

Antifungal Potential of Indigenous Medicinal Plants against *Myrothecium* Leaf Spot of Bitter Gourd (*Momordica charantia* L.)

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ABSTRACT

Bitter gourd is of great importance due to its usage against the treatment of numerous ailments in human beings. A comprehensive survey at four localities of Southern Punjab, Pakistan was carried out to determine the severity of *Myrothecium* leaf spot. Maximum disease severity was at C₁ (Chak 11/NP) and least at C₂ (Kot Mehtab). Among isolated species *Myrothecium roridum* was found more prevalent and pathogenic as compared to *M. verrucaria*. Antifungal activity using solvent extracts of five medicinal plants (*Mangifera indica*, *Melia azedarach*, *Nicotiana tabacum*, *Moringa oleifera* and *Eucalyptus globosum*) were evaluated against isolated species by agar well diffusion method at various concentrations (0.01, 0.10, 1.0 and 10.0 µg / mL). *N. tabacum* revealed maximum zone size (13.40 mm and 8.28 mm) with ethanol and chloroform solvents respectively followed by *M. azedarach* (9.00mm and 6.48mm). However, least inhibition was observed with ethanol and chloroform extracts of *E. globosum* (6.04mm and 3.88mm zone size respectively). Ethanol extracts showed highest activity when compared to chloroform extracts. Qualitative phytochemical analysis showed that all the selected plants are rich in chemical compounds such as alkaloids, terpenoids, flavonoids and phenols whereas Saponins was only present in *N. tabacum* while absent in rest of the extracts.

Key words: *Momordica charantia* L.; secondary compounds; solvent plant extracts.

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INTRODUCTION

Bitter gourd (*Momordica charantia* L.), also known as “bitter melon” is a tropical vine of the family Cucurbitaceae, widely cultivated in natives of tropical regions of Asia with worldwide distribution in China, Japan and Africa¹. Bitter gourd is economically important plant having medicinal value and use against cancer, diabetes and many infectious diseases and also considered as a powerful weapon against immunodeficiency virus (HIV)^{2, 3, 4}. The crop is having problems such as low seed germination, small sized and deformed fruit, low yield, Asynchronous flowering and attack of pests and diseases². A number of diseases attack bitter gourd but *Myrothecium* leaf spot caused by *M. roridum* Tode ex Fr. and *M. verrucaria* (Alb. Schew) Ditm ex Fr., is a serious pathosystem in cultivated areas. The genus *Myrothecium* includes eight reported species⁵, out of which many are saprophyte soil inhabitants⁶. *M. roridum* considered as an important plant pathogen, affecting more than 200 plant species of different botanical families^{7, 8} and has been reported as a key pathogen of cucurbits causing leaf spot / blight^{9, 10}. The isolation of *M. roridum* has been reported from seeds of bottle-gourd¹¹, Indian gourd, red gourd, sponge gourd¹², pumpkin¹³ and melon¹⁴. As compared to other species *M. verrucaria* has showed a high incidence on seeds of watermelon¹⁵ and also responsible for leaf spot disease in bitter gourd¹⁶. Both *M. roridum* and *M. verrucaria* are soil inhabiting as well as seed transmitted pathogens of wide host range. Murakami *et al.* (1999)¹⁷ reported that *M. roridum* develops large necrotic lesions which verified the ability of *M. roridum* to produce toxins¹⁸.

Chemical fungicides are being used by farmers world over for the management of fungal disease resulted in increasing production cost and pose threat to human health and environment¹⁹. However, botanicals or biopesticides are considered to be one of the best substitutes of expensive and hazardous fungicides²⁰. As an alternative to chemical fungicides, botanicals have been reported against fungal diseases of plants. Since plant products being rich in active chemical components can be obtained from any plant part viz., seeds, roots, leaves, flowers, bark etc.^{21, 22}. Phytochemical screening to obtain the knowledge of chemical constituents present in plants and their antimicrobial activities have been reported by earlier scientists^{23, 24, 25}. These secondary metabolites defend host plants against attack of microorganisms, insects and herbivores²⁶. In Pakistan, to our best of knowledge no work is reported towards the ecofriendly management of leaf spot disease of bitter gourd. The aims of present investigations were to evaluate the inhibitory effects of organic solvent extracts of indigenous medicinal plants against leaf spot disease of bitter gourd for disparity in their phytochemical properties.

MATERIALS AND METHODS

Sample collection

A comprehensive survey of rural areas of District Rahim Yar Khan, Pakistan (27°40'-29°16' north latitudes and 60°45'-70°01' east longitudes) from four different localities (C1= Chak 11/NP, C2= Kot Mehtab, C3= Muhammad Pur and C4= Chak 165/P) was conducted during 2014-15. From each locality 20 plants were randomly selected and tagged, naturally infected bitter gourd leaves showing typical symptoms of leaf spot disease were preserved in clean brown paper bags and transported to the Mycology laboratory of Plant Pathology Department, Multan.

Disease Severity

Management of Myrothecium leaf spot

Forty infected leaves from already tagged plants were randomly selected from each locality for the assessment of disease severity with the help of 0-4 disease rating scale suggested by Vir and Grewel (1974)²⁷ where 0= no symptoms on leaves, 1= <5% infection on leaves, 2 = 5-25% infection on leaves, 3=25-75% infection on leaves, 4= >75% infection on leaves. Percent Disease Index was calculated by the modified formulae suggested by Sultana & Ghaffar (2009)¹⁶:

$$\text{Percent Disease index (PDI)} = \frac{\text{Sum of all numerical rating}}{\text{Number of leaves assessed}} \times \frac{100}{\text{Maximum disease grade}}$$

Isolation and morphological characterization of fungal strains

The infected bitter gourd leaf segments (1cm x1cm) were washed with tap water, surface sterilized with 70% ethanol for 1 min and rinsed in five changes of sterile distilled water. Isolation procedure was carried out on Potato Dextrose Agar medium (PDA- Difco) as described by Dhingra & Sinclair (1985)²⁸. Five leaf segments were aseptically plated on solidified PDA and incubated at 25°C for one week to facilitate the mycellial growth. Purification of culture was done by single hyphal tip technique, stored at 4°C on PDA slants and identified according to the cultural and morphological characters. Morphological characterization of sporodochia, phialides and conidia of resultant fungi were also studied. Size of conidia were measured (length x width) and compared with characters illustrated by Barnett & Hunter (1987)²⁹. Pathogenicity test was also carried out on healthy seedlings to confirm Koch's postulates.

Plant materials

Fresh and healthy leaves of selected medicinal plants (*Mangifera indica*, *Melia azedarach*, *Nicotiana tabacum*, *Moringa oleifera* and *Eucalyptus globosum*) were collected from local area, identified and authenticated from Department of Botany, Bahauddin Zakariya University, Multan.

Extraction

The leaves samples were thoroughly washed with tap water, followed by distilled water and finally with ethanol (Merck) to eliminate any traces of contaminants. Blot dried leaves of each sample were then dried in the oven at 50°C for 24 hours, homogenized to fine powder and stored in airtight bottles. Ten gm of powdered material was used in Soxhlet apparatus (J.P.Selecta-Spain) with 100 mL solvents (ethanol and chloroform) for 48 hours. The extract solutions were centrifuged at 6000 rpm for 10 min, filtered with filter paper (Whatman No.1) and concentrated over a water bath at 40°C. After complete solvent evaporation extract residues were sealed in dark bottles at 4°C for further use by Modified method of Nycee *et al.* (2012)³⁰.

Agar well diffusion assay

Antifungal tests were carried out by Agar well diffusion method (Perez *et al.*, 1990)³¹. An aliquot of 100µL spore suspension (10⁶spores/mL) was streaked on the surface of petri plates having PDA medium. Wells of 8 mm in diameter were punched in the seeded agar plates with the help of sterile cork borer. About 100µL of plant extracts alone and in combination (1:1v/v) with certain concentrations (0.01, 0.10, 1.0 and 10.0 µg / mL) were added into each well and allowed to diffuse for 1hr at room temperature. Dimethyl Sulfoxide (DMSO) was used as a control and treated plates were incubated at 25°C for 72 hours. Antifungal activity was evaluated by

measuring the size of zone with a ruler (mm). All the tests were performed in a Completely Randomized Design (CRD) with five replicates.

Phytochemical Screening

Plant extracts were subjected to phytochemical screening using the methods of Sofowora, (1993)³² and Harborne, (1973)³³.

Alkaloids (Wagner's test): 20mg of ethanolic extract was warmed with 2% sulphuric acid (H₂SO₄) for 1-2 min, filtered and treated with few drops of wagner's reagent. Presence of reddish brown precipitation or turbidity indicated the presence of alkaloids.

Tannins (Ferric chloride test): About 20mg plant extract was dissolved in ethanol, few drops of 0.1% ferric chloride added and observed for formation of blue black coloration.

Steroids: 2mL of acetic anhydride and conc. H₂SO₄ added to 50mg ethanolic plant extract. Blue green ring indicates the presence of steroids.

Terpenoids (Salkowski Test): About 5mL extract of each sample was mixed in (2mL) chloroform and (3mL) conc. H₂SO₄ was added carefully to form a layer. Formation of reddish brown coloration at interface indicates the presence of terpenoids.

Saponins: 20mg of powdered sample was boiled in 5ml distilled water and shaken vigorously for a stable persistent froth. Three drops of olive oil was mixed vigorously with frothing and observed for formation of emulsion.

Flavonoids: Powdered sample (20mg) was heated with 10ml of ethyl acetate for 3min and filtered. 4mL of filtrate was mixed with 1mL of dilute ammonia solution. A yellow coloration that disappears on addition of conc. Hydrochloric acid indicated the presence of flavonoids.

STATISTICAL ANALYSIS

The datasets of disease severity, isolation frequency and size of inhibition zone were subjected to analysis of variance (ANOVA) and treatment means were evaluated with Fisher's Least Significance Difference (LSD) test at 5% level of significance (Steel *et al.*, 1997)³⁴ using Analytical Software (2005).

RESULTS

Four different locations of District Rahim Yar Khan were evaluated for the disease severity of *Myrothecium* leaf spot and disease symptoms were observed as: yellowing, chlorosis and appearance of brown concentric rings on necrotic areas of leaves, which later coalesce to give the blighted appearance. In advance stages necrotic and chlorotic areas increased in size resulting into curling and death of leaves. Isolates of each species were identified on the basis of morphological characteristics of conidia, conidiophores, sporodochia and phialides.

Leaflets with characteristics leaf spot symptoms yielded two isolates; *M. roridum* and *M. verrucaria* with varying percentages of PDI. Laboratory experiments confirmed that isolates of *M. roridum* and *M. verrucaria* were pathogenic. Maximum disease severity 63.80% was observed at locality C₁ (Chak 11/ NP) followed by 37.50, 23.80 and 20.60 % at C₃, C₄ (Muhammad Pur, Chak 165 P) and C₂ (Kot Mehtab) respectively (Fig 1).

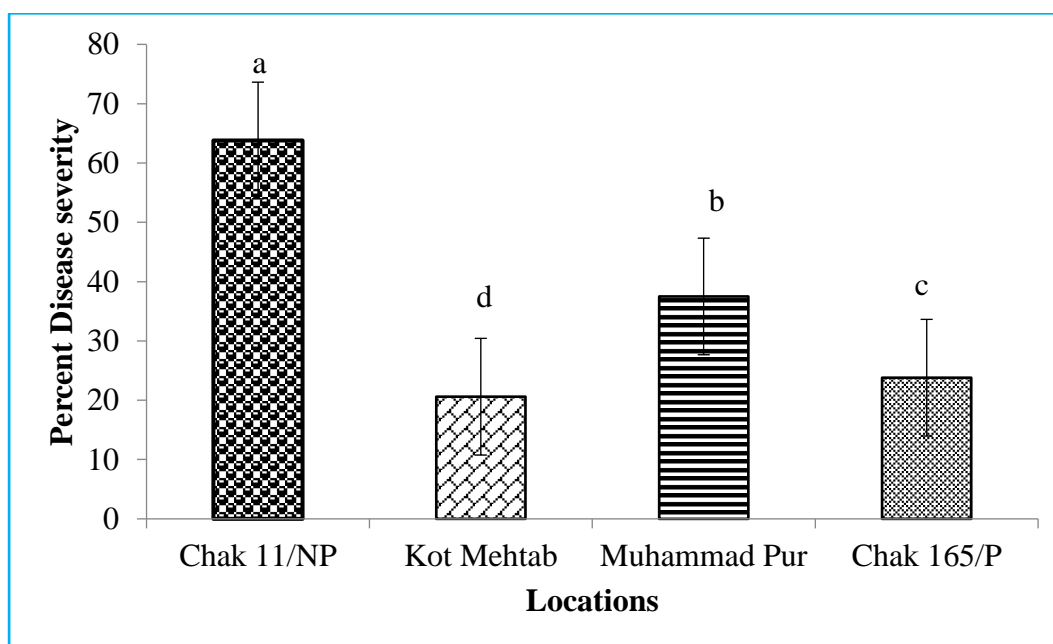
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Fig 1: Percent disease severity of *Myrothecium* leaf spots on different locations

Considering the isolation frequency of leaf samples collected from four different localities yielded two isolates of *Myrothecium* species. Leaflets from C₁ and C₃ have both species of *Myrothecium* (Myr1, Myr2, Myv1, and Myv2) whereas locality C₂ samples possess only Myr1 and Myr2. Overall maximum frequency percentage of Myrv1 was isolated from samples of locality C₂ followed by C₃, C₄ and C₁ with 80.00, 65.00, 60.00 and 55.00 % respectively while least was of Myv2 only from two localities C₂ and C₁ with 10 and 5% respectively (Table 1).

Table 1: Frequency isolation (%) of leaf spot disease of bitter gourd form infected plants

*Locality	***Frequency of Isolation (%)			
	**Myr1	**Myr2	**Myv1	**Myv2
C ₁ (Chak 11/ NP)	55.00 d	30.00 e	10.00 i	5.00 j
C ₂ (Kot Mehtab)	80.00 a	20.00 g	0.00 k	0.00 k
C ₃ (Muhammad Pur)	65.00 b	20.00 g	5.00 j	10.00 i
C ₄ (Chak 165/ P)	60.00 c	25.00 f	15.00 h	0.00 k

Myr1= *Myrothecium roridum* Myr2= *Myrothecium roridum*, Myv1=*Myrothecium verrucaria*, Myv2=*Myrothecium verrucaria*. * Data are means of four replications of two consecutive years. Means showing different letters are statistically significant. LSD_{0.05} value for comparison of means is 2.52.

The organic solvent extracts had antifungal effects on the growth of *M. roridum*. Ethanol extraction was superior as compared to chloroform. Ethanol extract of *Nicotiana tabacum*; in particular, showed maximum inhibition zone size (22.20 mm) of *M. roridum* using 10 µL concentration followed by 13.80, 13.60, 12.80 and 12.20 mm zones of inhibition formed by *Melia azedarach*, *Moringa oleifera*, *Mangifera indica* and *Eucalyptus globosum* respectively, compared to control (Dimethyl sulphoxide) with zero inhibition zones. It was observed that the zone size increased with an increase in concentration of the extracts. Overall *N. tabacum* showed maximum inhibitory effects towards mycelia growth of *M. roridum* at all concentrations with 13.40 mm inhibition zone followed by 9.00, 7.64, 6.32 and 6.04

mm zone of inhibition in *M. azedarach*, *M. oleifera*, *M. indica* and *E. globosum* respectively (table 2).

Table 2: Inhibitory effect of various ethanolic plant extracts at different concentrations on mycelial growth of *Myrothecium roridum* using agar well diffusion method.

Concentrations	Size of zone (mm)					Mean
	<i>Mangifera indica</i>	<i>Nicotiana tabacum</i>	<i>Eucalyptus globosum</i>	<i>Melia azedarach</i>	<i>Moringa oleifera</i>	
0.01µl	0.00 j	11.80 f-h	0.00 j	8.20 i	7.60 i	5.52 D
0.1 µl	7.60 i	15.20 c	7.40 i	10.40 h	8.20 i	9.76 C
1 µl	11.20 gh	17.80 b	10.60 h	12.60 d-g	8.80 i	12.20 B
10 µl	12.80 d-f	22.20 a	12.20 e-g	13.80 cd	13.60 de	14.92 A
DMSO	0.00 j	0.00 j	0.00 j	0.00 j	0.00 j	0.00 E
Mean	6.32 D	13.40 A	6.04 D	9.00 B	7.64 C	

Values are means of five replications. Lower case letters after values represent comparison across inhibition percentage and capital letters represent comparison across concentrations and plant extracts. LSD_{0.05} value for inhibition percentage= 1.43, for Concentrations = 0.62 and for Plant extracts = 0.72.

Correspondingly chloroform extracts of *N. tabacum* also showed better inhibitory effects on mycelia growth of *M. roridum* with maximum (17.2 mm) inhibition zone at 10 µL concentration followed by 12.4, 12.2, 11.2 and 10.2 mm inhibition zone size produced by *M. azedarach*, *M. oleifera*, *E. globosum*, and *M. indica* respectively compared to untreated control. In general *N. tabacum* performed best forming 8.28 mm inhibition zone followed by *M. azedarach* (6.48 mm), *M. oleifera* (5.88 mm), *M. indica* (5.16 mm) and *E. globosum* (3.88 mm) inhibition zones (table 3).

Table 3: Inhibitory effect of various plants extracts at different concentrations extracted with chloroform on mycelial growth of *Myrothecium roridum* using agar well diffusion method.

Concentration	Size of zone (mm)					Mean
	<i>Moringa oleifera</i>	<i>Nicotiana tabacum</i>	<i>Eucalyptus globosum</i>	<i>Melia azedarach</i>	<i>Mangifera indica</i>	
0.01 µl	0.00 i	0.00 i	0.00 i	0.00 i	0.00 i	0.00 D
0.1 µl	7.40 gh	11.40 cd	0.00 i	8.80 f	7.20 h	6.96 C
1 µl	9.80 e	12.80 b	8.20 fg	11.20 d	8.40 f	10.08 B
10 µl	12.20 bc	17.20 a	11.20 d	12.40 b	10.20 e	12.64 A
DMSO	0.00 i	0.00 i	0.00 i	0.00 i	0.00 i	0.00 D
Mean	5.88 C	8.28 A	3.88 E	6.48 B	5.16 D	

Values are means of five replications. Lower case letters after values represent comparison across inhibition percentage and capital letters represent comparison across concentrations and plant extracts. LSD_{0.05} value for inhibition percentage= 0.96, for Concentrations = 0.38 and for Plant extracts = 0.44.

Preliminary qualitative phytochemical analysis showed that plant extracts have fair amount of secondary metabolites. Considering the presence or absence of these compounds in medicinal plants utilized, it was observed that alkaloids, terpenoids and flavonoids were present in all plants extracts except *E. globosum* while tannins were found in *N. tabacum*, *M. indica* and *E. globosum*. Steroids were present in *M. azedarach* and *M. oleifera*, whereas, saponins were only present in *N. tabacum* but absent in rest of the plant extracts (table 4).

Table 4: Analysis of phytochemicals extracted from medicinal plant extracts.

Plants extracts	Alkaloids	Tannins	Steroids	Terpenoids	Saponins	Flavonoids
<i>Mangifera indica</i>	+	+	-	+	-	+
<i>Melia azedarach</i>	+	-	+	+	-	+
<i>Nicotiana tabacum</i>	+	+	-	+	+	+
<i>Moringa oleifera</i>	+	-	+	+	-	+
<i>Eucalyptus globosum</i>	-	+	-	-	-	-

+ = Present

- = Absent

DISCUSSION

Myrothecium leaf spot of bitter gourd is considered to be the most serious pathosystem of cucurbit in the cultivated areas. During the current research, *Myrothecium* infestation on various locations of the District Rahim Yar Khan was assessed. We observed *Myrothecium* species to produce brown to dark brown leaf spots which later on coalesce to give blighted symptoms in the field on the host plants and it seems³⁵ a serious threat to vegetable growers of the area. During the in vitro studies of the collected diseases samples of the different locations we found *M. roridum* and *M. verrucaria* both pathogenic and responsible for leaf spot in bitter gourd. We calculated less isolation frequency of *M. verrucaria* revealed that it is a weak pathogen of bitter gourd grown in this area. Our findings are in agreement with the results of earlier workers whom reported *M. roridum* and *M. verrucaria* both as plant pathogens while later is a weaker one⁷. *Myrothecium* species are soil-inhabiting fungi but due to facultative parasitic nature also able to cause disease in aerial parts of economically important host plants^{6, 36, 37, 38}. However, *M. roridum* also identified as a virulent seedborne pathogen¹⁵. Rain splashes, irrigation water and dew are dissemination agencies of both *Myrothecium* species. Prolonged hot and humid weather favors the foliar attack of *Myrothecium*³⁹. Two isolates; *M. roridum* and *M. verrucaria* were isolated and identified and compared with work of earlier scientists³⁶. According to some scientists large necrotic lesions are due to necrotrophic nature of fungi that produce toxins in host tissues resulting into death of host tissues and the fungal pathogen colonize over these necrotic areas^{40, 8}. Though virulence of *M. roridum* was demonstrated on large number of plants but different *M. roridum* isolates revealed variability in virulence but highly aggressive behavior was observed in tomato and cucumber⁴¹. *N. tabacum* was found rich in all compounds except steroids and thus possessed excellent fungicidal activity (Taiga et al., 2008)⁴² which proved that *N. tabacum* was more fungitoxic compared to other plant extract due to the subsistence of tannins in addition to Saponins and flavonoids. These results corroborated with the outcome of other scientists^{43, 42}. Furthermore, phytochemical studies of various plant extracts showed that fungicidal activity is due to presence of tannins, alkaloids, flavonoids and saponins^{32, 44}. Out of five plants extracts used, all of them showed antimicrobial activity but *N. tabacum* and *M. azedarach* were found more promising^{45, 46, 47}. Plants with more phenolic contents show efficient antioxidant activity⁴⁸. Similarly, according to Ali et al., (2008)⁴⁹ plant possesses natural antioxidants in the form of phenolic compounds such as phenolic acids, tocopherol, flavonoids etc. These results are substantiated with the outcome of

this study. There is a need to explore more plants possessing antimicrobial potential to use in our organic farming system for cleaner environment.

CONCLUSION

Use of plant extracts to control fungal pathogen responsible for blight of bitter gourd is ecofriendly approach and an effective alternative to toxic chemical fungicides. Studies are in progress, in the greenhouse and in open fields, to evaluate antifungal effect of these natural substances on bitter gourd development and on qualitative and quantitative fruit production.

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CONFLICT OF INTEREST DISCLOSER

There is no conflict of interest among authors regarding the current manuscript.

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Erratum

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