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PHYTOCHEMICAL CHARACTERIZATION AND ANTIFUNGAL ABILITY OF THREE MELIACEAE SPECIES AGAINST *Bipolaris oryzae*

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Brown spot (BS) of rice caused by *Bipolaris oryzae* can occur from seedling to flowering stage, resulting in significant economic yield losses worldwide (Imran *et al*., 2020).This disease is more common in rice growing areas of Asian countries, including China, Japan, Burma, Bangladesh, Philippines, Saudi Arabia and Thailand (Goel *et al.,* 2006). Brown spot disease is characterised by dark oval spots on leaves and these spots are smaller on older leaves as compared to young leaves. Black to dark oval spots can also be seen on infected panicles and on glumes, as well as discoloration of the entire surface. The pathogen can also penetrate grains resulting in "pecky rice', a term used to explain discoloured and shrivelled grains. The pathogen produces both intra- and inter-cellular mycelium, which appears as dark brown to greyish brown mat on the infected tissues, but in culture it is olive to grey or black in colour (Satija *et al.,* 2005). The massive use of synthetic fungicides in crop management to control pathogenic fungi had a detrimental environmental impact. The inappropriate use of such fungicides has adverse side effects on ecosystems and fungicidal resistance has become a constraint.

In the current scenario, the prospect of managing the agricultural ecosystem in an eco-friendly and profitable manner is a profound target of interest in order to meet the global food demand as a consequence of fungal diseases (Nguefack *et al*., 2013). *Azadirachta indica* is one of the most important medicinal trees belonging to the family Meliaceae and is native to the Indian subcontinent (Kumar *et al.,* 2021). Neem consists of a wide variety of biologically active compounds that are structurally variable and chemically diverse, with more than 140 compounds isolated from various parts of this tree. Neem has considerable potential and is a potential candidate for commercial control agents because of its low toxicity (Gurjar *et al.,* 2012). Preliminary phytochemical screening of *Melia azedarach* showed the presence of a number of secondary metabolites like carbohydrates, proteins, terpenoids, alkaloids, saponins, flavonoids, steroids, amino acids, anthraquinones and tannins (Yoon *et al.,* 2013). Carpinella *et al.* (2003) evaluated the botanical extracts prepared from seed kernels, leaves and fruits of *M. azedarach* which also manifested higher antifungal activity against *Bipolaris oryzae, Aspergillus flavus, Sclerotinia sclerotiorum* and *Fusarium verticillioides*. Another species *Toona ciliata* has been the less explored tree, having carbohydrates, proteins, sterols, flavonoids, glycosides, tannins and phenolic compounds.

Phytochemical compounds found in botanical extracts exert biological activity against plant fungal pathogens in vitro and in vivo and can be used as bio-fungicidal products (Romanazzi *et al.,* 2012). The secondary metabolites found in botanicals cause toxicity to membranes and organelles, inhibiting the mycelial development of the targeted pathogen (Nagamma *et al.,* 2019). These are also recognised to have antifungal properties and their efficacy is being tested against various phyto-pathogenic fungi for the production of natural plant products as antifungal agents (Cowan, 1999). These natural plant products play a vital role in plant disease management. Hence, there is a dire need to develop botanical bio-fungicides with improved performance and wide applications. Therefore, the present study aims to systematically investigate the crude extracts of leaf, bark and fruit for their phytochemical properties and antifungal activities against the brown spot pathogen.

The leaves and bark of the three selected species were collected from multiple trees ranging in age from 11-13 years growing at the Research Farm, Department of Forestry and Natural Resources, Punjab Agricultural University, during the months of March to August. The fully ripened fruits of *Melia azedarach* (Dhrek) and *Toona ciliata* (Toon) were collected in the months of May-June, while the fruits of *Azadirachta indica* (Neem) were collected in July-August during the peak fruiting period. They were washed 2–3 times in tap water and then washed again with sterile distilled water. Samples were dried in a hot-air oven at 60°C for one week and then ground in an electric grinder so as to obtain fine powder. The extracts were obtained by adding dry powdered tissues to distilled water/fractionated 50% ethanol in a 1:1 w/v proportion for 48 hours at 4°C. Then the extract was filtered through double-layered muslin cloth and then centrifuged at 5000 rpm for 30 min; the supernatant was filtered through Whatman No.

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1 filter paper and sterilised at 100°C for 30 min. The extract was preserved aseptically in a brown bottle at 5°C until further use. The obtained extracts served as crude extracts (100% concentration) and were used as a stock solution (Hussain *et al.,* 2012).

A phytochemical analysis of the different crude extracts was conducted at the PG laboratory of the Department of Botany, PAU, Ludhiana. The method of Balabaa *et al.* (1974) was adopted for estimating total flavonoid content. The 0.2 ml of crude solution was mixed with methanol (3 ml), aluminium chloride (0.2 ml, 10%), potassium acetate (0.2 ml, 1 M) and distilled water (5.6 ml) and the mixture was incubated for 30 min at room temperature. Then the absorbance was determined at 415 nm against a blank. Methanol (1 ml) in place of the sample solution was used as the blank, and Quercetin was used as the standard solution. The data was calculated using a standard curve obtained from various standard concentrations and was expressed in units mg/g DW.

The determination of total phenolic content was done using the procedure given by Bray and Thorpe (1954). 0.2 ml of the crude extract of each sample was pipetted out in test tubes. With the help of distilled water, the final volume in each test tube was reduced to 3 ml. 0.5 ml of Folin-Ciocalteau reagent was added and incubated at room temperature for 3 minutes, and then 2 ml of sodium carbonate was added to it with gentle mixing. Test tubes were then kept in a boiling water bath for 1-2 minutes and rapidly cooled under tap water. The absorbance was measured at 720 nm. Gallic acid was used as a standard and expressed in units mg/g DW.

Total tannins content was determined using Sadasivam and Manickam (1992) methodology. A 0.2 ml of the crude sample was taken, followed by the addition of 5.5 ml distilled water and 0.5 ml of the Folin-Denis reagent. Folin-Denis reagent was prepared by dissolving 100 g sodium tungstate and 20 g phosphomolybdic acid in 750 ml distilled water and 50 ml phosphoric acid was added. After 2 hours of refluxing, the volume was reduced to 1 litre with distilled water. After 5 minutes, 1 ml of a 35% sodium carbonate solution was added. With distilled water, the final volume was reduced to 10 ml and thoroughly shaken on a cyclomixer. The intensity of colour so developed was measured at 760 nm after 30 minutes against a reagent blank. The standard curve was prepared by using tannic acid in the range of 0-100µg and was expressed in units mg/g DW.

Total terpenoid content was determined using the standard protocol of Ghorai *et al.* (2017). Five ml of chloroform was added to 0.2 ml of each crude sample. The samples were mixed thoroughly, and 0.1 ml of concentrated sulphuric acid was added to each sample. The samples were incubated in the dark for 1.5–2 hours. Each sample contained a reddish brown precipitate. The reaction mixture's supernatant was then decanted without disturbing the precipitate. 1.5 ml of 95% (v/v) methanol was added to the precipitates obtained from the reaction mixture, and the precipitate was vortexed to completely dissolve in methanol. The intensity of the reddish brown colour was observed at 538 nm against the 95% methanol blank. The amount of total terpenoids was calculated from the standard curve using linalool (4-14 µg) as a standard and expressed in units mg/g DW.

The methodology of Baccou *et al.* (1977) was employed to estimate the total saponin content. To remove the alcohol, 0.2 ml of the crude extract was placed in new tubes and placed in a boiling water bath. After removing it from the water bath, it was allowed to cool down. Later, 2 mL of ethyl acetate, 1 ml each of Reagent A (0.5 ml of anisaldehyde and 99.5 ml of ethyl acetate) and Reagent B (concentrated H_2SO_4) was added. The solution was vortexed and incubated at room temperature for 10 minutes. At 430 nm, the formed red complex was measured against a blank reagent. The amount of saponin was quantified from the saponin standard curve (10-50 µg) and expressed in units mg/g DW.

The fungus was isolated from brown spot-infected leaves collected from a rice growing area, which were washed with sterile water to remove the dirt from the surface, and the infected portion of the leaf along with some of the green portion was cut into small pieces. These were dipped in 1% sodium hypochlorite for one minute, followed by three washings with distilled water to remove excess sodium hypochlorite. Then the leaf bits were transferred into the sterile petri dishes containing potato dextrose agar medium amended with streptomycin sulphate to avoid bacterial contamination under aseptic conditions. The isolated PDA plates were incubated at 24±2ºC for 3 days and the actively growing mycelium was sub cultured. A portion of culture was taken on a slide and observed under a compound microscope, where the pathogenic structures were identified with the help of relevant literature (Mew and Gonazales, 2002). The fungus, thus purified, was confirmed through microscopy and kept in the refrigerator until use. All these procedures were done aseptically in the laminar air flow chamber. The pure culture was examined at 40x magnification to study the conidial characters. The pure culture was viewed under a scanning electron microscope available at NIPER, Mohali, Chandigarh to examine the fine surface features of fungal conidia and the hyphal behaviour of the test fungi.

The *in vitro* experiment was conducted in the laboratories of the Department of Plant Pathology, PAU,

Fig. 1. Pure culture and conidia of *Bipolaris oryzae* as observed under Leica Bright Field Research Microscope.

Ludhiana. The antifungal activity of these botanical extracts was tested at different concentrations on potato dextrose agar medium, employing the poisoned food technique against *B. oryzae*. To determine final concentrations, required amounts of double-strength, sterile PDA medium were amended with varying amounts of plant extracts and aseptically poured into sterile plates. Similarly, control was maintained by placing a 5-mm disc of the test fungi in the centre of the non-poisoned PDA medium. The extracts were incorporated into PDA at a high temperature to avoid microbial contamination on plates. Three replications were maintained for each concentration and inoculated plates were then incubated at 28±2°C and colony diameter was measured till the mycelial growth in the control covered the entire plate. The efficacy was expressed as a percent inhibition of mycelial growth, which was calculated using the formula given by Vincent (1947).

The experimental design of the study was kept as CRD and all experiments were repeated independently three times with at least three independent replicates. Using SPSS statistical software, analyses were performed by analysis of variance in conjunction with Tukey's post hoc test ($p \leq 0.05$).

The *B. oryzae* colonies were fast growing, velvety, compact, and abundant with short aerial mycelium (Fig. 1). The surface is initially green and becomes black with a raised periphery. The pathogen produces both intra- and inter-cellular mycelium, which appears dark olive to grey or black in colour. Microscopic examination demonstrated a simple brown conidium in a sympodial pattern (Fig. 2). Conidiophores are produced in small groups or alone and are geniculate, thick, and erect. Conidiophores are dark olivaceous at the base and

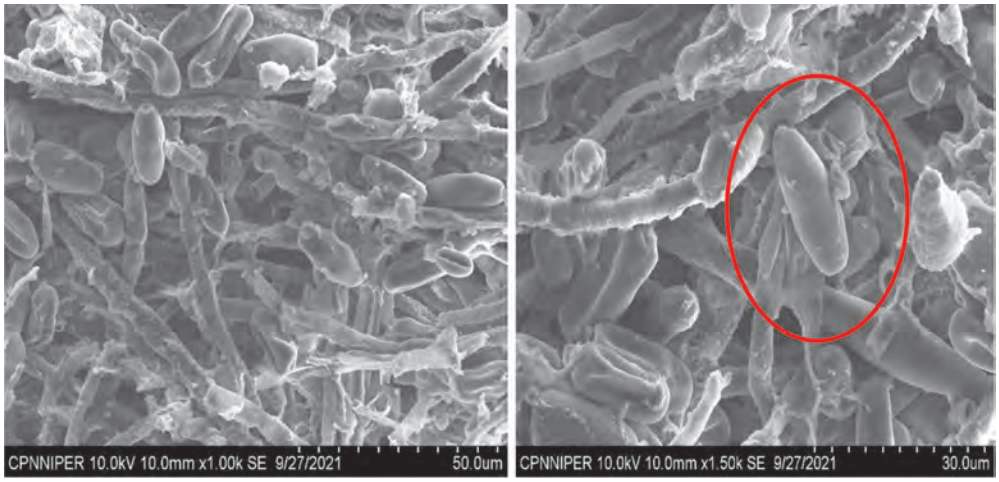

Fig. 2. Scanning electron micrograph of *B. oryzae* at different resolutions revealing surface morphology (scale bar: 50µm; 30µm)

Table 1. Phytochemical analysis and secondary metabolites composition of botanical extracts of selected tree species

Tree species	Botanical extract		Total Phenol content (mg/g DW)	Total Flavonoids (mg/g DW)	Total Tannins (mg/g DW)	Total Saponin content (mg/g DW)	Total Terpenoid content (mg/g DW)
Azadirachta indica (neem)	Leaf	Aqueous	31.30^{b}±0.55	14.33^{cd}±1.05	18.63^{b}±0.55	9.88^{def}±0.46	12.56^{fghijk}±.48
	Leaf	50% Ethanol	34.62^{b}±1.58	19.28^{ab}±1.00	21.95^{ab}±1.58	13.34^{b}±1.00	16.30^{bcd}±.95
	Fruit	Aqueous	34.02^{b}±1.07	11.70^{def}±1.30	21.34^{b}±1.06	10.53^{cde}±0.27	16.45^{bc}±1.23
	Fruit	50% Ethanol	38.23^{a}±1.27	11.61^{def}±2.09	25.56^{a}±1.27	12.37^{bc}±0.02	19.47^{a}±0.56
	Bark	Aqueous	22.84^{de}±.16	7.59^{g}±0.61	10.17^{def}±0.15	7.25^{gh}±0.12	12.87^{fghij}±1.23
	Bark	50% Ethanol	25.29^{cd}±0.56	10.45^{efg}±1.59	12.62^{cd}±0.55	8.73^{efg}±0.48	15.98^{bcde}±1.53
M. azedarach (Dhrek)	Leaf	Aqueous	20.26^{efg}+4.28	11.08^{def}±2.71	6.36^{efgh}±5.18	8.22^{efg}±1.41	9.71^{k}±0.48
	Leaf	50% Ethanol	18.54^{fgh}±1.05	13.15^{de}±1.05	6.53^{efgh}±1.23	8.46^{efg}±1.05	13.45^{defgh}±0.94
	Fruit	Aqueous	22.01^{defg}±1.58	16.61^{bc}±1.58	10.76^{cde}±1.73	11.91^{bcd}±1.58	13.60^{cdefg}±1.23
	Fruit	50% Ethanol	27.22^{c}±0.64	21.82^{a}±0.64	14.43^{c}±1.54	17.12^{a}±0.64	16.62^{b}±0.56
	Bark	Aqueous	15.54^{hi}±0.92	10.13^{efg}±0.92	7.10^{efg}±0.79	5.44^{hi}±0.92	10.02^{jk}±1.23
	Bark	50% Ethanol	16.94^{ghi}±0.85	11.54^{def}±0.85	9.95^{def}±0.02	6.84^{gh}±0.85	13.13^{efghi}±1.53
Toona ciliata (Toon)	Leaf	Aqueous	17.96^{gh}±0.02	12.56^{de}±0.02	5.92^{fgh}±0.64	7.86^{fg}±0.02	6.86^{l}±0.48
	Leaf	50% Ethanol	18.56^{fgh}±0.10	13.16^{de}±0.10	9.91^{def}±1.25	8.45^{efg}±0.10	10.60^{hijk}±0.95
	Fruit	Aqueous	13.93^{i}±1.05	8.53^{fg}±1.05	2.37^{h}±1.22	3.83^{i}±1.05	10.75^{ghijk}±1.23
	Fruit	50% Ethanol	13.75^{i}±.49	8.35^{fg}±0.49	2.82^{gh}±0.55	3.65^{i}±0.49	13.77^{bcdef}±0.56
	Bark	Aqueous	17.04^{ghi}±.71	11.64^{def}±0.71	4.51^{gh}±1.06	6.94^{gh}±0.71	7.17^{l}±1.23
	Bark	50% Ethanol	17.29^{ghi}±1.26	11.89^{def}±1.26	6.33^{efgh}±1.15	7.19^{gh}±1.26	10.28^{ijk}±1.53

Values depicted by same letter are not significantly different as per Tukey's HSD test ($p \leq 0.05$).

lighter at the apex (Fig. 2). Typically, conidia are septate, curved, fusoid and navicular. Fully mature conidia are brown and germinate from their two polar ends (Fig. 1), while less mature, subhyaline conidia produce germ tubes from intermediate segments (Imran *et al.,* 2020).

Secondary metabolites are biologically active forms of compounds found in plants that contribute to a variety of antimicrobial plant properties (Ngadze 2014). The perusal of data from Table 1 revealed the phytochemical content in different botanical extracts prepared from various parts, viz., leaves, fruit and bark of three selected tree species namely *A. indica*, *M. azedarach* and *T. ciliata* belonging to the family Meliaceae*. A. indica* contained the maximum phenolic content followed by *M. azedarach ciliata*. The fruits of *A. indica* had the highest phenolic content, followed by its leaves and bark. The ethanol extracts possessed higher phenol content than aqueous extracts, with maximum phenol content in the ethanol fraction of *A. indica* fruits and minimum in *T. ciliata* fruit extract. Phenolic compounds act on various pathogens related to fungal diseases via the mechanism of inhibiting essential enzymes of the pathogen through specific interactions with sulfhydryl groups and non-specific interactions with other fungal proteins (Chakraborty and Chakraborty, 2010).

Significant variation was noticed for flavonoid content among different botanical extracts and a variable response was recorded. Maximum flavonoid content was observed in the fruits of *M. azedarach* followed by *A. indica* and *T. ciliata*. They also possess antimicrobial properties against a wide range of microorganisms. It is a known fact that they have been found under *in vitro* conditions to be effective antimicrobial agents against numerous phytopathogens (Cowan 1999).

Tannins are astringent in nature and possess the ability to arrest or inhibit the growth and development of several microorganisms. Maximum tannin content was found in *A. indica* followed by *M. azedarach* and *T. ciliata*. Significantly higher tannins were recorded in the fruits and leaves of *A. indica* and the lowest in the fruits of *T. ciliata*. The mode of antimicrobial action of tannins is potentially due to inactivation of microbial adhesins and cell envelope transport proteins (Singh *et al.,* 2008). This is achieved by either protein precipitation or by restricting the availability of nutritional proteins to growing fungi.

M. azedarach fruit extracts had the highest saponin content, followed by *A. indica* and *T. ciliata*. The antifungal activity of saponins has been known for decades and their activity against fungal plant

Table 2. Effect of different botanical extracts prepared from trees of family Meliaceae on mycelial inhibition (%) of brown spot pathogen

Tree species	Botanical extracts		*Bipolaris oryzae*					
			10%	20%	30%	40%	50%	MEAN
Azadirachta indica (Neem)	Leaf	Aqueous	19.38	27.14	41.23	52.62	64.25	40.92^{d}
	Leaf	50% Ethanolic	23.17	30.56	51.16	61.21	67.40	46.70^{bc}
	Fruit	Aqueous	24.54	34.88	47.45	61.64	71.87	48.07^{b}
	Fruit	50% Ethanolic	31.03	41.07	52.72	64.25	76.34	53.08^{a}
	Bark	Aqueous	7.65	12.3	19.59	28.27	36.15	20.79^{i}
	Bark	50% Ethanolic	11.28	17.41	25.18	33.91	41.2	25.79^{h}
Melia azedarach (Dhrek)	Leaf	Aqueous	12.82	18.51	25.58	35.52	44.32	27.35^{g}
	Leaf	50% Ethanolic	16.63	23.58	27.98	38.27	50.62	31.41^{f}
	Fruit	Aqueous	24.63	32.77	44.77	48.79	57.31	41.65^{d}
	Fruit	50% Ethanolic	29.11	34.85	47.57	55.46	63.96	46.19^{c}
	Bark	Aqueous	6.91	12.25	19.53	21.43	25.68	17.16^{j}
	Bark	50% Ethanolic	9.94	20.08	26.66	32.51	39.1	25.65^{h}
Toona ciliata (Toon)	Leaf	Aqueous	10.91	16.75	23.5	33.74	40.37	25.054^{h}
	Leaf	50% Ethanolic	19.04	20.72	28.74	39.04	49.12	31.33^{f}
	Fruit	Aqueous	9.35	14.93	21.16	25.16	33.81	20.88^{i}
	Fruit	50% Ethanolic	13.81	20.72	27.03	35.04	40.85	27.49^{g}
	Bark	Aqueous	18.47	25.81	31.34	37.26	47.91	32.15^{f}
	Bark	50% Ethanolic	20.92	29.41	38.64	48.52	56.72	38.84^{e}

Values depicted by same letter are not significantly different as per Tukey's HSD test ($p \leq 0.05$).

pathogens of crops has been reported many times in the literature (Kumar *et al.,* 2021).

Terpenoid content was higher in ethanol extracts than in aqueous extracts. This shows that some compounds are more effective and extraction is more efficient in an ethanolic solvent as compared to an aqueous one. *A. indica* contained the highest terpene content, followed by *M. azedarach* and *T. ciliata*. The fruits of *A. indica* followed by its leaves and bark contained the most content. *T. ciliata* retained the lowest fraction of soluble terpenoids as compared to the other two species. The secondary metabolites found in botanicals cause toxicity to the cell walls, membranes and organelles of the targeted pathogen (Nagamma *et al*., 2019) employing the mechanism of spore germination inhibition and reduced mycelial development.

Data regarding the effect of botanical extracts from different parts of tree species on the *in vitro* growth of *B. oryzae* is illustrated in Table 2. The tested botanicals exhibited differential antifungal activity and significantly inhibited the growth of *B. oryzae* at different concentrations (Table 2). The increase in concentration of botanical extracts in the amended PDA increased the inhibition of mycelial growth. Botanical extracts showed mostly linear concentration dependent inhibitory effects on fungi growth in our experimental set up (Sarda *et al.,* 2020). Ethanol leaf (67.40%) and fruit extracts (76.34%) of *A. indica* significantly suppressed the colony growth of the tested fungal species. In contrast to that, bark extracts, whether aqueous (20.79%) or ethanolic (25.79%), did not show any pronounced antifungal effects. In general, the ethanol extracts showed better mycelial growth inhibition than the aqueous extracts at every concentration tested**.** Among *A. indica*, fruit extracts (Fig. 3) showed significantly higher growth inhibition than their other counterparts, like leaf and bark. Maximum % growth inhibition was recorded with ethanolic neem fruit extract at 50% concentration (76.34%) followed by aqueous neem fruit extract at 50% concentration (71.87%). Neem contains various metabolites like nimbanene, nimbin, 6-desacetylnimbinene, nimbolide, nimbandiol and ascorbic acid which showed inhibitory activity against various fungal pathogens (Ara *et al.,* 1990). Azadirachtin is the main limnoid phytoconstituent obtained from the seeds of the neem tree (Akhila and Rani 1999). Botanical extracts can be made from any part of the tree, but the seeds contain the highest concentrations of azadirachtin (Yoon *et al.,* 2013).

Among *M. azedarach*, fruit extracts at 50% concentration performed significantly better than leaf

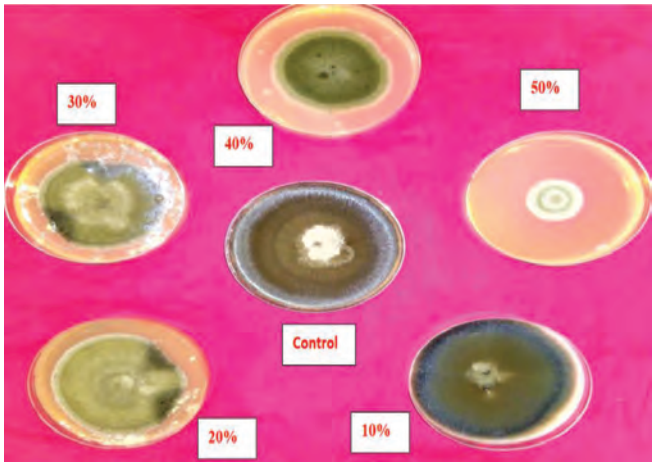

Fig. 3. Antifungal activity of ethanolic fruit extracts of *A. indica* against *Bipolaris oryzae*

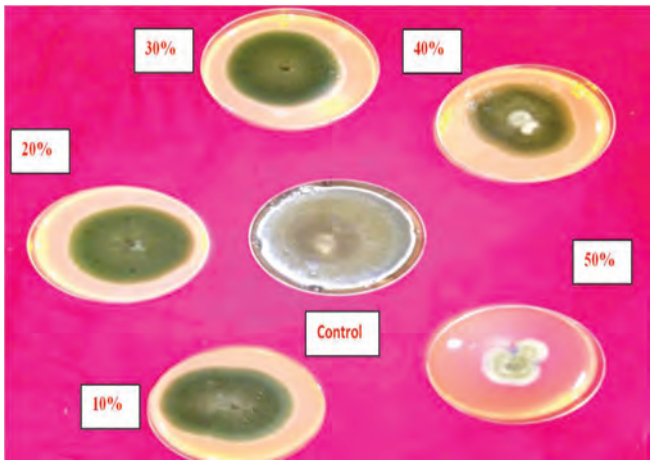

Fig. 4. Antifungal activity of ethanolic fruit extracts of *M. azedarach* against *Bipolaris oryzae*

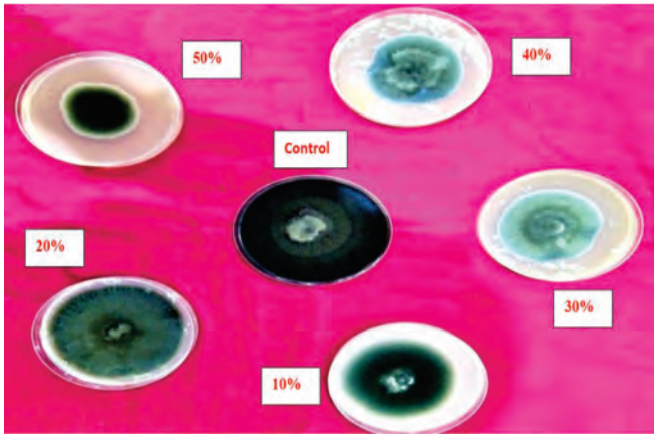

Fig. 5. Antifungal activity of ethanolic bark extracts of *T. ciliata* against *Bipolaris oryzae*

and bark. There was a gradual reduction in fungal colony diameter as the fruit extract concentration was increased to 50%. There was a maximum reduction of 63.96% in fungal colony diameter due to 50% ethanol fruit extract (Fig. 4). Aqueous fruit extracts also resulted in 24.6–57.31% mycelial growth inhibition. Lower concentrations of 10% leaf extracts exhibited a 12–16% reduction in fungal colony growth. However, the highest concentration of 50% reduced fungal growth by 44%-50%. Different plant parts showed variable antifungal activity. Bark extracts were found to be comparatively less effective against the target fungal pathogen as compared to extracts of other parts of *M. azedarach*. Aqueous bark extracts of *M. azedarach* (25.68%) exhibited the least percent inhibition of mycelial growth inhibition as compared to other treatments. Our results are in conformity with Carpinella *et al.,* (2003) who reported that botanical extracts prepared from seed kernels, leaves and fruits of *M. azedarach* manifested higher antifungal activity against *B. oryzae.*

Among *T. ciliata*, it was found that there is an inverse relationship between the concentration of the botanical extracts and the linear growth of *B. oryzae,* i.e., as the concentration increases, the linear growth decreases. Bark extracts were the most effective inhibitors for the growth of *B. oryzae* compared with the control (Fig. 5). Ethanol bark extracts at 50% concentration (56.72%) recorded significantly higher percent mycelial inhibition as compared to their leaf (49.12%) and fruit (40.85%) extracts. Though, the percent inhibition was statistically lower than *A. indica* and *M. azedarach* extracts. Our results are in accordance with Kumar *et al.*, (2012), who reported that natural tetratriterpenoids like cedrelone obtained from the powdered bark of *T. ciliata* had fungicidal activity against the groundnut rust pathogen (*Puccinia arachidis*).

The current study shows that the bark, leaf, and fruit of all three tree species had tested positive for different phytochemical compounds. All of the botanical extract concentrations tested had an inhibitory effect on colony growth, but the highest concentrations had a particularly pronounced effect on *B. oryzae* growth.

A. indica provided excellent control followed by *M. azedarach* and *T. ciliata*. Commercial exploitation of the compounds found in these botanicals could be used to screen and develop such novel types of natural bio-fungicides for controlling brown spot.

Authors' contribution

Conceptualization of research work and designing of experiments (RS, AK, VKS, ST); Execution of field/lab experiments and data collection (RC); Analysis of data and interpretation (RC, AK); Preparation of manuscript (RC, ST).

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