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Table: 1, Figs : 4

Fungicidal potential of leaf extracts of *Datura metel* L. to control *Sclerotium rolfii* Sacc.

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ABSTRACT

Sclerotium rolfii Sacc. causes collar rot disease in bell pepper (*Capsicum annuum* L.) that results in significant yield losses. Since use of fungicides causes environmental pollution and health hazards, therefore, an alternative environment friendly strategy was used to control this pathogen. Methanolic leaf extract of weed *Datura metel* L. (family Solanaceae), was evaluated for its efficacy in controlling *in-vitro* growth of *S. rolfii*. Extract concentrations ranging from 0.5 % to 4.0 %, significantly controlled the fungal growth by 29-88 % over control. Based on the polarity, various constituents of methanolic leaf extract were partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. Bioassays with 3.125 to 200 mg mL^{-1} concentrations of these fractions revealed that chloroform fraction was the most antifungal followed by ethyl acetate fraction that reduced the biomass of *S. rolfsii* by 36-47 % and 5-43 %, respectively. The best antifungal chloroform fraction was subjected to GC-MS analysis to identify its antifungal constituents. A total of 19 compounds were identified in this fraction. The most abundant compound were 1-hexacosanol (12.87 %), 1-octadecene (10.69 %), 2-methyl-3-phenyl-2-propenal (8.72 %), 1-eicosanol (6.80 %). 1-heptadecene (6.66 %), 1-octadecanol (6.62 %), 1,3(15),10-bisabolatriene (6.41 %) and 1,6,10-farnesatrien-3-ol (6.38 %). This study concluded that chloroform soluble fraction of methanolic leaf extract contained potent antifungal compounds to control *S. rolfsii*.

Keywords: Antifungal, bell pepper, bioassay, *Capsicum annuum*, collar rot, *Datura metel*, leaf extract, methanolic extract, natural fungicides, *Sclerotium rolfsii*.

INTRODUCTION

Bell pepper (*Capsicum annuum* L.) is an important vegetable native to Central America, northern South America and Mexico (25). Now, it is grown in Central Asia, Southeast Asia and Europe. It is rich in minerals, proteins, and vitamins B6, C, E and K1 (23). Its production is severely affected by highly destructive soil-borne fungus *Sclerotium rolfsii*, causing collar rot (28). The pathogen causes significant economic losses in over 500 host plant species in 100 families (10). High temperature (25-30 °C), low organic matter and soil moisture are conducive factors for disease development. The pathogen produces white hyphae, which later turns to dark-brown/black spherical sclerotia around the infected plants stem. These sclerotial bodies can remain active for many years even under adverse environmental conditions (6).

Control of collar rot pathogen is major challenge due to its wide adaptability in diverse agro-ecological zones. Many cultural, physical and biological management strategies are used for its control but each has some limitations (26). In modern agriculture system, farmers rely on chemical fungicides but their indiscriminate usage has created numerous problems such as fungal resistance to fungicides, reappearance of target and non-target microflora, damage of beneficial microorganisms, and chemical residues in

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food, feed and fodder (3). Increased awareness about these harmful effects has led to the development of alternate environment friendly disease control strategies (1,29). Natural products are important to control soil-and seed-borne fungal pathogens (*S. rolfsii*, *Macrophomina phaseolina* and *Ascochyta rabiei*) (5,17-19).

Datura metel L. (Figure 1) (family Solanaceae), is a medicinal plant rich in antifungal, antibacterial, insecticidal, antioxidant, herbicidal, anticancer, anti-inflammatory and anti-rheumatoid activities (13,32). It is used to treat skin rashes, allergies, ulcers, bronchitis, diabetes, eczema, painful tumors and glandular inflammation such as mumps (21). It is also rich in metabolites (alkaloids and various active phytochemicals), which are effective to manage pathogenic organisms (2). However, studies regarding antifungal activity of *D. metel* against plant pathogenic fungi especially *S. rolfsii* are rare. Recently, Jabeen *et al.* (16) reported that soil amendment with 2.5 % *D. metel* biomass can completely control the collar rot disease in chili. Therefore, the present study was done to assess fungicidal potential of leaf extract of *D. metel* to control *in-vitro* growth of *S. rolfsii*, and to identify possible antifungal constituents through GC-MS analysis.

Figure 1. *Datura metel* plant bearing fruits

MATERIALS AND METHODS

Isolation of pathogen

S. rolfsii infected bell pepper plant samples were collected and cut into small pieces (5 mm size) and were surface sterilized with 3 % NaOCl solution for one minute followed by three consecutive washings with distilled water. After that, the diseased samples were placed on freshly prepared malt extract agar containing Petri plates (9-cm dia) in an incubator at 28 °C. After 7 days, the plates became full of mycelium and small tan colored sclerotia appeared on it. The mature fungal culture was confirmed from Fungal Culture Bank of Pakistan and after identification it was sub-cultured on malt extract agar (MEA) plates and stored at 4 °C to be used for further experimental studies.

Bioassays with methanolic leaf extract

Leaves were collected from a mature *D. metel* plant at fruiting stage during June 2017 from Lahore, Pakistan. Lahore is located at an altitude of 217 m, 31°15 - 31°45′ N

and 74°01′- 74°39′ E, with annual rainfall of 838.8 mm. Maximum and minimum temperatures during June are 40 °C and 28 °C , respectively. The leaves were washed in tap water and completely dried in shade. Five kg of crushed leaves were dipped in 10 L methanol for 2 weeks and filtered. The resultant leachate was evaporated under reduced pressure on a rotary evaporator at 45 °C to yield crude methanolic extract of *D. metel* leaves.

To prepare stock solution, 14.4 g crude methanolic extract was mixed in 5 mL of dimethyl sulphoxide (DMSO) and raised the volume to 18 mL by adding sterilized distilled water. Likewise, control solution contained 5 mL of DMSO and 13 mL of distilled water. Malt extract broth (76 mL) was autoclaved in 250-mL volume flasks. Nine test treatments (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 %) were made by mixing stock and control solutions in appropriate quantities (0.0:4.0, 0.5:3.5, 1.0:3.0, …, 4.0:0.0) to prepare a mixture of 4 mL that was added to 76 mL broth to make the final volume up to 80 mL. It was equally divided and poured into 100-mL flasks to prepare four replicates of each treatment. Seven-day-old pure culture of *S. rolfsii* was used to prepare the fungal plugs (5 mm size) using a sterilized cork borer. Each flask was inoculated and incubated at 28 °C for one week. Fungal mats were separated on filter papers and weighed after drying (15).

Bioassay with different fractions of methanolic leaf extract

D. metel methanolic leaf extract was mixed in 500 mL distilled water and transferred to a separating funnel and successively extracted with *n*-hexane, chloroform, ethyl acetate and *n*-butanol on the basis of increase in polarities. After extraction, all the organic solvents were evaporated under vacuum in a rotary evaporator to get 147 g of *n*-hexane, 318 g of chloroform, 34.78 g of ethyl acetate, 49.44 g of *n*-butanol and 137 g of aqueous sub-fraction.

Antifungal activity of all the organic fractions was investigated *in-vitro* against *S. rolfsii.* Each organic fraction (1.2 g) was dissolved separately in 0.5 mL of DMSO followed by the addition of 5.5 mL autoclaved malt extract broth to prepare a 200 mg mL^{-1} stock solution. It was then serially double diluted by adding the malt extract broth to prepare lower concentrations of 3.125, 6.25, 12.50, 25, 50 and 100 mg mL^{-1}. For control, 0.5 mL of DMSO was mixed in 5.5 mL of malt extract broth and serially double diluted to get corresponding concentrations of DMSO in different concentrations of the organic fractions. Three replicates of each concentration were prepared by adding 1 mL of medium in each 10-mL volume test tube. *S. rolfsii* spore suspension was prepared using autoclaved water and 20 µL of this suspension was added to each test tube. The test tubes were arranged in a completely randomized design in an incubator set at 28 °C and continuous dark. Fungal biomass from each treatment was collected on filter papers after seven days growth, dried and weighed (22).

GC-MS analysis

GC-MS analysis of chloroform fraction was done to identify the antifungal phyto-constituents present in *D. metel* leaves. For this, 2 µL of chloroform fraction was injected into GC 2010 coupled with 30 m × 0.25 µm × 0.25 µm capillary column with 1.33 mL min^{-1} of flow rate. Helium was used as a carrier gas. Sample injection split ratio was 10:1 with 100 kPa pressure. Oven temperature was 100 °C for 3 min that was raised at the rate of 10 °C min^{-1} and hold up to 325 °C. Injector temperature was maintained at 200 °C. Total

GC running time was 25 min. NIST 02 library was attached to the GC-MS instrument for chromatogram interpretation. The compounds name, molecular weight, molecular formula and their structures were determined after comparison with library compounds.

Statistical analysis

All the data were analyzed on Statistics 8.1 software by using ANOVA followed by LSD test at P≤ 0.05.

RESULTS AND DISCUSSION

Antifungal activity of methanolic leaf extract

All the concentrations of methanolic leaf extracts were inhibitory to *S. rolfsii* (Figure 2). However, the inhibitory effects of 2-lowest concentrations (*viz.* 0.5 and 1.0 %) concentrations on the fungal biomass were less only 29 % and 30 %, respectively. Concentrations above 1 % were highly antifungal and reduced the fungal biomass by 81-87 %. Leaf extracts effectively suppressed the growth of *S. rolfsii.* A 4 % extract reduced fungal biomass production up to 87 %. The present findings are in line with the results of Fakai *et al.* (11) who reported that the higher concentrations of *D. metel* significantly inhibited the growth of targeted fungal pathogens (*Rhizopus oryzae*, *Aspergillus flavus*,

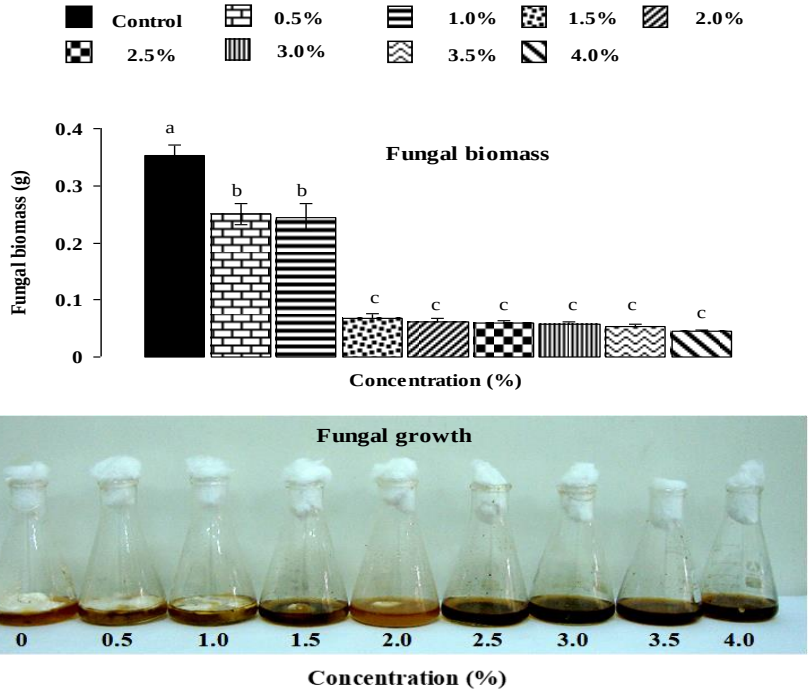

Figure 2. Effects of methanolic leaf extract of *Datura metel* on growth and biomass production of *Sclerotium rolfsii*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤ 0.05) as determined by LSD Test.

A. niger and *A. fumigatus*). *D. metel* is a natural source of phytochemicals and secondary metabolites having potent antifungal activities resulting in the inability of the fungal pathogens to survive and reproduce (8). Karim *at al.* (20) recommended that 1.5 % crude seed extract of *D. metel* arrested the mycelial growth of *Colletotrichum gloeosporioides* by 80 %.

Antifungal activity of fractions of methanolic leaf extract

The effects of different sub-fractions of methanolic leaf extracts on biomass of *S. rolfsii* are shown in Figure 3 and 4. DMSO used to dissolve the fractions was slightly inhibitory to fungal growth but less than the fractions. Different sub-fractions showed variable antifungal effects against *S. rolfsii*.

(i). Chloroform fraction: It showed the highest antifungal activity. Its different concentrations suppressed fungal growth by 36-47 %.

(ii). Ethyl acetate fraction: It was second most inhibitory fraction**,** which reduced fungal biomass by 5-43 %. Earlier, Sharma (30) worked on different polar and non-polar extracts of *D. metel* against pathogenic species of *Aspergilli*. He reported that higher concentrations of chloroform fraction suppressed the growth of *A. niger*, *A. flavus* and *A. fumigatus.*

(iii). *n*-hexane fraction: Its antifungal effect was less (10-29 % suppression in fungal growth) by various applied concentrations.

(iv). *n*-butanol fraction: This highly polar fraction at higher concentrations (12.50 mg mL^{-1} and above) decreased the fungal growth biomass by 17-36 % and 0-36 % over control. However, its lower concentrations (3.125 and 6.25 mg mL^{-1}) stimulated the biomass (11-40 %) of *S. rolfsii* than control.

Compounds identification through GC-MS analysis

GC-MS analysis of chloroform fraction revealed the presence of 19 major and minor phyto-constituents (Table 1).

(i). Higher concentrations : 1-Hexacosanol (12.87 %), 1-octadecene (10.69 %), 2 methyl-3-phenyl-2 propenal (8.72 %), 1-eicosanol (6.80 %), 1-heptadecene (6.66 %), 1-octadecanol (6.62 %), 1,3(15),10-bisabolatriene (6.41 %), and 1,6,10-farnesatrien- 3-ol (6.38 %).

(ii). Moderate concentrations : Naphthalene, 1,2,3,4-tetrahydro-5-nitro-(5.45 %), 1-pentadecanol (4.86 %) and 3-hexadecene, (Z)- (4.86 %)

(iii). Lower concentrations : Octadecanoic acid, methyl ester (3.71 %), 2,6,10-dodecatrien-1-ol, 3,7,11-trimethyl (3.22 %), 7-tetradecene (2.15 %), 1-pentadecene (2.37 %), 1-hexadecene (2.37 %), 1-tetradecanol (2.36 %), hexadacanoic acid, methyl ester (2.04 %) and 1-docosene (0.93 %).

Literature survey showed that some of these identified compounds had antifungal properties against a variety of fungal species and thus might be responsible for control of *S. rolfsii* growth in the present study. Among these, the most abundant compound 1-hexacosanol was previously isolated from hexane fraction of a medicinal plant *Cirsium italicum* and tested against *Penicillium chrysogenum*, *Aspergillus fumigatus*, *Candida krusei* and *C. albicans.* It showed the highest antifungal potential against *Candida* species

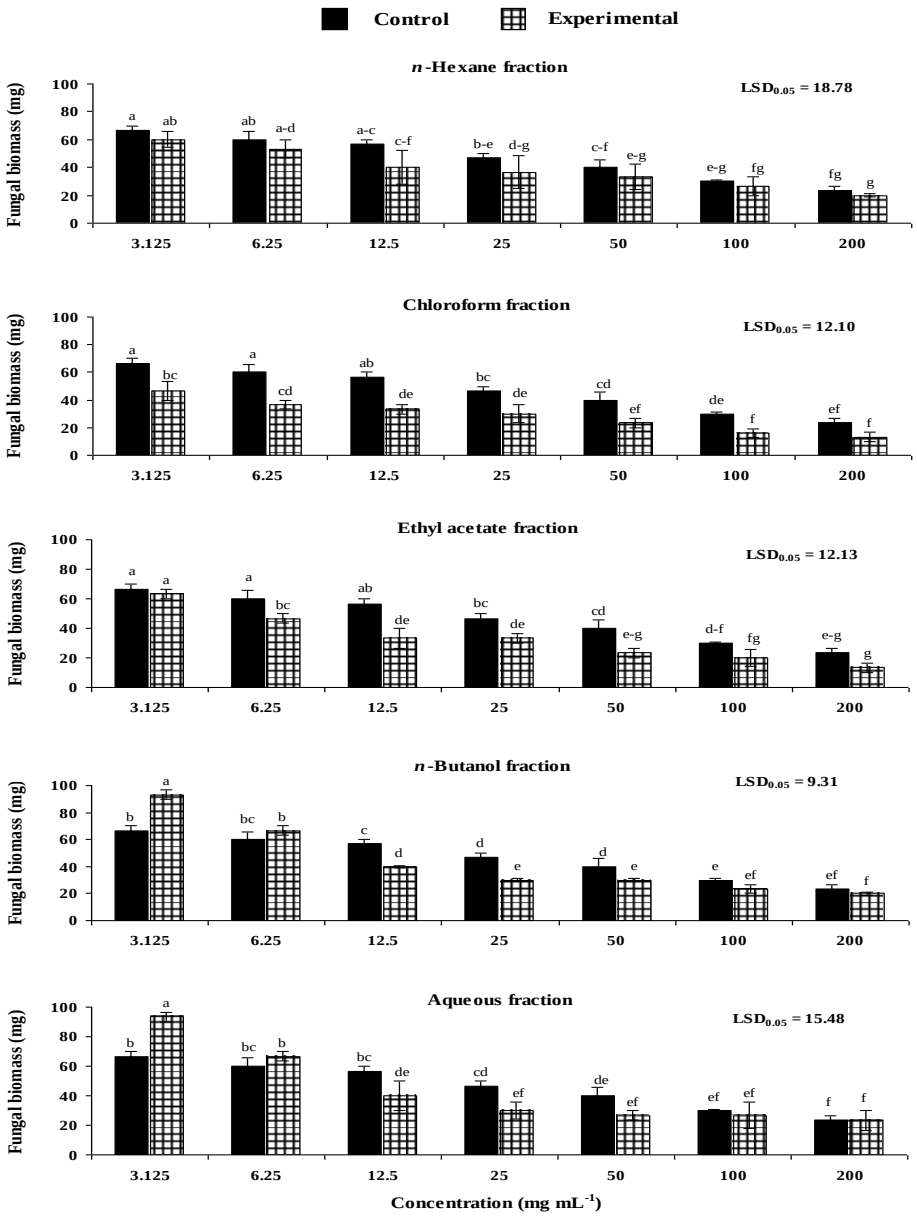

Figure 3. Effects of different fractions of methanolic leaf extract of *Datura metel* on biomass of *Sclerotium rolfsii*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by LSD Test.

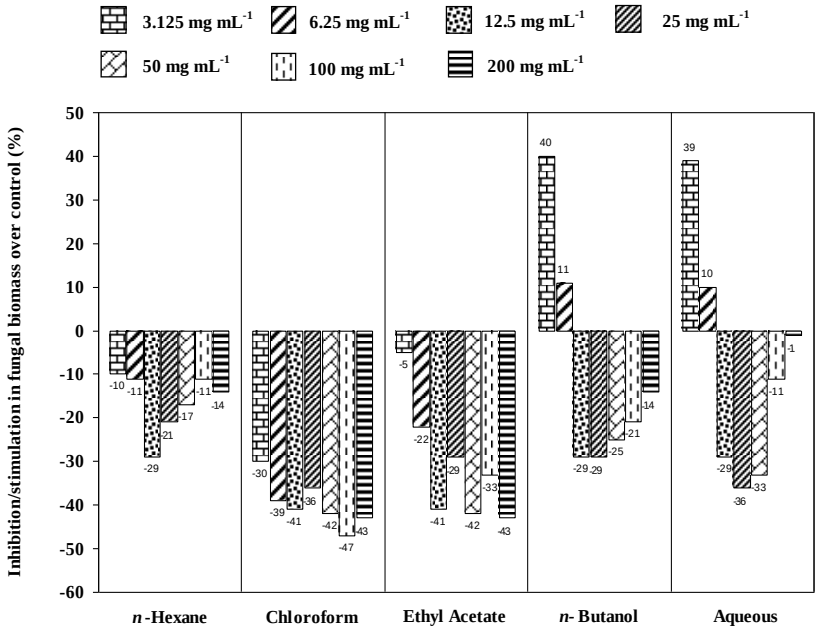

Figure 4. Inhibitory/ Stimulatory effects of fractions of methanolic leaf extracts of *Datura metel* on biomass of *Sclerotium rolfsii*

Table 1. Phytoconstituents identified from chloroform fraction of methanolic leaf extract of *D. metel*

S. No.	Names of compounds	Molecular formula	Molecular weight	Peak area (%)
1	2 Methyl-3-phenyl-2-propenal	$C_{10}H_{10}O$	146	8.72
2	1,6,10-Farnesatrien-3-ol	$C_{15}H_{26}O$	222	6.38
3	1,3(15),10-Bisabolatriene	$C_{15}H_{24}$	204	6.41
4	1-Docosene	$C_{22}H_{44}$	308	0.93
5	1-Octadecanol	$C_{18}H_{38}O$	270	6.62
6	1-Heptadecene	$C_{17}H_{34}$	238	6.66
7	1-Octadecene	$C_{18}H_{36}$	252	10.69
8	1-Hexadecene	$C_{16}H_{32}$	224	2.37
9	1-Tetradecanol	$C_{14}H_{30}O$	214	2.36
10	Hexadacanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	2.04
11	1-Pentadecanol	$C_{15}H_{32}O$	228	4.86
12	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	3.71
13	3-Hexadecene, (Z)-	$C_{16}H_{32}$	224	4.86
14	Naphthalene, 1,2,3,4-tetrahydro-5-nitro-	$C_{10}H_{11}NO_2$	177	5.45
15	7-Tetradecene	$C_{14}H_{28}$	196	2.56
16	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl	$C_{15}H_{26}O$	222	3.22
17	1-Eicosanol	$C_{20}H_{42}O$	298	6.80
18	1-Hexacosanol	$C_{26}H_{54}O$	382	12.87
19	1-Pentadecene	$C_{15}H_{30}$	210	2.37

(12). Shrestha and Timilsina (31) identified 1-heptadecene from the ethyl acetate fraction of *Rumex nepalensis* with strong antifungal activities against *A. flavus* and *Fusarium* spp. Similarly, 1-eicosanol was effective against *Candida kruzi*, *C. tropicalisn* and *C. albicans* (9). Two compounds namely 1-octadecene and octadecanoic acid, methyl ester were previously identified from the root extract of *Lupinus termis* (14) and leaf extract of *Cynodon dactylon* (4), respectively, with strong growth inhibitory potential against *A. flavus, A. niger* and *C. albicans*. Similarly, 1-octadecanol, 1-heptadecene and 1-pentadecanol have been tested against *C. albicans* with promising results (7,12,27). Kumari *et al.* (24) reported that 1-tetradecanol; hexadacanoic acid, methyl ester and 1-pentadecene were also effective against *A. niger, A. terreus* and *A. flavus.*

CONCLUSIONS

This study concluded that leaf extracts of *D. metel* were highly antifungal to *S. rolfsii.* A 4 % methanolic extract can control growth of the target fungus up to 88 %. Bioassays with four organic solvents of different polarities showed that antifungal compounds in leaf extract of this medicinal plant were mostly present in its chloroform soluble fraction. The lowest concentration of this fraction (3.125 mg mL^{-1}) significantly controlled growth of fungal pathogen. GC-MS analysis showed that the most antifungal compounds present in this fraction were 1-hexacosanol; 1-eicosanol; 1-octadecene; 1-octadecanol and 1-heptadecene.

DECLARATION

We declare that all authors of this Ms have made substantial contributions. We have not excluded any author that substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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PUBLISHER NOTE