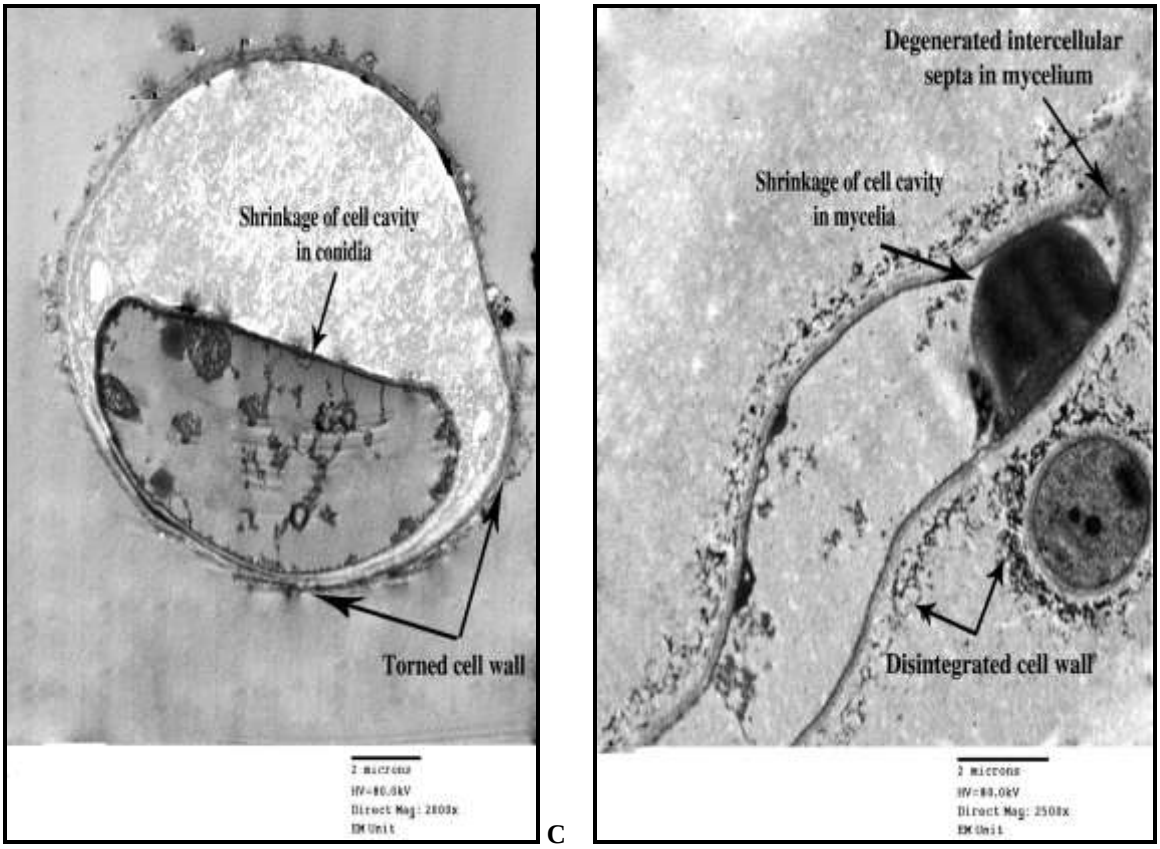


Figure 2**:** Control (A), effect of petroleum ether (B) and ethanol extract (C) of Moringa oleifera seeds on the cell wall of Aspergillus flavus by transmission electron microscope

Changes in cell wall of fungus; external, disintegration and irregular cell wall was found under TEM treated with Petroleum ether extract and ethanol extract of *Moringa oleifera* seeds. Also there are some deformation inside cell and shrinkage of cell cavity this may be due to presence of antioxidant properties of tested extract which are effective superoxide antioxidants with ability to inhibit mycelial growth by reacting with cell wall components (Chandrasekaran et al., 2011). From research findings, it has been noted that n-Hexadecanoic acid, Octadecanoic acid and Tetradecanoic acid found on *Moringa oleifera* seed extracts may have target site on the cell wall of this fungus. The mode of action may possibly be by attack on the sugar residues on the cell wall of fungal species according to (Rahuman et al., 2000). Antifungal activity of *Moringa oleifera* seed could be correlated to its scavenging action on superoxide and hydroxyl radical which could be part of cellular metabolism of the enteropathogens (Mahmood et al., 2009).

5. CONCLUSION

Dermatophytes, non dermatophyte fungi are the cause of human skin and nail infections, While the dermatophytes are predominant in skin infections non dermatophyte fungi are more common in onychomycosis (nail infections).
While macromorphological and micromorphological as well as cultivation on differential media identified all isolated molds (derematophyte and non dermatophye molds), cultivation on corn meal media and chromogenic Candida agar could identified isolated yeasts.
EME is have more antifungal activity than PME on all tested fungi. Dermatophytes are the more sensitive fungi for the activity of Moringa olifera extracts. Except Candida krusei all tested yeasts were sensitive for EME. Moringa olifera extracts is active against dermatophyte,non-dermatophyte and yeasts , therefore.it could be used as an alternate source for the treatment skin diseases caused by fungi.

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