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Antifungal Activity of *Citrullus colocynthis* against *Fusarium oxysporum, Alternaria alternata, Macrophomina phaseolina* and *Colletotrichum musae*

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Four important plant pathogenic fungi were subjected to evaluate the antifungal potentiality of *Citrullus colocynthis*. It was recorded that the growth of *Fusarium oxysporum, Alternaria alternata, Macrophomina phaseolina* and *Colletotrichum musae* were significantly (Pd" 0.01) reduced by extract of *C. colocynthis* obtained by various solvents (water, acetone, ethanol, methanol and chloroform). Ethanolic extract gave the most promising results against all tested fungi. *M. phaseolina* was observed to be highly sensitive to the ethanol extract and zone of inhibition was 26.5 mm. Ethanolic extract rendered highest the percent growth reduction of *M. phaseolina* (41.67%). The growth *F. oxysporum, C. musae* and *A. alternata* were reduced by 37.2%, 35.67% and 28.935% respectively by the ethanolic extract of *C. colocynthis*. GC-MS analysis of crude extract showed that the major components of the crude extract were l-(+)-Ascorbic acid 2,6-dihexadecanoate (94.2%), Eicosanoic acid (92.3%) and 2-Heptadecenal (91.8%).

Key words: Antifungal activity, *Citrullus colocynthis*, Plant pathogenic fungi, GC-MS.

Plant pathogenic fungi are responsible for pre and post-harvest diseases and are regarded as the major cause of yield losses in numerous economically important crops[1]. Species of *Fusarium, Colletotrichum, Macrophomina* and *Alternaria* cause various plant diseases such as blight, rots, dieback, anthracnose and wilt. *Fusarium oxysporum,* found in its many pathogenic forms, is the most damaging species of the genus. *F. oxysporum* cause severe loses in a wide variety of crop plants[2]. The genus *Alternaria* is widely distributed in nature and its species are among the most common fungi on the phyllosphere[3]. It includes both plant-pathogenic and plant-saprophytic species that may damage crops in the field or cause post-harvest decay[4]. *Colletotrichum* species are fungal pathogens that devastate crop plants worldwide. The genus *Colletotrichum* comprises ~600 species attacking over 3,200 species of monocot and dicot plants[5]. Although many cultivated fruit crops are affected by infection of *Colletotrichum* species, the most significant economic losses are incurred when the fruiting stage is attacked[6]. *Macrophomina phaseolina*, a soil inhabiting fungus is an important root pathogen and causes dry root rot, stem canker, stalk rot or charcoal rot of over 400 plant species[7].

Despite good plant protection practices, synthetic fungicides are regarded as main tool in controlling the fungi and in maximize yields[8].

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Concerns have been raised about both the environmental impact and the potential health risks related to the use of these compounds. Hence, there is a great demand for novel antifungal agent having properties like a wide range of structural classes, selectively acting on new targets and fewer side effects[9]. Awareness of organic farming and its products has encouraged plant pathologist to assess the use of natural compounds as an alternative of fungicides.

Plants produce a wide variety of bioactive metabolites which are the part of plant defensive mechanisms against pathogens. Some secondary metabolites give plants their odors (terpenoides), some are responsible for plant pigments (quinines and tannins) and others (e.g., some of terpenoids) are responsible for plant flavor. These antimicrobial bioactive compounds are divided by Cowan[10] into 5 main classes consisting: terpenoids and essential oils; phenolics and polyphenols; alkaloids; polypeptides and mixtures (crude extract). The additive or synergetic effect of the mixtures, the increase in the antimicrobial spectrum of the extract and the decreased risk for pathogen resistance to mixture are the advantages of using crude extract[11].

Citrullus colocynthis is an important medicinal plant belonging to the family of Cucurbitaceae. *C. colocynthis* is a small scarbid perennial creeping herb with prostate or climbing stem, bearing smooth spherical fruits which are mottled green when young and somewhat yellow when ripe[12]. The plant is well known for its medicinal uses[13]. However, use of *C. colocynthis* metabolites for the management of plant pathogenic fungi has not been investigated extensively, thus in the present study four important plant pathogenic fungi were subjected to evaluate the antifungal activity of the crude extract of *C. colocynthis*. Gas chromatography-mass spectrometry (GC-MS) was also performed to find out the chemical composition of biologically active compounds in the crude extract.

MATERIALS AND METHODS

Collection and Storage of Plant Samples

Plants of *C. colocynthis* were collected locally from the desert areas of Riyadh, Saudi Arabia. The freshly collected whole plants of *C. colocynthis*, were stored at 4°C at the Department of Botany and Microbiology, College of Science, King Saud University.

Preparation of Leaf Extract

Fresh leaf parts of the plant material were washed under running tap water to remove soil particles and other dirt. Samples were washed twice with distilled water and were air dried for about 1 week, and then were homogenized to fine powder. Samples were stored in airtight bottle. The air dried and powdered leaf material (20g) was extracted with 200 ml acetone, methanol, ethanol, chloroform and distilled water using Erlenmeyer flask in a shaking incubator at 28°C for 2 days. The prepared extract was filtered through whatman filter paper (no. 41) and evaporated until dryness. The extract was stored (maximum for two days) at 4°C until antifungal assay was carried out.

Test Organisms

Pure cultures of fungal strains, *Fusarium oxysporum, Alternaria alternata, Macrophomina phaseolina* and *Colletotrichum musae* have been used in the present study. Stock cultures of test fungi were maintained on potato dextrose agar slants (PDA, Scharlau Chemie, Spain) and were stored at 4 °C.

Antifungal Assay by Well Diffusion

Antifungal assay of water, acetone, ethanol, chloroform and methanol extracts of leaves were evaluated against pathogenic fungi by measuring the diameter of the inhibition zone formed around the well. Test fungal suspension prepared in sterilized distilled water *(*1.0×10^6 spores/ml) was spread on PDA with the help of sterilized cotton swab. The extracts (50µl/well) were placed in wells made on the pathogen inoculated agar plates. Respective solvents were used as control. Plates were incubated for 3 days at 25°C, and inhibition zones of mycelial growth around the wells were measured. Each extract was analyzed in triplicate

Antifungal Assay by Agar Dilution

To determine the percent growth reduction of plant pathogenic fungi by crude extracts of tested plant, agar dilution method was employed. Crude extract (4 ml) was placed in sterilized petri dish which was immediately followed by pouring 16 ml of PDA, so as to make the final concentration of crude extract to 20%. After the agar solidified, mycelial discs of the tested fungi (5 mm) obtained from actively growing colonies were

placed in the centre of the agar plates. The Petri dishes were incubated at 25°C for 4 days and after that the percent inhibition in the radial colony growth was calculated. The diameter of mycelial colony developed on the crude extract containing PDA plates was compared with the diameter of colony obtained on control plates (devoid of the crude extract). The inhibition of fungal growth was calculated by the following formula:

$$I = (C-T/C) \times 100$$

Where, I = inhibition (%), C = colony diameter in control plate and T = colony diameter in treated plate.

GC-MS Analysis of Crude Extract

The crude extract which showed strong positive antifungal activity was analyzed for its chemical composition. The analysis was done by using Perkin Elmer (Clarus 500, USA) gas chromatography coupled with (Clarus 500, USA) mass spectrometer (MS) equipped with RTx-5 column (30x0.32nm). The oven temperature was initially held at 75°C for 2 min, then increased to 75 to 175°C at a rate of 50°C per min and finally held at 175°C for 7 min. Helium (3 ml/min) was used as a carrier gas. Neither internal, nor external chemical standards were used in this chromatographic analysis. Interpretation of the resultant mass spectra were made using a computerized library-searching program (NIST database) and by studying the fragmentation pattern of such compound resulted from mass spectrometry analysis. Concentration of compound was calculated by the following formula:

Compound concentration percentage= [P1/P2] x 100

Where, P1 is the peak area of the compound and P2 is whole peak areas in the fractionated extracts.

Statistical Analysis

Data were analyzed by least significant difference (L.S.D.) test at probability of 0.01 to identify significant effect of a treatment. Duncan Multiple Range Test was used to evaluate the significant differences between treatments (P d" 0.01). Analysis of variance (ANOVA) analysis was done with the SPSS statistics software.

RESULTS

Antifungal activity of water, acetone, ethanol, methanol and chloroform extract of *C. colocynthis* leaves was evaluated against four important plant pathogenic fungi viz.; *F. oxysporum, A. alternata, M. phaseolina* and *C. musae.* The results of well diffusion assay showed that all crude extracts of *C. colocynthis* had inhibitory effects (10 to 26.5 mm) on the tested plant pathogenic fungi as represented in Table 1. *F. oxysporum* was inhibited maximum by ethanol extract (20.5 mm) whereas methanol, acetone and water moderately inhibited the growth of this pathogen and were non- significantly (Pd" 0.01) different in their effect. A very weak zone of inhibition was observed by the chloroform extract (10 mm) against *F. oxysporum*. In case of *C. musae* the ethanol extract showed highest zone of

Table 1. Antifungal activity of crude extracts of *C. colocynthis* against *F. oxysporum, A. alternata, M. phaseolina* and *C. musae* evaluated by well diffusion method

Extracts	Plant pathogenic fungi			
	F. oxysporum	*C. musae*	*A. alternata*	*M. phaseolina*
	Diameter of zone of inhibition (mm)			
Water	14.5 ±0.58^{b}	10 ±0.00^{a}	13.5 ±1.73^{bc}	11.0 ±1.15^{ab}
Acetone	14.5 ±1.00^{b}	14.5 ±0.06^{c}	13.0 ±1.73^{ab}	12.0 ±0.00^{b}
Methanol	15.0 ±1.00^{b}	12.0 ±1.00^{b}	12.5 ±0.58^{ab}	17.0 ±1.00^{c}
Ethanol	20.5 ±0.58^{c}	23.5 ±1.00^{d}	16.0 ±1.15^{c}	26.0 ±1.00^{d}
Chloroform	10 ±1.73^{a}	10 ±0.00^{a}	11 ±1.15^{a}	10.00±1.15^{a}

Zone: mean ±SD for N = 3.
Data followed by different letters in the column are significantly different (P ≤0.01) according to Duncan's multiple range test.

inhibition (23.5 mm) whereas acetone, methanol showed moderate zone of inhibition (14.5 mm and 12.0 mm respectively). Water and chloroform extracts weakly inhibited the fungus *C. musae* (10 mm) and the results were not different significantly at Pd" 0.01. *M. phaseolina* was observed to be highly sensitive to the ethanol extract and zone of inhibition was 26.5 mm. Methanol extract moderately inhibited (17.0 mm) the growth of *M. phaseolina* whereas water, acetone and chloroform weakly inhibited this fungus (12.0, 11.0 and 10.0 mm respectively). *A. alternata* was noticed to be least affected by extracts of *C. colocynthis,* maximal zone of inhibition was induced by ethanol extract (16.0 mm). Other extracts i.e. water, acetone, methanol and chloroform showed 13.5, 13.0, 12.5 and 11.0 mm zone of inhibition respectively.

Table 2 depicts the percent growth reduction of *F. oxysporum, A. alternata, M. phaseolina* and *C. musae* by the extracts of *C. colocynthis*. The results of percent growth reduction are commensurable with the results of Table 1. Percent growth reduction was highest of *M. phaseolina* (41.67%) by the ethanol extract. Whereas, methanol extract reduced the growth of *M. phaseolina* by 34.33%. Maximum reduction in the growth of *F. oxysporum* was induced by ethanol extract (37.20%). Similarly radial growth of *C. musae* was highly reduced by the ethanol extract (35.67%). Maximum reduction in the radial growth of *A. alternata* was achieved by ethanol extract and methanol extract was found be at par with it. Rest of the extract were also able to reduce the growth of pathogenic fungi by varying degree. However, from the present results it can be figured out that ethanol extract of *C. colocynthis* was most effective in reducing the growth of tested plant pathogenic fungi. Thus, the chemical composition of the ethanolic crude extract of *C. colocynthis* was assayed by employing gas chromatography coupled with mass spectrometer (GC-MS). Table 3 represents the data of major chemicals detected in

Table 2. Effect of crude extracts of *C. colocynthis* on percent growth reduction of *F. oxysporum, A. alternata, M. phaseolina* and *C. musae* evaluated by agar dilution method.

Extracts	Plant pathogenic fungi			
	F. oxysporum	*C. musae*	*A. alternata*	*M. phaseolina*
	Reduction in the radial growth (%)			
Water	25.56 ±1.11[b]	20.74 ±0.64[a]	20.67 ±0.58[a]	21.07 ±0.06[b]
Acetone	26.29 ±0.64[b]	26.30 ±0.64[b]	23.96 ±0.83[b]	23.33 ±0.00[c]
Methanol	34.22 ±.70[c]	29.13 ±0.51[b]	27.67 ±0.58[c]	34.33 ±0.58[d]
Ethanol	37.20 ±0.72[d]	35.67 ±1.15[c]	28.93 ±0.06[c]	41.67 ±0.58[e]
Chloroform	10 ±0.61[a]	10 ±0.64[d]	10.74 ±0.64[d]	19.63 ±0.64[a]

Zone: mean ±SD for N = 3.
Data followed by different letters in the column are significantly different (P≤0.01) according to Duncan's multiple range test.

Table 3. Major chemical components detected in the crude extract of *C. colocynthis* analysed by gas chromatography-mass spectrometry (GC-MS)

Compound name	Mw[a]	Formula	%(Rev) [b]
l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	$C_{38}H_{68}O_8$	94.2
Eicosanoic acid	312	$C_{20}H_{40}O_2$	92.3
2-Heptadecenal	252	$C_{17}H_{32}O$	91.8
2-(1-4-Cyano- 1,2,3,4etrahydronaphthyl)propanenitrile	210	$C_{14}H_{14}N_2$	91.7
N-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	90.9

[a]Molecular weight
[b](Rev), % of the compound concentration in the total extract.

the crude extract. The main components in the crude extract were l-(+)-Ascorbic acid 2,6-dihexadecanoate (94.2%), Eicosanoic acid (92.3%) and 2-Heptadecenal (91.8%).

DISCUSSION

The results of present investigation revealed that the growth of *F. oxysporum, A. alternata, M. phaseolina* and *C. musae* were significantly reduced by extracts of *C. colocynthis.* Ethanolic extract was found to be the most promising extract against all tested fungi. Plant-derived compounds are regarded as a substantial source for novel lead structures to develop medicines and biocides natural products**.** *C. colocynthis* has been studied extensively for pharmacological activities[13]. However, the utilization of *C. colocynthis* in the management of plant pathogenic diseases has not been explored widely. Hadizadeh et al.[14] observed that ethanolic extract of *C. colocynthis* was most effective in reducing the growth of *A. alternata* and *Rhizoctonia solani*. In another study methanolic plant extract of *C. colocynthis* inhibited 85.67% of *Aspergilus flavus* growth and more than 90% of aflatoxin[15]. Organic solvent extract of *C. colocynthis* was recorded highly effective against *F. oxysporum* f. sp. *lupine*[16]. Ethyl acetate crude extract of *C. colocynthis* was recorded most active against *Fusarium graminearum* and *A. alternata*, with inhibition zone of 13 to 15 mm[17]. Our results are in line with these reports.

Gas chromatography coupled with mass spectrometer (GC-MS) revealed that the main components in the crude extract were l-(+)-Ascorbic acid 2,6-dihexadecanoate, Eicosanoic acid and 2-Heptadecenal. Known compounds found in *C. colocynthis* include glycosides (Estrols classified to groups A, B, K, L, and E curcurbitacins), alkaloids, and flavonoids[18]. It can be noted that l-(+)-Ascorbic acid 2,6-dihexadecanoate is a known hyaluronidase inhibitor[19] and it may be responsible for the inhibiting the growth of tested plant pathogenic fungi.

CONCLUSION

Present study is the preliminary investigation to evaluate the antifungal potentiality of *C. colocynthis* against *F. oxysporum, A. alternata, M. phaseolina* and *C. musae*. The results showed that *C. colocynthis* can be utilized as a source of non-pollutive fungicides for the management of important plant pathogenic fungi. However further investigation can be conducted to purify the bioactive compound through column chromatography, to test the efficacy of the extract *in vivo* and to make it in the form of applied.

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