Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Ul-Haq et al., J Plant Pathol Microb 2014, 5:2
DOI: 10.4172/2157-7471.1000224

Research Article Open Access

Antifungal Properties of Phytoextracts of Certain Medicinal Plants Against Leaf Spot Disease of Mulberry, *Morus* spp.

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Abstract

The fungi toxicant use checks the pathogen, yet its use in the organic farming system is least permitted because of their eco-toxic properties. Hence, the use of plants for their anti-fungal properties which could be used against the pathogen in the organic farming system becomes an area of interest for the eco-friendly mode of disease management. Six commonly available medicinal plants were selected based on their wide application in the state and for their valuable use as ethano-medicines. Cold water extracts of the six plants were tested in vitro against three identified leaf spot pathogens, *Cercospora moricola* Cooke, *Alternaria alternata* and *Cladosporium cladosporiodes* and extracts of plants viz. *Artemisia absanthemum, Allium sativa L, Euphorbia ligularia* Roxb, *Zingiber officinale* and *Datura metel* showed more than 85% conidial inhibition *in vitro* with a 94.56% in *A. absanthemum* and more than 50% decrease in leaf spot disease incidence and severity in field conditions except *A. sativa* L and *E. ligularia* Roxb. at 0.05% decrease in PDI. All the plant extracts screened showed more than 50% percent mycelial inhibition with respect to control (water). The highest mycelial inhibition of more than 70% was found in *E. ligularia* Roxb. Followed by *Z. officinale* in all the three pathogens. So these plants with anti-fungal properties could be utilized against these pathogens, at least to lessen the impact of these pathogens. Similar eco-friendly means of disease control has been appreciated by the present environment conscious generation. Thus exploring new plants for their anti-fungal activity would bring about more resource base for use in eco-friendly and sustainable mode of agriculture especially in organic farming.

Keywords: Antifungal; Eco-organic; Phytoextracts; Leaf spot disease; *Morus* spp.,; Organic farming

Introduction

Mulberry (*Morus* spp.) leaves forms the only food material for the silkworm, *Bombyx mori* L. For the development of silk industry, production of high quality silkworm cocoons is must. To achieve the goal of production of good quality silkworm cocoon crop, certain factors play important role. The most important factor is the mulberry leaf, contributing about 38.2% followed by climate (37.0%), rearing techniques (9.3%), silkworm race (4.2%), silkworm egg (3.1%) and other factors (8.2%) in producing good quality cocoons. Hence, quality of mulberry leaf is one of the basic prerequisite of sericulture and plays a pivotal role for successful silkworm cocoon crop [1]. Healthy mulberry leaves influences the growth, development and quality of cocoons formed and thus decide the superiority of silk to a greater extent. Mulberry is exposed to the ravages of different pests and diseases. All mulberry varieties are attacked by one or more fungi that cause scattered, rather definite, round to oval, angular, or irregularly shaped spots on the leaves. These spots usually become conspicuous from late June through August. Leaf spots are the most common diseases of mulberry plants. The diseases development is favored by cool weather, light and frequent rains, fog or heavy dews, high humidity, and crowded or shady plantings. A few spots on the leaves do little harm to a mulberry plants and are far more unsightly than they are injurious. However, leaf spot infections that start early in the growing season can lead to premature defoliation. If it occurs over two or more successive years, it can seriously weaken a tree, reduce its growth, and increase its susceptibility to bark borers, winter injury, and other diseases. Leaf spots commonly increase in number and size in late summer and early autumn as the leaves begin to senesce. The occurrence of a leaf spot disease late in the growing season generally does not seriously affect the health of a tree. Certain leaf spots with special names, such as anthracnose, black spot, downy spot or white mold, ink spot, spot anthracnose, leaf blister or curl, scab, shot-hole, sooty blotch, and tar spot, all occur in the mulberry plantations by the diversity of pathogens.

Leaf spot disease of mulberry is an important fungal disease of mulberry plantation causing considerable damage to the rearing and ultimately to the cocoon crop parameters. Various methods for the management of the disease have been studied by various workers in other states. There are reports that foliar spray of carbendazim and Mancozeb 70% WP in the ratio of 0.1% and 0.2% were most effective in reducing the disease. However due to residual effect of synthetic fungicides, there is demand for more ecofriendly substances like bio pesticides [2]. As such exploration of plant resources for their antifungal potential against the pathogen is quite inevitable for a sustainable and ecofriendly management of the pathogen. Further these plant extracts could be readily used by the farmers to lessen the impact of the pathogen on their mulberry plantation. Using plant resources for its antifungal activity is an attractive avenue for the development of sustainable mode of moriculture in organic farming system. Hence, new plants especially locally available, need to be explored for their antifungal property. Thus six plants locally used in medicinal purposes were selected based on

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Received: February 21, 2014; **Accepted** April 28, 2014; **Published** May 07, 2014

Citation: Ul-Haq S, Hasan SS, Dhar A, Mital V, Sahaf KA (2014) Antifungal Properties of Phytoextracts of Certain Medicinal Plants Against Leaf Spot Disease of Mulberry, *Morus* spp. J Plant Pathol Microb 5: 224. doi:10.4172/2157-7471.1000224

their abundant availability during the growing season and for their ethano-economic use [3-5].

Materials and Methods

Pathogen isolation

The pathogen was isolated from the diseased leaf as small, scattered, circular to oval dead areas in the leaves; usually tan, dark brown, yellow, gray, purple, or black. Some spots are raised, shiny, and coal black, others may drop out leaving ragged holes; some are marked with light and dark concentric zones. Numerous spots develop yellow, purple, red, or reddish brown to black margins; and later, in damp weather, increase in size and number and merge into large, angular to irregular dead areas. Dark areas and speck-sized, fungus-fruiting bodies (known as pycnidia, acervuli, and perithecia) commonly form in the dead tissues of many older spots. Heavily infected leaves may turn yellow to brown, wither, and drop early, weakening the tree. Occasionally, some leaf spotting fungi deform or kill leaf tissues, buds, twigs, or even small branches. The pathogen was first isolated on PDA plates at 25 ± 5°C in BOD incubator and transferred on fresh PDA slants at regular interval for pure culturing and for further study.

Plant extracts preparation

Five plants viz. *Artemisia absanthemum*, *Allium sativa* L, *Euphorbia lingularia* Roxb., *Zingiber officinale* and *Datura metel* were selected for the study. Healthy non infected leaves, seeds, rhizomes and bulbs of the six plants were collected from the local area i,e. karewa lands of Panzgam, Pulwama Kashmir and the *zingiber officinale* procured from the local market of Pampore. Extracts were prepared from different plant species (Table 1). Fresh leaves were collected and thoroughly washed in sterilized water before preparing their extract whereas bulbs and rhizomes were used in case of Garlic and Ginger, respectively. The extracts were prepared as per the method of Awuah. The leaves of the selected plants were collected and cleaned with distilled water and dried under shade. Individual samples were ground with the help of mortar

S. No	Botanicals	Pathogen spp.	Treatments #									%I/C
			0.1%			0.2%			0.5%			
			TNO	TNG	% I (χ^2)	TNO	TNG	% I (χ^2)	TNO	TNG	% I (χ^2)	
01	*Artemesia absanthemum*	*C. moricola*	63.3 (52.7)	17.7 (24.8)	72.11 {2.28}	72.0 (58.7)	13.3 (21.3)	81.48 {2.41}	68.0 (56.0)	9.67 (18.0)	85.78 {3.43}	94.56
		C. cladosporioides	62.7 (52.6)	15.0 (22.7)	76.06 {2.31}	70.3 (52.6)	13.0 (21.0)	81.52 {3.40}	86.7 (69.0)	15.0 (22.6)	82.69 {3.51}	91.53
		A. alternata	73.3 (58.9)	13.7 (21.6)	81.36 {8.42}	68.3 (58.9)	41.3 (40.0)	39.51 {2.09}	67.7 (56.0)	18.7 (25.5)	72.41 {1.30}	91.24
02	*Allium sativa L.*	*C. moricola*	89.0 (70.6)	65.0 (53.7)	26.97 {1.05}	79.0 (70.6)	47.3 (43.4)	40.08 {8.12}	83.0 (65.6)	64.0 (54.6)	22.89 {9.12}	79.6
		C. cladosporioides	85.3 (67.5)	65.3 (53.9)	23.44 {3.04}	86.0 (67.5)	47.3 (43.4)	44.96 {1.15}	83.7 (66.5)	40.0 (39.1)	52.19 {5.20}	86.59
		A. alternata	82.7 (66.3)	72.3 (58.5)	12.50 {0.15}	77.0 (66.3)	53.0 (46.7)	31.17 {4.07}	83.7 (67.9)	49.0 (44.4)	41.43 {1.12}	84.7
03	*Euphorbia ligularia Roxb*	*C. moricola*	92.0 (74.5)	21.7 (27.7)	76.45 {3.46}	87.3 (74.5)	17.3 (24.5)	80.15 {2.48}	86.0 (68.0)	10.7 (19.0)	87.60 {6.57}	94.67
		C. cladosporioides	82.7 (65.5)	16.3 (23.8)	80.24 {7.46}	78.0 (65.5)	13.7 (21.6)	82.48 {9.46}	70.3 (57.4)	11.0 (19.3)	84.36 {3.43}	91.7
		A. alternata	89.7 (71.4)	36.3 (37.0)	59.48 {1.27}	74.3 (71.4)	24.7 (29.7)	66.82 {1.28}	58.3 (50.0)	16.7 (24.0)	71.43 {4.26}	91.12
04	*Datura metel*	*C. moricola*	78.7 (62.6)	46.7 (43.0)	40.68 {9.12}	89.0 (2.6)	43.0 (40.9)	51.69 {9.21}	74.3 (60.0)	34.3 (35.7)	53.81 {2.19}	91.32
		C. cladosporioides	75.0 (60.3)	24.3 (29.3)	67.56 {12.29}	77.3 (60.3)	21.7 (27.7)	71.98 {2.34}	49.7 (44.8)	16.0 (23.6)	67.79 {1.19}	89.67
		A. alternata	94.0 (76.5)	42.0 (40.3)	55.32 {8.25}	83.7 (76.5)	24.0 (29.3)	71.31 {1.36}	80.0 (63.8)	15.0 (22.6)	81.25 {1.45}	92.2
05	*Zingiber officinale*	*C. moricola*	68.3 (56.4)	33.0 (34.8)	51.71 {7.16}	75.7 (56.4)	31.0 (33.8)	59.03 {9.23}	82.3 (65.1)	25.0 (29.9)	69.64 {2.34}	93.29
		C. cladosporioides	9.00 (72.7)	64.7 (53.5)	28.15 {3.06}	92.3 (72.7)	41.0 (39.8)	55.6 {1.24}	71.3 (58.6)	29.0 (32.4)	59.35 {2.22}	88.2
		A. alternata	69.7 (39.5)	58.3 (49.8)	16.26 {0.77}	78.7 (59.5)	48.3 (44.0)	38.56 {3.11}	71.3 (58.2)	30.0 (33.1)	57.94 {1.21}	89.06
06	**Control/water**	***C. moricola***	69.0 (55.4)	64.3 (53.1)	4.67 {0.02}							
		C. cladosporioides	87.3 (68.2)	80.3 (60.5)	7.00 {0.15}							
		A. alternata	93.3 (70.1)	87.0 (58.7)	6.34 {0.35}							
SEm ± /F-test			5.01/*	2.14/**		5.45/ns	1.77/**		5.27/**	3.75/**		
Cd at 5%			14.4	6.20		5.75	5.11		15.2	10.8		
CV (%)			13.4	9.70		14.7	9.05		15.0	21.9		
SD ±			13.40	21.16		11.63	14.62		14.33	14.92		

TNO=total no. of spores observed, TNG=total no. of spores germinated, %I/C=Percent inhibition over control (0.5%) and #=avg. of 10 replications and %I/ {χ^2}=percent inhibition and chi test. values in bracket(s) are arc sine values

Table 1: Effect of different Botanicals on the conidial germination of identified leaf spot disease causing pathogens of mulberry.

and pestle or by using the automated grinder followed by addition of sterile distilled water (1.00 ml/g). Then this material was taken in a beaker and boiled at 80°C for ten minutes in a hot water bath. The material was homogenized for five minutes and filtered through muslin cloth. The filtrate was centrifuged at 5000 RPM for fifteen minutes and the clear supernatant was collected [6,7]. This was taken as 100% basic stock solution and further diluted to desired concentrations (2.50, 5.00 and 10.00%) with distilled water before use. Then they were mixed with PDA medium and sterilized as per the method of poisoned food technique. Varying amounts of plant extract were added to PDA to get a final concentration of 5%, 10%, 15%and 20% to access their effect on the mycelial growth of the test pathogen [8-10].

Inoculation

The PDA mixed with the plant extracts were poured in Petri plates and allowed to set. Then, one disc (7 mm) of the test fungus taken from the margin of five days old culture were taken and placed in the reversed orientation at the centre of the Petri plates. Three replications were set up for each treatment. The whole set up is placed in BOD incubator with temperature set at 25°C for five days. Pathogen grown on PDA plates with no plant extracts but with only distilled water acts as control plate. Percent inhibition is calculated as,

The inhibition percent was calculated by the formula given by Vincent.

$$I = \frac{(C\text{-}T)}{C} \times 100 \quad (1)$$

Where C=growth in control: T=Growth in treated groups and I=inhibition percent.

Average of four replications of each test is taken for calculations.

In vitro effect of phytoextracts

Spores of three pathogens were taken from 7 day-old cultures on PDA. Spore suspension (103 conidia/ml) were made separately against three different concentrations of the phytoextracts 0.1, 0.2, and 0.5%) when the plates were thoroughly covered with mycelium and spores. The spores were removed and put in triplicate in phytoextracts solution or suspensions in sterile water where different concentrations of each phytoextracts were used. Five ml suspensions of each were taken in small sterilized Petri dishes (65 mm) and kept at 28°C for 30 min. Then a drop of lacto phenol cotton blue was added to conidial suspension on the slides [11]. The slides were finally examined under microscope (×400) for recording the percentage of conidial germination.

Field trials

Field trials were conducted for two consecutive growing seasons (2012 & 2013) at the Central Sericutural Research and Training Institute, Gallander, Pampore, India (TS1) temperate humid, located at an altitude of 1574 meters above mean sea level, 74.93° E longitude and 34.02° N latitude [12]. Mulberry plants raised by following the standard cultural practice. The field shows leaf spot disease incidence during 2011 growing season. Hence experiment is carried out at the natural inoculums potential of the soil. In order to understand the relative distribution of disease and its impact on leaf yield, an experiment was conducted under field conditions for a period of two years (2012 & 2013). Five selected varieties were undertaken by following the paired plot technique with a plant gap of 3×3 ft having three replications. Treatment plots were sprayed with extracts of plants viz. *A. absanthemum*, *A. sativa L, E. ligularia, Z. officinale* and *D. metel* in 0.2%, 0.1% and 0.05% concentration for the control of leaf spot disease after the initial appearance of disease symptoms. Water sprayed plots were kept as control for comparison. Data were recorded after one week in protected and un-protected plots [13]. Disease severity was calculated at random from 10 plants of each replication. In each plant, all the leaves from three branches, one each at top, middle and bottom position, were counted for recording the disease incidence by using the following grading scale and 0-5 is used for statistical analysis [14-16].

$$\text{Disease incience (DI)} = \frac{\text{No. of infected leaves}}{\text{Total No. of leaves observed}} \times 100 \quad (2)$$

$$\text{Precent Disease Index (PDI)} = \frac{\text{Sum of all numerical values}}{\text{Total no. of leaves graded x Maximum grade (5)}} \times 100 \quad (3)$$

Statistical analysis

The data obtained was analyzed using technique of ANOVA as given by Ronald E Walpole to test the effectiveness of the plant extracts and if there is any significant difference in the antifungal properties of the plant extracts.

Result

In vitro mycelia inhibition

The results as presented in Table 1 shows that the plant extracts were effective in significantly reducing the conidial germination as compared to control where 7%, 6.34% and 4.67% inhibition was observed in *C. moricola, C. cladosporiodes* and *A. alternata* respectively. More than 90% conidial germination inhibitions as compared with control plates were observed with 0.5% aqueous extracts of the plants viz. *A. absanthemum, A. sativa L., E. ligularia, Z. officinale* and D. metel. Again 72.7% mycelial growth inhibition was observed by using followed by *Z. officinale, E. ligularia,* which showed the 71.6% inhibition. The least mycelial growth inhibition was found in *D. metel,* which showed 45.5% followed by 48.9% inhibition by *A. sativa* L. in *C. moricola* and *A. alternata.*

Disease incidence

The decrease over percent disease index (PDI) was highest in all the five phytoextracts except *A. sativa* L. in all the three concentrations (0.05, 0.1 and 0.2%) and E. ligularia at 0.05% (Table 2). The application of the phytoextracts of *D. metel* showed 76.3% followed by 71.9% in *E. ligularia* showed decrease in the disease incidence and severity. The finding showed the antifungal effect of the five plants extract against leaf spot disease pathogens (Table 3).

Discussion

Leaf spot disease pathogens are necrotropic foliar pathogens with high competitive saprophytic and biotrophic activity. Hence regular application of the fungicide is needed for the chronic disease of mulberry. Synthetic chemicals might successfully control the disease but their application is against the logic of eco-organic moriculture. Hence exploration of alternative antifungal agents, especially the plant extracts has merits. Plant extracts as potential antifungal substance has been explored against several fungal diseases. In our study, six plants showed 50% or above fungal mycelium inhibition activity against the pathogens in *in vitro* experiment. These plants have been reported to possess antifungal properties against different fungi. Although none of the plants under study showed 100% mycelia inhibition plant yet most of them showed antifungal activity against leaf spot disease. From the in vitro and field results, it can be safely concluded that the aqueous extracts of the six plants could be used in the organic farming

S. No	Antagonists	Agar Plate method at 0.5% conc.			% Inhibition over control (%)		
		C.m	*C.c*	*A.a*	*C.m*	*C.c*	*A.a*
01	*Artemesia absanthemum*	12.3 (20.5)	12.0 (20.2)	12.0 (20.2)	58.0	61.7	59.1
02	*Allium sativa L.*	15.0 (22.7)	12.0 (20.2)	15.0 (22.7)	48.9	61.7	48.9
03	*Euphorbia ligularia Roxb*	8.0 (16.4)	9.33 (17.7)	8.0 (16.0)	72.7	70.2	72.7
04	*Datura metel*	16.0 (23.5)	15.0 (20.7)	13.3 (21.3)	45.5	52.1	54.5
05	*Zingiber officinale*	8.33 (16.5)	9.67 (18.0)	11.7 (19.8)	71.6	69.1	60.2
10	Control	29.3 (32.7)	31.3 (34.0)	29.3 (32.7)			
	Cd at 1%	2.92	2.60	4.52			
	Cd at 5%	7.44	6.60	11.4			
	SEm ± /F-test	0.95/**	0.84/**	1.47/**			

Cc=Cerrcospora moricola, Cc=cladosporium cladosporiodes and Aa=alternaria alternate.
Values in Brackets are sine transformed values

Table 2: *In vitro* botanical control on leaf spot disease pathogens at CSR&TI, Pampore in August 2012.

S No	Treatment	Conc.	GSH		KNG		IC N		TR-10		CW	
			Mean PDI	% D/C	Mean PDI	% D/C	Mean PDI	% D/C	Mean PDI	% D/C	Mean PDI	% D/C
01	*Artemesia absanthemum*	0.20	1.51 (7.03)	70.6	3.31 (10.4)	55.29	3.61 (10.9)	61.9	5.98 (14.1)	53.0	6.95 (15.2)	58.81
		0.10	2.24 (8.60)	56.4	4.03 (11.5)	45.52	4.24 (11.8)	55.3	6.73 (15.0)	47.1	7.65 (16.2)	54.64
		0.05	3.17 (10.2)	38.4	4.48 (12.2)	39.53	5.44 (13.4)	42.5	7.2 (15.5)	43.4	8.51 (16.9)	49.56
02	*Allium sativa L.*	0.20	3.77 (11.1)	26.7	5.25 (13.2)	29.13	5.01 (12.8)	47.1	7.31 (15.5)	42.5	8.67 (17.0)	48.61
		0.10	4.24 (11.8)	17.6	5.10 (13.0)	31.16	5.15 (13.0)	45.7	7.63 (15.9)	40.0	9.71 (18.0)	42.42
		0.05	4.36 (12.04)	15.2	5.39 (13.3)	27.24	6.38 (14.5)	32.6	9.55 (17.9)	24.9	10.7 (18.9)	36.83
03	*Euphorbia ligularia Roxb.*	0.20	1.22 (6.33)	76.3	3.08 (10.1)	58.4	4.02 (11.5)	57.6	4.77 (12.2)	62.5	7.03 (15.3)	58.29
		0.10	2.60 (9.28)	49.4	4.06 (11.6)	45.11	4.99 (13.3)	47.4	6.39 (14.6)	49.8	7.63 (16.0)	54.77
		0.05	3.24 (10.3)	36.9	5.29 (13.2)	28.55	6.12 (14.3)	35.4	7.27 (15.6)	42.9	10.9 (19.3)	35.58
04	*Datura metel*	0.20	1.44 (6.88)	71.9	3.29 (10.4)	55.56	5.12 (13.0)	46.0	5.96 (14.6)	53.1	6.80 (15.0)	59.68
		0.10	2.44 (8.97)	52.6	4.15 (11.7)	43.99	6.52 (14.7)	31.1	7.29 (14.1)	42.7	7.87 (16.2)	53.33
		0.05	2.99 (9.94)	41.9	5.30 (13.3)	28.41	7.62 (15.9)	19.5	7.53 (15.6)	40.8	9.25 (17.7)	45.15
05	*Zingiber officinale*	0.20	1.91 (7.93)	62.8	3.22 (10.3)	56.55	3.46 (10.7)	63.4	5.82 (15.9)	54.2	6.96 (15.2)	58.75
		0.10	2.62 (9.31)	49.0	4.08 (11.6)	44.89	4.76 (12.5)	49.7	7.55 (13.9)	40.6	6.76 (15.0)	59.93
		0.05	3.30 (10.1)	35.7	5.46 (13.5)	26.25	5.17 (13.1)	45.4	7.94 (14.9)	37.6	7.06 (15.4)	58.13
06	**Control/water**		5.10 (15.7)		7.40 (17.9)		9.50 (17.6)		13.0 (16.3)		17.0 (24.2)	
Cd at 5%			0.39		0.78		1.57		2.03		2.40	
CV (%)			8.26		10.41		17.30		16.69		16.63	
SEm±			0.13		0.27		0.54		0.70		0.83	

Table 3: Field control of different botanicals on the leaf spot disease of mulberry at CSR&TI, Pampore.

environment to lessen the impact of the pathogens on mulberry leaf crop, although complete control could not be attained. Yet based on their wide availability and ease of application it could be used in wide scale in the moriculture. Even though more useful oils and other components could be extracted through the use of other synthetic solvent and refined techniques yet, their use by the marginal farmers in the organic environment is limited. Hence the use of aqueous extracts has merits and is simple and could be easily followed even by a layman. This study would benefit the farmers who wish to lessen the impact of leaf spot disease and enhance the cocoon crop. More and more plants, locally available need to be explored for a fruitful sustainable moriculture.

Conclusions and Forward Look

From the results some plants under study showed significant inhibitory effect even though none of the plant extracts shows cent percent mycelia inhibition. They are widely available in the state. Hence these plants could be used in the organic farming environment to lessen the impact of the leaf spot disease at global level and mostly in the temperate region of Jammu and Kashmir. More novel plants

need to be explored to increase the resource base for use in eco-organic moriculture in a sustainable mode.

Acknowledgements

Authors are highly thankful to Dr. K. A Sahaf, Director CSR&TI, Govt. of India, Pampore, Kashmir for providing all the necessary facilities and encouragement for the present work. We duly acknowledge the contributions of Prof. Vijayshri, Director, School of Sciences, IGNOU, Main Campus, New Delhi and Dr. Irfan Illahi, Scientist-B CSR&TI, Pampore and Prof. Neera Kapoor, (Coordinator School of Sciences, IGNOU), Prof. Amrita Nigam and Prof. Jaswant Sokhi eminent professors of the School of Sciences, IGNOU, Main Campus, Maidan Garhi, New Delhi for their generous and enthusiastic help and constructive suggestions.

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