

Potential of *Zygothellium coccineum* Extracts in Inhibiting the Growth of Plant Pathogenic Fungi

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The antifungal activity of acetone, methanol, ethanol, chloroform, and water leaf extract of *Zygothellium coccineum* was evaluated against *Aspergillus flavus*, *Cladosporium* sp., *Alternaria alternata* and *Fusarium solani*. The extracts exhibit numerous degree of inhibition pattern on tested fungal species. Well diffusion assay showed that in general methanol extract was highly effective against all the tested pathogens whereas, acetone and ethanol inhibited the tested pathogens moderately. While, the inhibitory effect of water extract was very weak on the tested fungi. However, chloroform extract failed to inhibit *F. solani* and *A. flavus*. The pathogen *Cladosporium* sp. was observed to be most sensitive fungus and maximum growth was inhibited by methanol extract (36.0 mm). Percent growth reduction of plant pathogenic fungi evaluated by agar dilution showed that Methanol extract reduced maximum the mycelial growth of *Cladosporium* sp. (55%) followed by *F. solani* (53%), *A. alternata* (51%) and *A. flavus* (43.5%) respectively. The major chemicals detected in the methanolic extract were 1-Hexyl-2-nitrocyclohexane (89.8%), 2-Octadecyl-propane-1,3-diol (88.9%), Octadecanal (88.6%) beside these several known terpenoids, saponins, phenols and glutaraldehyde (75.2%) were also present in the extract. Thus it can be concluded that *Z. coccineum* has the potential to be utilized as an antifungal agent.

Key words: Antifungal activity, *Zygothellium coccineum*, Plant pathogenic fungi, GC-MS.

Fungi causing diseases to plants are one of the components responsible for the losses in agricultural production. Species of *Fusarium*, *Aspergillus*, *Cladosporium* and *Alternaria* cause various plant diseases such as blight, fruit rot, root crown rot, dieback and wilt. *Fusarium* spp. cause most of the damage during food production. *F. solani* is widely found in soil and constitutes one of the most important phytopathogens in agriculture. The genus *Alternaria* is distributed all over in nature and its species are among the

most common fungi on the phyllosphere¹. It includes both plant-pathogenic and plant-saprophytic species that may damage crops in the field or cause post-harvest decay². *Aspergillus flavus* also produce highly toxic aflatoxins that contaminate cotton seed, corn, peanuts and tree nuts during harvesting or storage³.

Despite good plant protection practices, synthetic fungicides are regarded as main tool in controlling the fungi and in maximize yields⁴. However, use of these fungicides has negative effects on human health as well as on environment. The shortcomings of synthetic fungicides has changed the approach of the agriculturist towards the use these synthetic fungicides. Now day's farmers as well as consumers prefer the agriculture products which are free of pesticides or organic. Hence, there is a great demand for novel antifungal

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agent having properties like a wide range of structural classes, selectively acting on new targets and fewer side effects⁵. The use of plant extracts against fungal pathogens may also reduce the chances of resistance development in pathogens due to the presence of new and different antifungal compounds.

The *Zygophyllum* is the leading genus in the family Zygophyllaceae. The plants of this genus are commonly found in Middle East, South Africa, Australia, South and central Asia. In Saudi Arabia, this group of plants is distributed in North Hijaz, South Hijaz, Northern region, Western Najd, and Eastern region. *Zygophyllum coccineum* is a desert shrub up to 75 cm high, green glabrous, leaves compound, with a pair of bright green, glabrous, cylindrical fleshy leaflets at least 10 mm long terminating a slightly longer, fleshy petiole⁶. *Z. coccineum* is known for its medicinal values, fruits of this plant are used in the treatment of rheumatism, gout, asthma, hypertension, as a diuretic and an antidiabetic⁷. Among the Arabs, the seeds are reputed as anthelmintic⁸. *Z. coccineum* was reported to produce zygophyllin, quinovic acid, saponins, tannins, resins, and wax⁹. Despite the fact that this plant has medicinal importance, the *Z. coccineum* has not been explored for its potential as an antifungal agent against plant pathogenic fungi¹⁰.

MATERIALS AND METHODS

Collection and Storage of Plant Samples

Plants were collected from the desert of Riyadh, Saudi Arabia. Collected fresh plant material was examined and the old, insect- and fungus-infected leaves were removed. Leaves were washed with tap water and were surface sterilized by dipping them in 0.1% sodium hypochlorite solution for one minute. After that leaves were washed with distilled water. Leaves were dried at room temperature (25 °C) for about a week on a laboratory desk. Leaves were covered with clean sheets of paper to avoid any deposition of dust. The dried material was ground to a fine powder using a grinding mill and stored in airtight bottles in the dark until extraction was done.

Preparation of Leaf Extract

Separate aliquots of finely ground plant material (10 g) were extracted with 100 ml of

solvents; water, chloroform, acetone, ethanol and methanol (technical grade-Merck) in 250ml conical flasks, while shaking vigorously for 3–5 min on a shaking machine (Labtec model LSB-015S) at high speed. After centrifuging at 3500 rpm for 5 min, the supernatants were decanted into labeled, weighed glass vials. The process was repeated three times and the extracts were combined. The solvent was removed under a stream of cold air at room temperature.

Fungal Strains and Inoculums Quantification

The four test fungal species, *A. flavus*, *Cladosporium* sp. *A. alternata* and *F. solani* were obtained from the Department of Botany and Microbiology, King Saud University, Saudi Arabia. All the fungi used in the present study are important plant pathogenic fungi with economical importance. Fungal cultures were maintained on Potato Dextrose agar (PDA). All these fungi were subculture on PDA at 26 °C for at least two to four days before being used in the screening assays.

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 the negative 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

T = colony diameter in treated plate.

GC-MS Analysis of Crude Extract

The crude extract which showed strong 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 (30x0.32mm). 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:

$$\text{Compound concentration percentage} = [P1/P2] \times 100$$

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 < 0.01). Analysis of variance (ANOVA) analysis was done with the SPSS statistics software.

RESULTS AND DISCUSSION

Water, acetone, ethanol, methanol and chloroform extract of *Z. coccineum* leaves were evaluated against four plant pathogenic fungi; *A. flavus*, *Cladosporium* sp., *A. alternata* and *F. solani*. The results of well diffusion assay presented in Table 1 show that extracts exhibit numerous degree of inhibition pattern on tested fungal species. In general it was observed that methanol extract gave the most promising results against the tested pathogenic fungi. Whereas, chloroform extract failed to inhibit *F. solani* and *A. flavus*. Among all the tested pathogens, *Cladosporium* sp. was the most sensitive fungus and maximum growth was inhibited by methanol extract (36.0 mm). The extracts prepared with acetone, ethanol and chloroform inhibited the above mentioned pathogen moderately. Whereas, the inhibitory effect of water extract was very weak on the same pathogen. *F. solani* was inhibited maximum by methanol extract followed by acetone, water and ethanol extracts respectively. Whereas, methanol and ethanol extracts showed a very good

Table 1. Antifungal activity of crude extracts of *Z. coccineum* against *A. flavus*, *Cladosporium* sp., *A. alternata* and *F. solani* evaluated by well diffusion method.

Extracts	Plant pathogenic fungi			
	<i>A. alternata</i>	<i>F. solani</i>	<i>Cladosporium</i> sp.	<i>A. flavus</i>
	Diameter of zone of inhibition (mm)			
Water	10.0±0 ^a	24.5±0.58 ^a	10.3±0.58 ^a	10.3±0.58 ^a
Acetone	28.0±0 ^c	27.0±1.15 ^c	28.0±0 ^c	25.0±0 ^b
Methanol	26.0±1.7 ^c	30.0±0 ^d	36.0±1.7 ^d	28.7±0.58 ^c
Ethanol	25.3±0.58 ^b	21.5±0.58 ^b	25.3±0.58 ^b	24.0±0 ^b
Chloroform	25.3±0.58 ^b	0.0±0 ^{N.S}	25.3±0.58 ^b	0.0±0 ^{N.S}

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.

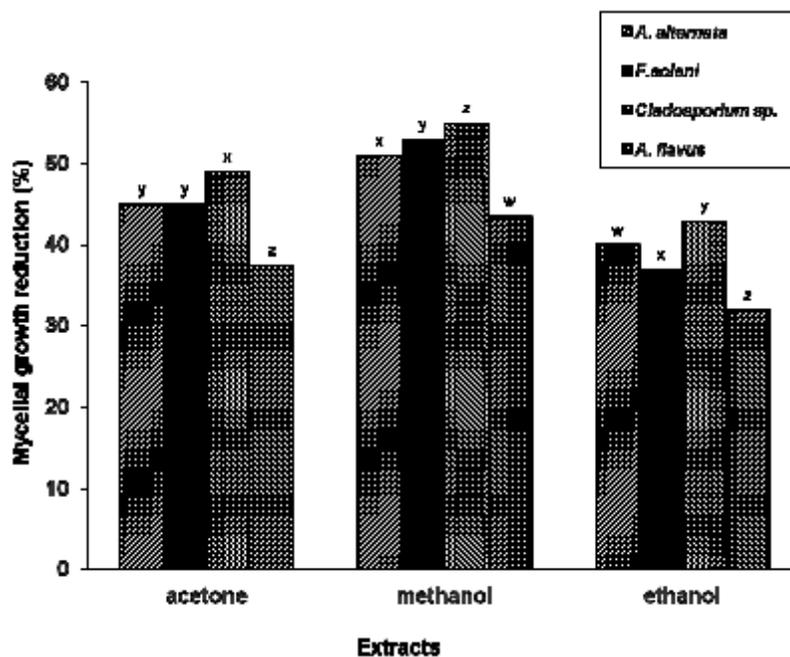
N.S. non significant

inhibitory activity against *A. alternata* and the effect of both extract on this pathogen was significantly not different. Ethanol and chloroform were significantly same in inhibiting moderately the pathogen *A. alternata* (25.3 mm). Similarly, *A. flavus* was inhibited maximum by methanol extract, while acetone and ethanol were significantly same in inhibiting moderately the pathogen. Water extract showed weak zone of inhibition against *A. flavus*.

The results of well diffusion assay clearly showed that the methanol, acetone and ethanol extracts of *Z. coccineum* had the potential to inhibit the growth of tested pathogenic fungi. Therefore these extract were further tested to find out their effect on the mycelial growth of pathogenic fungi. Figure 1 depicts the percent growth reduction of *A. flavus*, *Cladosporium* sp., *A. alternata* and *F. solani* caused by the methanol, acetone and ethanol extracts. As expected all these extract were able to reduce the growth of tested fungi significantly. Methanol extract gave very promising results against all tested pathogenic fungi; next to it was acetone and then ethanol. It

has been observed that methanol extract reduced the growth of most of the tested fungi more than fifty percent. Methanol extract reduced maximum the mycelial growth of *Cladosporium* sp. (55%) followed by *F. solani* (53%), *A. alternata* (51%) and *A. flavus* (43.5%) respectively. Similarly, acetone extract reduced highest the growth of *Cladosporium* sp. (49%) followed by *F. solani* (45.3%), *A. alternata* (45%), and *A. flavus* (37.5%) respectively. Whereas, the ethanol extract reduced maximum the growth of *Cladosporium* sp. (42.7%), followed by *A. alternata* (40%), *F. solani* (37%) and *A. flavus* (32%) respectively.

Z. coccineum has been studied extensively for pharmacological activities¹¹⁻¹³. However, it has not been explored for its antifungal potential. In a study it was reported that bioactive secondary metabolites from *Z. coccineum* inhibited *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *Botrytis cinerea*, *Phomopsis obscurans*, *P. viticola* and *F. oxysporum*¹⁰. Crude extract contains mixture of several compounds/secondary metabolites, the additive or synergetic



Each value is an average of three replicates. Bar's showing different letters are significantly different ($P \leq 0.05$) according to Duncan's multiple range tests

Fig. 1. Effect of methanol, ethanol, and acetone crude extracts of *Z. coccineum* on percent growth reduction of *A. flavus*, *Cladosporium* sp., *A. alternata* and *F. solani* evaluated by agar dilution method

effect of the mixtures increases the antimicrobial spectrum of the extract¹⁴.

The data of the present results showed that methanol extract of *Z. coccineum* leaves gave the most promising results against *A. flavus*, *Cladosporium* sp., *A. alternata* and *F. solani*. Thus, the chemical composition of the methanolic extract of *Z. coccineum* was assayed by employing gas chromatography coupled with mass

spectrometer (GC-MS). The major chemicals detected in the crude extract were 1-Hexyl-2-nitrocyclohexane (89.8%), 2-Octadecyl-propane-1,3-diol (88.9%), Octadecanal (88.6%) beside these several known terpenoids, saponins, phenols and glutaraldehyde (75.2%) were also detected (Table 2). *Z. coccineum*, is a well known saponin-rich plant. The saponins, terpenoids and other secondary metabolites and compounds of this

Table 2. Major chemical components detected in the crude extract of *Z. coccineum* analyzed by gas chromatography-mass spectrometry (GC-MS)

Compound name	Mw ^a	Formula	% (Rev) ^b
1-Hexyl-2-nitrocyclohexane	213	C ₁₂ H ₂₃ O ₂ N	89.8
2-Octadecyl-propane-1,3-diol	328	C ₂₁ H ₄₄ O ₂	88.9
Octadecanal	268	C ₁₈ H ₃₆ O	88.6
Cyclohexane,1-(1,5-dimethylhexyl)-4-(4-methylpentyl)	280	C ₂₀ H ₄₀	87.9
Tetradecanal	212	C ₁₄ H ₂₈ O	87.6
Oxirane,heptadecyl-	282	C ₁₉ H ₃₈ O	86.9
2(3h)-Furanone,3-(15-hexadecynylidene)	334	C ₂₁ H ₃₄ O ₃	85.0

^aMolecular weight

^b(Rev), % of the compound concentration in the total extract.

genus have been reported earlier¹⁵⁻²¹. The presence of many compounds such as glutaraldehyde, polyphenols, alkaloids; terpenoids and essential oils in the methanolic extract of *Z. coccineum* may be responsible for antifungal activity²². These compound may have worked synergistically or additively to reduce the growth of pathogenic fungi. Moreover, the mixture of these compounds may have broadened its potential to render the growth of more than one pathogen.

From the present study it can be concluded that methanolic extract of *Z. coccineum* has the potential to be utilized as an antifungal agent. The use of plant extracts in protecting crops against fungal pathogens may inhibit the development of resistance in the pathogen population due to the different antifungal compounds it contains. Further, the use of crude plant extract to protect crops against fungal attack may also be acceptable in the organic production of crops.

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