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Antifungal Activity of Ethanol Extracts of Fourteen Wild Indigenous Plants Against Four Phytopathogenic Fungi

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Abstract

An experiment was conducted to assess the biological activities of ethanolic extracts of fourteen wild indigenous plant obtained from the Abu-Tartur Phosphate mining area. Such wild plant extracts were tested against four economically important phytopathogenic fungi, *Fusarium oxysporum*; *Alternaria alternata, Macrophomina phaseolina* and *Penicillium italicum*. Results, *in vitro,* suggested that ethanolic extract of *Fagonia arabica* L. had highest inhibition zones against tested phytopathogenic fungi (28.00 mm, 36.00 mm, 24.73 mm and 39.733 mm), respectively. In addition, different concentrations of *F. arabica* extracts (0.5, 1.5, 2.5, 3.5 mg/ml) were mixed with the media of pathogenic fungi *P. italicum* and compared with dimethyl sulfoxide as control. Highest mycelial growth inhibition was measured in *vitro* at concentration 3.5 mg/ml of *F. arabica* extract. The antioxidant inhibition was calculated against *F. oxyspoum* (48.2%), *A. alternata* (68.4%), *M. phaseolina* (67.0%) and *P. italicum* (70.8%). Also, the highest values of total phenol contents (90.174 mg/g) in *F. arabica* were recorded. Electron micrographs showed the impacts of extracts on morphological as well as ultrastructural modifications of cytoplasm and cell wall in treated cells compared to the control hyphae. The treated mycelial micrographs (3.5 mg/ml extract) showed aberrant morphology: partial distortion, partial loss of nuclear membranes and disappearance of hyphal cytoplasm. It can be concluded that the addition of plant extracts to the growth media negatively affected fungal growth and the use of *F. arabica* extracts may be considered as a botanical fungicide.

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Introduction

Human and plants have been closely linked throughout civilization's development. Archaeological data suggest that different plant extracts can be employed as medicines since ancient times [1]. Contamination of toxigenic, allergenic and pathogenic fungi may pose a significant risk to human life [2]. Just about 15,000 of the approximate 1.5 million fungal species on Earth are plant pathogens, the majority of which belong to the Ascomycetes and Basidiomycetes classes [3]. While less than 10 percent of all known fungi growing colonize living plants, 70 percent of all known plant diseases are collectively responsible for phytopathogenic fungi [4-6]. There are numerous factors contributing to the role of fungi as a main category of plant pathogens; many phytopathogenic fungi are capable of infecting any plant tissue at any stage of growth. Most have complex cycles of life / infection involving multiple (up to five) stages, each of which may occur in another plant host and may be characterized by specific reproductive strategies [7].

Traditionally, these pathogens have been treated with a variety of synthetic fungicides, such as anilinopyrimidines, hydroxianilides and azoles. Nevertheless, indiscriminate use of these chemicals has improved plant fungal resistance. In addition, the application of several synthetic fungicides has now been limited due to their undesirable characteristics including acute toxicities, low degradation rate, and the ability to accumulate in the foods as well as their ability to cause toxic effects on beneficial microorganisms [8-9]. Natural products can be used to control fungi as an alternative to these synthetic fungicides, because they are not harmful to humans. Since the plant extracts are biodegradable to non-toxic materials, and can be used as active agents for controlling the fungal phytopathogens and they have the potential to be used in the integrated pest management programmers [10-14].

The Egyptian flora is rich in variety, and numerous plant species have been used as a medicine since ancient times, when ancient Egyptians have documented several plant species as well as their use in the Ebers Papyrus [15-16]. Egypt has about 384 plant species which can be used as medicine and these species have been explored in the Mediterranean coastal region, the western and eastern deserts of the Nile and Sinai Peninsula. The wild plants studied are grown in arid regions of the Saharo Arabian-Saharo Sendian area and are registered and collected from the Abu-Tartur mining area. Abu Tartar is one of the largest phosphate mining areas (1000 million tons) in the Middle East. The land used for mining activities is situated in the western desert of Egypt, approximately 60 km from El-Kharga City as well as 10 km from the main road between El-Karga and El-Dakhla Oasis [17]. The flora of wild medicinal plants in the phosphate mining area have been explored by [18-20].

The fourteen studied wild taxa including: *Trigonella hamosa* L., *Astragalus vogelii* (Webb) Bornm, *Melilotus indicus* (L.) All., *Senna italica* Mill. are members of family Leguminosaeae, *Polygonum equisetiforme* Sm., *Rumex vesicaricus* L. are members of family Polygonaceae, *Farsetia aegyptia* Turra, *Schouwia purpurea* (Forssk.) Schweinf., *Morettia philaeana* (Delile) DC, are members of family Cruciferae, *Trichodesma africanum* (L) is a member of the family Boraginaceae, *Citrullus colocynthis* (L.) Schrad. Belonging to family Cucurbitaceae, *Reseda decursiva* Forssk, is a member of family Resedaceae, and *Fagonia arabica* (family Zygophyllaceae) is an important herb, represented in Egypt by eighteen species [21].

Fagonia arabica-complex consists of four species (*F. arabica* L.; *F. acerosa* Boiss; *F. zilloides* Humbert and *F. taeckholmiana* Hadidi). *F. arabica* (L.) is known to be widespread, ranging in distribution from the North Africa Sahara to South Arabica and Pakistan [22]. Several biological researches have been studied on *F. arabica* [23-27]. The whole plant is used for medicinal purposes; popularly known in hilly areas as a fever remedy. Infusion is useful as a coolant in stomatitis. It is believed to purify blood and also serves as a deobstructive agent [28]. *Fagonia arabica* can be used as a potential cost-effective and safe alternative to commonly approved thrombolytic drug [29]. *Fagonia* extracts demonstrated good antibacterial and antifungal activity [30]. *Fagonia arabica* is considered to have antibacterial activity [31] against two strains of bacteria, namely *Escherichia coli*; *Staphylococcus aureus* [32].

In vitro antifungal activities of a specific extract from ten native Bahraini plants have been studied against four filamentous fungi using standard antifungal disks. The highest ethanolic extract activity was observed with the extract of *Cressa cretica* against *Penicillium citrinum*. Chloroform extracts of *Emex spinosa* Campd. showed the potential against *Alternaria alternata* (Fries)[33].

Fagonia arabica; because of its antioxidant capacity, decreases oxidative stress caused by ischemia-reperfusion and helps cells sustain cell ATP and lactic acid levels, thereby preventing cell death due to ischemia/reperfusion. Total Polyphenol Content (TPC) and antioxidant potential of *F. arabica* was assessed; which effect on neuroprotection and energy metabolism. The ischemic injury was characterized by impaired energy status as demonstrated by decreased ATP levels in the cells and increased lactic acid content. Both adjustments reacted favorably to *F. arabica* provided significant neuroprotection against ischemia and helped to preserve cell viability and mitochondrial integrity of the cells. *F. arabica* has demonstrated a large amount of TPC and antioxidant activity [34].

The prime objective of this experiment was to explore the antifungal activities of the ethanolic extracts of fourteen wild indigenous plants in Abu Tartur Phosphate mining region. The extracts were analyzed for antioxidants and total phenolic compounds. The four important toxigenic, allergenic and pathogenic fungi (*Fusarium oxysporum*, *Alternaria alternata*, *Macorphmina phaseolina* and *Penicillium italicum*) were selected that pose medical, health-related as well as economic risks. Moreover, the ultrastructure of *Penicillium italicum* treated with ethanolic *Fagonia arabica* (Dhamasa) extract was also studied.

Materials and methods

Study area

The Western Desert of Egypt has an area of more than 400,000 km^2. It's one of the most arid areas on Earth, and it's almost devoid of people today. It is made up of two large physiographic provinces separated by a prominent Eocene scarp. The southern portion of the Eocene scarp is known as the Sandstone Desert of Nubia. Abu Tartur Phosphate is one of the biggest phosphate mining areas (1000 million tons) in the Middle East. The mining land is situated in the Western Desert, approximately 60 km from El-Kharga City as well as 10 km from the main road between El-Karga and El-Dakhla Oasis [17]. Plant materials were collected from wild plant populations growing in sandy soils of the phosphate mining region at Abu-Tartur, approximately 700 km South west of Cairo at 25° 24' 417" north

and 30° 04′ 661″ east.

Plant material

Fresh materials of 14 native plant species have been collected from the mining area. Life span and life form of the species studied have been reported **(Table 1)**. The collection of all plants was brought to the Mansoura University, Egypt. The identification of these specimens has been achieved by [35].

Table 1: List of studied species, families, life span and life form.

Species	Families	Life span	Life form
Trigonella hamosa L.	Leguminosaeae	Annual	Herb
Astraglus vogelii (Webb)Bornm.		Annual	Herb
Melilotus indicus (L.) All.		Annual	Herb
Senna italica Mill.		Perennial	Sub-shrub
Polygonum equisetiforme Sm.	Polygonaceae	Annual	Herb
Rumex vesicaricus L.		Annual	Herb
Farsetia aegyptia Turra	Brassicaceae	Perennial	Herb
Schouwia purpurea (Forssk.) Schweinf.		Annual	Herb
Morettia philaeana (Delile) DC.		Perennial	Sub-shrub
Bassia muricata (L.) Asch.	Chenopodiaceae	Annual	Herb
Trichodesma africanum (L.) R. Br.	Boraginaceae	Annual	Herb
Citrullus colocynthis (L.) Schrad.	Cucurbitaceae	Perennial	Herb
Reseda decursiva Forssk.	Resedaceae	Annual	Herb
Fagonia arabica L.	Zygophyllaceae	Perennial	Herb

Preparation of the plant crude extract

Plant samples collected from the study area were air dried under the shade for two weeks, macerated to fine powder with a mortar followed by a potable mechanical grinder (model DFH 48). 100 grams of powdered shoot systems of study taxa were extracted by soaking in 500 ml ethanol for 72 hours [36]. The extracts were filtered through a cheese cloth under heavy hand pressure and the solvent was dried in a vacuum chamber at 60-65 °C with a rotary evaporator. The removed residue has been dissolved in DMSO, dimethyl sulfoxide (0.1%). The raw plant extracts were processed under refrigeration to prevent decomposition and later used for various biological activity studies [37].

Measurement of total phenolic content of the studied taxa

For studying biological activities, the total phenolic content is evaluated. 0.5 ml of the plant extract was mixed with 2 ml of the Folin-Ciocalteu reagent (diluted 1:10 with deionized water) and were neutralized with 4 ml of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 60 min and the absorbance of the blue color development was recorded at 760 nm using UV/V is spectrophotometer (Shimadzu PC-1650, Kyoto, Japan). Distilled water was used as a blank. Gallic acid (GA) was used as standard for plotting calibration curve and total phenolic contents were denoted as mg GA equivalent/gram of extract [38].

Measurement of antioxidant activity: Free radical scavenging method (DPPH)

Antioxidant activity of ethanol extracts for studied plants as well as the standard antioxidant ascorbic acid was measured with respect to the radical scavenging efficiency of the stable 2,2- Diphenyl-1-Picrylhydrazyl (DPPH) free radical activity [39]. The standard used for this purpose was ascorbic acid in 1- 100 µg/ml solution. The absorbance of the solution was recorded at 515 nm per minute, for a total period of 30 min. The scavenging activity against DPPH was measured by the following formula:

Scavenging activity (%) = [(A-B)/A] x100

In the equation; A is devoted to the absorbance of control (only DPPH solution) and B is devoted the absorbance of DPPH solution with sample.

Screening of different plant extracts for antifungal activities

Fourteen plant extracts were examined for their inhibitory activities against selected phytopathogenic fungi (Fusarium oxysporum; Alternaria alternata, Macrophomina phaseolina and Penicillium italicum) from Mycology Center of Assiut University by using the agar diffusion technique. The Potato Dextrose Agar (PDA) media with potato, glucose, agar, and distilled water containing 200 g, 20 g, 20 g and 1000 ml were used. The pH of the media was maintained at 5.6 either with 1N HCl or NAOH. A test agar medium was prepared by using 20 ml of the media in a petri dish [40]. All petri-plates were incubated at 28 ± 2°C for 72 h. The inhibition zone diameter was determined for all tested fungi [41-43].

The reduction (measured in percentage) (M_r) of colony diameter in the presence of each extract was computed following the formula [44]:

$$M_r = (M_1 - M_2) / M_1 \times 100$$

In the equation; M_r, M_1, and M_2 are devoted to the percentage reduction in colony diameter, colony diameter of the control and the treated medium, respectively.

Effect of *Fagonia arabica* extract on fungal linear growth

In this experiment the probable effect of Fagonia arabica extract was tested for the growth of tested fungi. The dimethyl sulfoxide (DMSO) was used to dissolve of the ethanolic extract, prepared at 1% on v/v basis, and this mixture was mixed with PDA and different concentrations (0.5, 1.5, 2.5 and 3.5 mg/ml) were obtained. A control containing only PDA plates with DMSO was maintained. Triplicate dishes have been used for each treatment. Daily measurement of linear growth was carried out. The inhibition in the growth of each treatment with respect to control was estimated by the following formula [45]:

Mycelial growth inhibition (%) = [(C –T) / C] x 100

In the formula C and T were devoted to the mycelial growth (mm) of control and treated cultures, respectively.

Transmission Electron Microscopy (TEM)

Because of the effective treatment of *Fagonia arabica* ethanolic extract in the reduction of *Penicillium italicum* mycelial growth, we studied this effect by using TEM. All fungal cultures were exposed to the extract with 20 mg/ml in PDA at a specific temperature (28 ± 2 °C). The DMSO-treated mycelia were considered as a control. Following the procedure described by [46] for TEM preparation, untreated and treated *P. italicum* was

fixed in glutaraldehyde (3%) in buffer (0.1 M at pH 7.0) of sodium cacodylate for 2h at room temperature, followed by rinsing by using the same buffer, and post- fixed in osmium tetroxide (1%) for 2h at room temperature. Different ethanol series (ranging from 10%) was used to dehydrate the samples for 15 minutes, followed by absolute ethanol for 30 min. Both epoxy resin and acetone were used to infiltrate the samples. The thin sections of the fungal cultures were examined with transmission electron microscope (JEOL model JEM-1230) at 70 kV.

Molecular identification of tested fungi

Fusarium oxysporium 9704 AUMC, *Alternaria alternata* 10301 AUMC and *Macorphmina phosealina* 10204 AUMC were obtained from Mycology Center Assiut University, but *Penicillium italicum* obtained from Plant Pathology Lab., Faculty of Agriculture, Mansoura University and were maintained in PDA medium, which used as test fungi for the measurement of antifungal activity.

The fungal DNA was extracted with the help of FastDNA® Spin Kit with specific instructions obtained from suppliers. The genomic DNA yield was recorded with a Nano Drop spectrophotometer with absorbance at 260 nm. The universal primers with high specificity for fungi 18FITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and 18RITS4 (5'- TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS1 region from DNA sample extracts in triplicate. For the preparation of PCR reaction, the solution and chemical used were as follow; 5 µl master mix, 20 µl each of primers ITS1 and ITS4, 3 µl of 50 mM $MgCl_2$, 0.5 µl of Ampli-Taq DNA polymerase, and 1 µl of genomic DNA and finally 50 µl volume were prepared by using distilled water. A thermo-cycler was used to perform PCR reaction in different temperature programs as follows; 94 °C for 5 min, 94 °C for 40 s, 55 °C for 45 s, 72 °C for 1.5 min, 72 °C for 7 min (35 cycles). The electrophoresis on 1.5% agarose gels (1.5% w/v; 30 min at 100 V, 1X TBE) was used for the determination of size of the PCR products. The 100 bp molecular size marker (MBI Fermentas, Lithuania) was used for the comparison of amplified fragments. The Qiagen II Agarose Gel Extraction Kit was used to purify the required products. The products obtained from PCR were stored at -20 °C. A thermocycler (Master cycler, Eppendorf, Hamburg, Germany) was used to perform sequencing reactions with a total volume of 10 µl in different temperature programs: 96 °C for 1 min, 96 °C for 30 s, 60 °C for 10 s, 60 °C for 4 min, 72 °C for 5 min (25 cycles) [47,48].

The Dye Ex 2.0 Spin Kit was used for the purification of products obtained from sequencing reactions followed drying the products with the help of vacuum centrifuge and finally analyzed with biosystems (ABI PRI SM Big Dye Terminator v1.1), sequencing kit and using ABI 3130 XL Genetic Analyzer (Applied Biosystems, Darmstadt) [49]. A Sequencher™ 4.8 Software was used for the annotation of the sequences. Different programs were used for DNA similarity searches such as BlastN program and the databases of European Molecular Biology Laboratory (EMBL) and GenBank from NCBI.

The phylogenetic as well as molecular evolutionary examinations were conducted by using 18S rRNA gene nucleotide sequences for sequence alignments with ClustalW and BioEdit 7.0.5.3 and implement in MEGA software version 6. The Neighbor-Joining algorithm method was used for the preparation of phylogenetic trees [50-51]. The Kimura Matrix was used for distance generation, and Bootstrap analysis was used for the tree stability (1000 replications).

Statistical analysis

All the data in the study were reported in triplicate with mean ± SD. The data were subjected to an analysis of variance by using SPSS 13.0 for Windows program. The means were compared by Duncan's multiple range test at $P < 0.05$ significant level and Least Significant Difference (LSD) was calculated for antifungal activity [52].

Results

Total phenolic contents of plants

Total phenol contents of ethanol extracts for the studied taxa were based on GA equivalents. Total phenolic contents were 13.42 mg/g GA equivalent. The observed results showed the highest values of total phenol contents (90.174 mg/g) in Fagonia arabica, while the lowest one was recorded for Astraglus vogelii extract (28.599 mg/g). Also, there was convergence of results between most plants under study as shown in **(Figure 1)**.

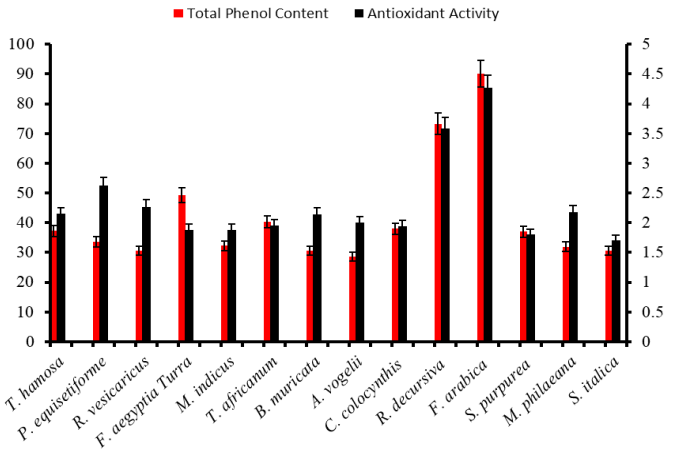

Figure 1: Total phenolic contents (mg/g) and antioxidant activity (%) of the studied plants of Abu Tartur Phosphate mining region of Western Desert, Egypt.

Antioxidant activity of studied taxa

The results related to antioxidant activity have been reported in Figure 1. The reducing powers of the examined extracts were moderately strong. Among the different studied taxa showed the highest scavenging activity values were 4.27 % for *Fagonia arabica* and 3.59 % for *Reseda decursiva* whereas the lowest scavenging activity were 1.71 % for *Senna italic* and 1.80 % for *Schouwia purpurea* which are very close to each other.

Antifungal activity of the ethanol extract for the studied taxa

The effect of fourteen plant ethanolic extracts on the growth of studied fungi differed significantly ($P < 0.05$) and the diameters colonies were recorded **(Table 2)**. It is evident from the result that most of the extracts obtained from studied plants showed significant inhibition of the fungal growth over the control. Antifungal activities of ethanol extracts of the tested plants, showed that: the most active plant extracts were *Fagonia arabica* (Barren Zone (BZ) =28.0 mm), *Reseda decurisva* (BZ=26.67 mm) and *Astragalus vogelii* (BZ=26.33 mm) against test phytopathogenic fungi *Fusarium oxysporum* while the lowest were *Citrullus colocynthis* (BZ =17.83 mm), *Bassia muricata* (BZ=19.10 mm) and *Morettia philaeana* (BZ=19.33 mm). For *Alternaria alternata*, the most active plant extracts were *F. arabica* (BZ=36.00 mm), *Polygonum equisetiforme* (BZ=27.07 mm) and *Reseda decurisra*

(BZ=25.33 mm), where, the lowest activation were for extracts of *Trichodesma africanum* (BZ=16.83 mm), *Citrullus colocynthis* (BZ=17.40 mm) and *Bassia muricata* (BZ=17.77 mm). The effect of plant extracts were in order: *F. arabica* (BZ=24.73 mm) *Reseda decurisva* (BZ=22.67 mm) and *Trigonella hamosa* (BZ=21.67 mm) and the lesser were for *Schouwia purpurea* (BZ=15.00 mm), *Melilotus indicus* (BZ=15.47 mm) and *Rumex vesicaricus* (BZ=16.50 mm) for *Macorphmina phaseolina*. Finally, for *Penicillium italicum*: *F. arabica* (BZ=39.73 mm), *Farsetia aegyptia* (BZ=30.367 mm) and *Polygonum equisetiforme* (BZ=26.67 mm) extracts were the most operative one and the lowest for *Schouwia purpurea* (BZ=19.77 mm), *Trigonella hamosa* (BZ=20.73 mm) and *Bassia muricata* (BZ=21.40 mm).

Each value represents the mean of three replicates (Average ± SD). Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test (P =0.01).

Effect of *Fagonia arabica* extract on mycelial growth

Table 3 showed the inhibition of mycelial growth of the studied fungi with increasing of *Fagonia arabica* extract concentration. The results obtained in the current study revealed that on the 6th day, the highest inhibition of fungal growth observed at concentration 3.5% of *F. arabica* extract against *F. oxyspoum* (48.2%), *A. alternata* (68.4%), *M. phaseolina* (67.0%) and *P. italicum* (70.8%).

Table 2: Antifungal activity and inhibition zone (mm) of different plant extracts against tested phytopathogenic fungi.

Studied Species	*Fusarium oxysporium*	*Alternaria alternate*	*Macorphmina phosealina*	*Penicillium itallicum*
Control (DMSO)	13.33 ± 0.58^{h}	14.83 ± 1.04^{h}	13.73 ± 0.63^{h}	15.37 ± 0.55^{h}
Trigonella hamosa L.	23.33 ± 1.53^{dc}	23.73 ± 1.10^{bcd}	21.67 ± 2.08^{b}	20.73 ± 0.64^{fg}
Astraglus vogelii (Webb) Bornm.	26.33 ± 3.21^{ab}	20.43 ± 0.51^{def}	17.53 ± 0.50^{efg}	22.40 ± 1.22d^{efg}
Melilotus indicus (L.) All.	22.67 ± 1.53^{cde}	22.67 ± 2.52^{cd}	15.47 ± 0.50^{gh}	26.00 ± 2.646^{c}
Senna italica Mill.	20.33 ± 2.08^{efg}	18.33 ± 1.53^{efg}	18.73 ± 0.10^{cde}	23.77 ± 0.68^{cdef}
Polygonum equisetiforme Sm.	21.33 ± 1.53^{def}	27.07 ± 3.41^{b}	17.73 ± 0.64^{def}	26.67 ± 1.53^{c}
Rumex vesicaricus L.	23.20 ± 0.10^{cde}	25.33 ± 1.16^{bc}	16.50 ± 0.50^{fg}	25.13 ± 1.63^{cd}
Farsetia aegyptia Turra	24.37 ± 1.18^{be}	20.67 ± 3.79^{def}	20.67 ± 3.51^{bc}	30.37 ± 0.55^{b}
Schouwia purpurea (Forssk.) Schweinf.	22.33 ± 2.52^{cde}	21.57 ± 0.40^{de}	15.00 ± 1.00^{gh}	19.77 ± 0.68^{g}
Morettia philaeana (Delile) DC.	19.33 ± 1.53^{fg}	22.33 ± 1.53^{cd}	18.20 ± 1.59^{def}	22.00 ± 3.00^{efg}
Bassia muricata (L.) Asch.	19.00 ± 1.00^{fg}	17.77 ± 0.68^{efgh}	19.67 ± 1.53^{bcd}	21.40 ± 0.53^{efg}
Trichodesma africanum (L.) R. Br.	21.33 ± 1.53^{def}	16.83 ± 0.76^{hg}	19.43 ± 1.25^{cde}	26.33 ± 3.51^{c}
Citrullus colocynthis (L.) Schrad.	17.83 ± 1.04^{g}	17.40 ± 0.53^{fgh}	17.47 ± 0.50^{efg}	24.33 ± 1.53^{cde}
Reseda decursiva Forssk.	26.67 ± 1.53^{ab}	25.33 ± 4.16^{bc}	22.67 ± 5.51^{cde}	26.50 ± 2.18^{c}
Fagonia arabica L.	28.00 ± 2.00^{a}	36.00 ± 1.00^{a}	24.73 ± 0.64^{a}	39.73 ± 2.83^{a}
Least Significant Difference (LSD)	2.90	3.48	2.33	3.078

Table 3: Effect of different concentrations of Fagonia arabica extract on mycelial growth of tested fungi.

Different concentrations of *F. arabica* extract (mg/ ml)	Diameter (mm) of mycelial growth of tested fungi after 6 days							
	Fusarium oxysporium		*Alternaria alternate*		*Macorphmina phosealina*		*Penicillium itallicum*	
	Mean of mycelial growth	% of inhibitions	Mean of mycelial growth	% of inhibitions	Mean of mycelial growth	% of inhibitions	Mean of mycelial growth	% of inhibitions
Control	62.3	-	65.6	-	90.0	-	81.3	-
0.5	45.7	26.2%	47.0	27.4%	47.3	46.3%	49.3	39.4%
1.5	41.3	23.7%	36.7	44.1%	38.3	57.4%	40.7	49.9%
2.5	36.7	41.1%	26.7	59.9%	36.0	60.0%	38.7	52.4%
3.5	32.3	48.2%	20.7	68.4%	29.7	67.0%	23.7	70.8%

Effects of *Fagonia arabica* extract on hyphal ultrastructure

TEM results showed that sub-cellular components of pathogens were covered with a distinct electron-dense wall as well as normal plasma lemma in the control treatment. Ultrastructural aspects of *P. italicum* hyphae were revealed a typical eukaryotic cytoplasmic component including: double membrane surrounded the nuclus and an Endoplasmic Reticulum (ER) was linked with nucleus, lipid droplets, Golgi bodies, vacuoles, as well as in mitochondria **(Figure 2)**. The cell membrane demonstrated as a sharp, electron dense phospholipid bilayer which was closely attached with the cell wall. Mitochondria demonstrated well-developed cristae. When the ethanolic extract of *Fagonia arabica* (3.5%) was applied to the pathogen's growth medium, several ultrastructural changes of the mycelium appeared. The transmission electron microscopy showed cytomorphological alterations of the hyphae, such as electron-transparency of cell walls, degeneration of cytoplasm, cytoplasmic vacuolation and the improvement in vacuoles numbers, lipid droplets as well as accumulation of Osmiophil bodies beneath the cell membrane. Also, some aberrant morphology was observed as demonstrated by the partial distortion, depletion of hyphal cytoplasm, partial loss of nuclear membrane and membranous organelles such as nuclei, endoplasmic reticulum and mitochondria **(Figure 2)**.

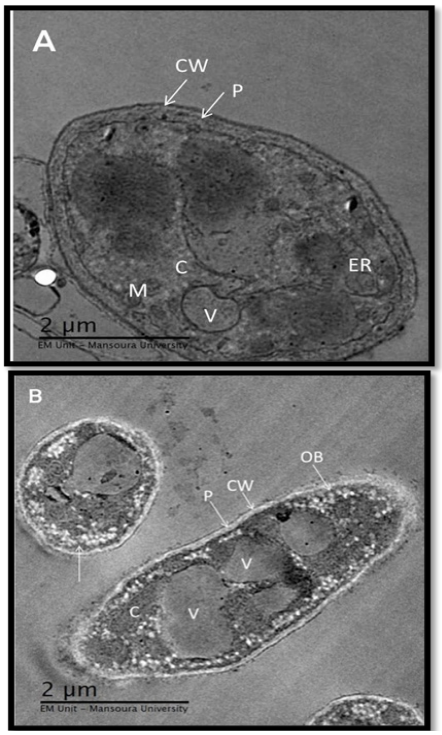

Figure 2: Transmission Electron micrographs of the ultrastructure of *Penicillium itallicum* hyphae. (a) A section of a hypha from untreated medium (control with DMSO) (b) A section of a hypha from medium treated with 3.5 mg/ml *Fagonia arabica* ethanolic extract; cell wall (CW) adjacent to the plasmalemma (P), cytoplasm (C), endoplasmic reticulum (ER), mitochondria (M), osmiophil bodies (OB) and vacuoles (V).

Phylogeny of fungal isolates

The 18S ribosomal RNA gene was employed as a eukaryotic marker for the fungi. The database at NCBI was used to compare the sequence analysis of 18S ribosomal RNA. The tested fungi were closely related to *Alternaria alternata* KU324792, *Fusarium oxysporum* MK929296, *Macrophomina phaseolina* FJ395243, and *Penicillium italicum* JX901397 **(Figure 3 & Table 4).**

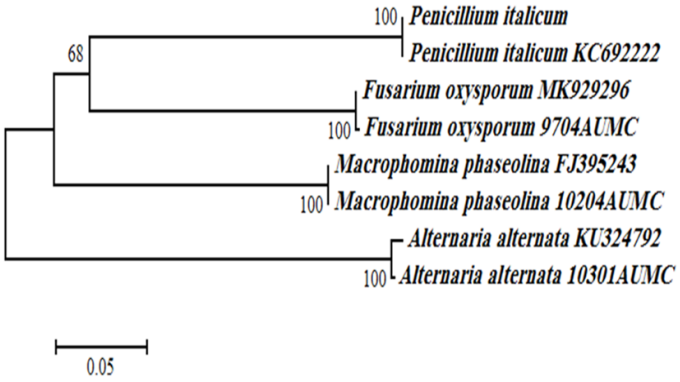

Figure 3: Phylogeny of tested fungal isolates. Multiple alignments of the sequences corresponding to the 18S rRNA gene of the studied isolates were carried out followed by neighbor joining clustering. Bootstrap values expressed as percentages of 1000 replications. Bar represents 0.1 substitutions per nucleotide position.

Table 4: Sequence homology of the 18S rRNA gene of the tested fungal isolates.

Tested fungi	Size (bp)	Closely related fungi	Accession number	Homology (%)
Alternaria alternate	540	*Alternaria alternata*	KU324792	99
Fusarium oxysporum	517	*Fusarium oxysporum*	MK929296	100
Macrophomina phaseolina	1421	*Macrophomina phaseolina*	FJ395243	100
Penicillium italicum	592	*Penicillium italicum*	JX901397	100

Discussion

Higher plants can be considered a good source of phytochemicals and better natural substitutes that serve as coveted medicines that may help to combat numerous deadly diseases around the globe. Plants often generate antimicrobial agents through secondary metabolism to defend themselves against pathogen attacks and many plant species, therefore, have significant antimicrobial activity that is used in diverse areas of research. In addition, flowering plants have the ability to inhibit the germination of spores and mycelial development [8]. Several higher plant metabolites have shown the effectiveness against various pathogens of the plants. Exploring the plants as sources of biologically active compounds therefore is worthwhile [53-54]. Few phytochemical compounds have been reported to have the antimicrobial activity such as tannin, glycoside, saponin, terpenoid, flavonoids, and alkaloids [55-57].

Various studies have recorded several polyphenol compounds in plants which have biological impacts such as antioxidant activity [58]. A compound's reduction capacity may be related to its ability to transfer electrons which can serve as an indication of its efficiency to have an antioxidant activity [59]. In this investigation, for their biological activities such as antioxidant and total phenol content, we analyzed the ethanol extracts of fourteen wild taxa collected from the phosphate mining area. Fagonia arabica's total phenolic content and antioxidant activity were coincident with its high inhibition on fungal activity. Antioxidants are useful for management of different diseases due to their scavenging activity. The quantitative determination of antioxidants has examined the Clausena heptaphylla is found to contain large concentrations of scavenging substances. CHET had substantial radical scavenging activity free of DPPH with an IC50 value (3.11 µg/ml) with high power of reduction [60].

Selected wild plants of Abu Tartur's Mining phosphate shown to have exceptional fungal toxicity against mycelial growth of various studied phytopathogenic fungi, particularly *F. arabica* which have the highest inhibition zones for the studied fungi. Similarly, **[**33] found *Fagonia indica* Burm f had the highest growth inhibition against *Penicillium citrinum* (29.3 mm). These findings are in line with other studies in which various plant extracts were assessed [61-62]. Mexico *Flourensia* spp. ethanol extracts can be used as an alternative of synthetic fungicides. *Flourensia* spp. ethanol extracts have demonstrated high efficacy in inhibiting the growth of *Fusarium oxysporum* and *Rhizopus stolonifera* [63]. In addition, [64]findings showed that *Periploca sepium* root barks demonstrated very strong antifungal activity against *Valsa mali* and *Fusarium graminearum*.

Several researchers have suggested that the efficiency of plant extracts on fungal growth may be related to the secondary metabolites (e.g., alkaloid, phenolic, terpenoids and flavonoid which could adversely affect the growth of pathogens [65]. Newly identified these compounds are considered as the best candidates for control of phytopathogenic fungi. However, one problem relating to the direct application of these preformed compounds in plants affects their low environmental durability, and they are even less bioavailable than conventional fungicides [66-67].

In an investigation, the efficiency of *F. arabica* extract to cause rearrangement of intracellular membranes, as well as other effects may be due to the presence of secondary metabolites (e.g., phenolic, alkaloids, flavonoids and terpenoids) which may affect pathogen development. The entire plants of different species of *Fagonia* were primarily examined for flavonol glycosides and terpenoid glycosides [68]. Analysis of volatile *F. arabica* constituents by GC / MS, revealed that twenty-eight compounds constituted 96.6% of the total volatile constituents of the identified compounds of *F. arabica* L. The major compounds are linalool (24.97%), phytol (8.5%), α-terpineol (4.45%), β-eudesmol (3.45%), α-damascone (3.24%) and δ-cadinene (3.05%). Furthermore, the essential oils obtained from eighteen Egyptian plants, namely, *Callistemon viminals, C. sinensis, C. lemon, C. paradisi, Cupressus macrocarpa, C. sempervirens, Pelargonium graveolens, Myrtuscommunis, Syzygium cumini, Citrus aurantifolia, Origanum vulgare, Rosmarinus officinalis, Schinus molle, S. terebinthifolius Artemisia judaica, A. monosperma,* showed the most positive effects on *F. oxysporum* in terms of antifungal activities except *S. molle* oil. The extracted oils have antimicrobial efficiency [69]. Moreover, the essential oils of The Amazonian Aniba species may have inhibitory effects on the conidial germination of *Aspergillus flavus, Aspergillus niger, Fusarium oxysporum, Fusarium solani, Alternaria alternata, Colletotrichum gloeosporioides, Colletotrichum musae* and *Colletotrichum* guaranicola [70].

The most frequent alternation observed at the ultrastructural level of treated *P. italicum* was related to the variation in the number as well as in the size of vacuoles. Similarly, 10 ml/L of aqueous garlic extract was observed in [45], which found alternations in the cell structures of all treated fungal species, and more marked at 100 ml/L. *Pythium ultimum* var. *ultimum* in addition to the rise in vacuolization, revealed a significant alteration in cytoplasmic membrane, which was partially disconnected from cell wall. [71]have observed ultrastructural alterations of the cytoplasmic membrane on some dematiaceous fungi following treatment with ajoene.

At the molecular level, the tested fungi described in this study can mainly be divided into 3 classes: Dothideomycetes, Sordariomycetes, Eurotiomycetes. The class Dothideomycetes contained 2 species which were closely related to *Alternaria alternata* KU324792 and *Macrophomina phaseolina* FJ395243. The class Sordariomycetes contained one species which was closely related to *Fusarium oxysporum* MK929296. The class Eurotiomycetes contained one species which was closely related to *Penicillium italicum* JX901397 [72-73] .

Conclusion

Since the studied plants were wild and accessible, they could be used as an economically and environmentally friendly source to controlled pathogen. Such extracts obtained from different plants have the ability to monitor such pathogens with ease of extraction methods. Natural plant extracts can be one of the attractive alternatives to combat fungi that attack agricultural crops, while preventing the application of chemical fungicides. This study revealed the potential for the antifungal action of *Fagonia arabica* crude extracts as alternatives to synthetic chemicals to combat various pathogens: *P. italicum, F. oxysporum, A. alternata* and *M. phaseolina*. We proposed using wild plants as a green technology to recover phytopathogenic fungi, as they were considered safe for the environment and for human health. Further work on the isolation and detection of bioactive components of the plant extracts using chromatography or other spectral measurement techniques should perform to monitor plant extracts to control these pathogens.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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