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Antifungal activities and chemical characterization of Neem leaf extracts on the growth of some selected fungal species in vitro culture medium.

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ABSTRACT: The efficacy of different extracts of neem leaf on seed borne fungi *Aspergillus*, *Rhizopus* and chemical characterization of the neem leaf extracts were studied in vitro on the culture medium. The growth of both the fungal species was inhibited significantly ($p<0.01$) and controlled with both alcoholic and water extract of all ages and of the concentrations used. The alcoholic extracts of neem leaf was most effective in comparison to aqueous extract for retarding the growth of *Rhizopus* and *Aspergillus*. The crude aqueous and alcoholic leaf extracts of neem was more effective in inhibitions of growth of the fungi *Aspergillus* in comparison to inhibitory effects on Rhizopus growth in the artificial culture medium. Leaf extracts of neem which are cheap and environmentally safe are promising for protecting crop species against the fungal infestation and leading towards improvement of the crop in terms of yield and productivity. @ JASEM

A large number of chemicals have been developed for the control of plant diseases. But due to over-growing awareness of the hazardous side effects of these chemicals, more and more emphasis is being given to the use of biocontrol agents. Now major challenge is felt in the field of plant pathology to introduce some ecofriendly and safe alternative control strategies for agriculture, which led researchers to turn their attention to plants and microorganisms as sources of biocontrol agents. As the sources of biocontrol agent, neem has already emerged at the top of the list of plants with the highest potential. The following species of neem trees of Meliaceae family have been the subject of botanical biocontrol research: *Azadirachta indica* A. Juss., *A. excelsa* Jack, *A. siamens* Valeton, *Melia azadirachta* L, *M. toosendon* Sicb. and Zucc. and *M. volkensii* Gurke. The Meliaceae specially *A. indica* (Indian neem tree), contains at least 35 biologically active principals of which Nimbin and azadirachtin (T.D. Pennigton *el al.,* 1981) are the most active insecticidal ingradients and are present predominantly in the seeds, leaves and other parts of the neem tree (Mulla *et at., 1999).*

In this study the effect of the leaf extracts of various ages of neem leaves along with different extractants. On in vitro culture medium of *Aspergillus* and *Rhizopus* were studied and subsequent chemical characterization of the need leaf extracts were mediated for its antifungal activity.

MATERIAL AND METHODS

Extraction of leaf extracts: Juvenile and mature leaves were collected separately from *Azadirachta indica* plants growing in University campus, Burdwan University. For antifungal and secondary metaibolite studies fresh leaves of 2-4 days old and 7-9 days old were collected during emergence time (February-March.). Collected fresh leaves *of A. indica* (Neem) were washed thoroughly in tap water and sterile distilled water, air-dried at 27°C, weighted (l00g) and ground in a sterile mortar. The paste was added to 100ml of sterile distilled water in 250 ml beaker, stirred vigorously and allow to stand for 1 hour and then filtered through four folds of sterile cheese cloth to obtain water extract.

Percentage inhibition of fungi growth by the leaf extracts was calculated using the formula:

$\% FG = Dc-Dr/Dc \times 100$

Where: %FG = % inhibition of fungi growth

D_c = diameter of control

D_r = diameter of test

In vitro tests: Species of *Aspergillus* and *Rhizopus* were collected from the Mycology and Plant Pathology Laboratory, Department of Botany, The University of Burdwan and are maintained in pure live on potato dextrose agar (PDA) slants at 4 ^{0}C. For evaluation of in vitro antifungal activity of the biocide (plant extract of *Azadirachta indica*) the phytoextracts were added to Potato Dextrose Agar (PDA) medium in different concentrations (0.1%; 0.5% and 1%) in separate sterilized petriplates. Each plate was inoculated with a mycelial disc (5mm diameter) taken from 7-day-old culture raised on PDA. The inoculated plates were incubated at 30 ± 1°C and the diameter of colony of the pathogen was measured in each case for successive 7 days (Dutta, 2001). The results are shown in table 1.

Table: l: Effect of crude aqueous and alcoholic leaf extracts of Neem *(A. indica*) of different ages on the growth of *Rhizopus* sp. and *Aspergillus* sp. in artificial culture medium.

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Experimental material	Nature of leaf extract	Ages of leaf (days)	Conc. of extract (%)	Measurement of percentage inhibition of Growth (mm) of fungi in artificial medium after hours of incubation						
				24	48	72	96	120	144	196
Aspergillus	Alcoholic Extract	Young 2-4 days	0.1	15.79	12.0	36.84	32.69	46.91	45.56	4.0
			0.5	31.58	24.0	47.36	36.54	49.38	48.89	42.22
			1.0	100.0	100.0	81.58	78.85	82.72	78.89	68.89
			SD(1%)	**1.30**	**1.071**	**1.08**	**1.21**	**1.28**	**1.29**	**1.31**
		Mature 7-9 Days	0.1	21.05	24.00	44.74	34.92	49.38	47.78	42.22
			0.5	42.10	36.0	5.00	38.46	56.79	57.78	51.11
			1.0	100.0	100.0	84.21	84.62	87.65	84.44	73.33
			LSD(1%)	**1.19**	**0.95**	**1.05**	**1.89**	**0.88**	**0.68**	**1.19**
	Water Extract	Young 2-4 days	0.1	0.0	4.0	28.95	34.62	39.51	6.67	0.0
			0.5	5.26	12.0	31.58	40.38	44.44	44.44	36.67
			1.0	26.32	2.0	44.74	5.0	59.26	46.67	38.89
			LSD(1%)	**0.74**	**0.68**	**1.41**	**3.16**	**1.59**	**1.29**	**1.27**
		Mature 7-9 Days	0.1	5.26	4.0	31.58	42.31	45.68	42.22	24.49
			0.5	15.79	8.0	36.84	42.31	51.85	53.33	44.44
			1.0	26.32	16.0	42.11	5.00	65.43	67.78	61.11
			LSD(1%)	**0.82**	**1.41**	**0.89**	**0.88**	**1.36**	**1.28**	**3.60**
		Control	0.0	19	25	38	52	81	90	90
Rhizopus	Alcoholic Extract	Young 2-4 days	0.1	16.67	13.33	30.95	22.22	26.79	11.11	0.0
			0.5	54.17	5.00	61.90	6.00	35.71	18.05	18.05
			1.0	100.0	100.0	90.48	71.11	67.86	61.11	63.33
			LSD(1%)	**1.79**	**0.99**	**2.34**	**0.89**	**0.94**	**1.07**	**0.73**
		Mature 7-9 days	0.1	37.50	4.0	47.62	42.22	30.36	8.33	10.00
			0.5	54.17	5.0	61.90	6.00	35.21	27.78	15.56
			1.0	100.0	100.0	90.48	82.22	78.57	68.05	64.44
			LSD(1%)	**0.96**	**0.56**	**1.81**	**1.38**	**2.18**	**1.25**	**1.54**
	Water Extract	Young 2-4 days	0.1	20.83	1.00	14.29	11.11	14.29	2.86	0.0
			0.5	37.50	23.33	19.05	17.78	23.21	4.17	6.67
			1.0	41.67	3.00	26.19	24.44	33.93	2.78	0.0
			LSD(1%)	**3.27**	**0.65**	**0.79**	**0.84**	**0.86**	**0.46**	**0.01**
		Mature 7-9 days	0.1	16.67	6.67	11.90	15.56	21.43	1.39	0.0
			0.5	25.00	16.67	21.43	2.00	25.0	9.72	0.0
			1.0	33.33	33.33	26.19	13.33	19.64	12.50	12.22
			LSD(1%)	**0.96**	**2.99**	**1.97**	**1.00**	**1.39**	**0.78**	**0.01**
		Control	0.0	24	30	42	45	56	72	90

Mean of 3 replicates.

Chemical characterization of Neem Isolates: For extraction, isolation and identification of active ingredients such as alkaloids, phenolics, terpenoides etc. solvent extraction procedure of Harborn, 1998 was adopted. Extracts obtained as above Harborn, 1998, were concentrated to 1ml and 20µl loaded on TLC plates (Silica gel G 0.2 ml) and developed by the following solvents: Acetic acid: Ethanol (1:3); Acetic acid: Water (1:10); Ethyl acetate: Ethanol (1:3); Hexane: Ethyl acetate (1:1); Methanoi: Toluene (8:2). The spot was observed on the TLC plates and R_f value was calculated by using the following formula :

R_f = distance traveled by center of component / distance traveled by solvent front

R_f value signifies the retention factor i.e., more the molecular weight the more will be the distance traveled by the isolates.

All chemicals were Anal R grade. Standard compound Nimbin was obtained from Himalaya Drug Chemicals as "Neem" (Ref: BPN 329).

RESULTS

In vitro tests: Results of the present investigation shows that the growth of both the saprophytic fungus *Rhizopua* and *Aspergillus* was inhibited with the crude aqueous and alcoholic extract of different aged leaves of *Azadirachla indica* (Table-1). From the result it is evident that the inhibition of growth of both the fungus was more pronounced with ethanolic leaf extracts as compared to aqueous leaf extracts. Significant inhibition of growth of *Rhizopus* and *Aspergillus* observed in the artificial culture media containing older leaf extracts *of Azadirachta indica.* Of the concentration of aqueous and alcoholic leaf extracts of different aged leaves it was observed that higher concentrations leaf extracts were more effective on growth inhibition of both *Rhizopus* and *Aspergillus* and it was also noted that from early period of incubation inhibition of growth occurred (Fig.-l & 2, Plate: 1).

* Corresponding author: *Mondall , N. K.

PLATE - 1

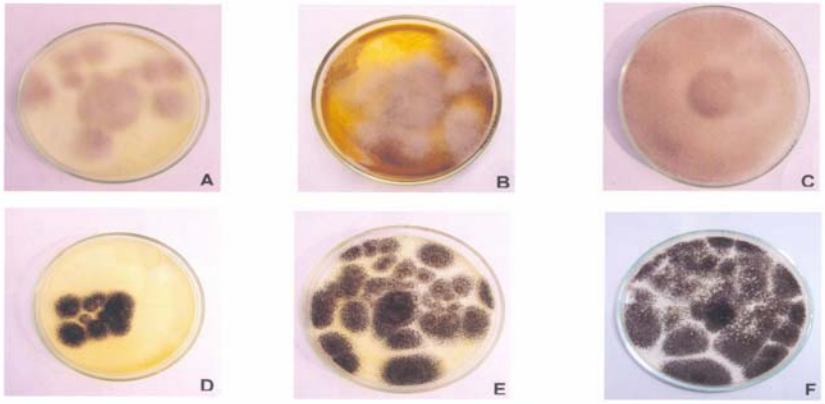

Plate 1 : Inhibitory effects of phytoextracts of neem (*Azadiachta indica*) on the growth of *Aspergillus* sp. and *Rhizopus* sp. in artificial culture medium.

a. Growth of *Rhizopus* sp. in 1% phytoextract (alcholoic) + PDA medium.
b. Growth of *Rhizopus* sp. in 1% phytoextract (water) + PDA medium.
c. Growth of *Rhizopus* sp. in PDA medium (control).
d. Growth of *Aspergillus* sp. in 1% phytoextract (alcholoic) + PDA medium.
e. Growth of *Aspergillus* sp. in 1% phytoextract (water) + PDA medium.
f. Growth of *Aspergillus* sp. in PDA medium (control).

PLATE - 2

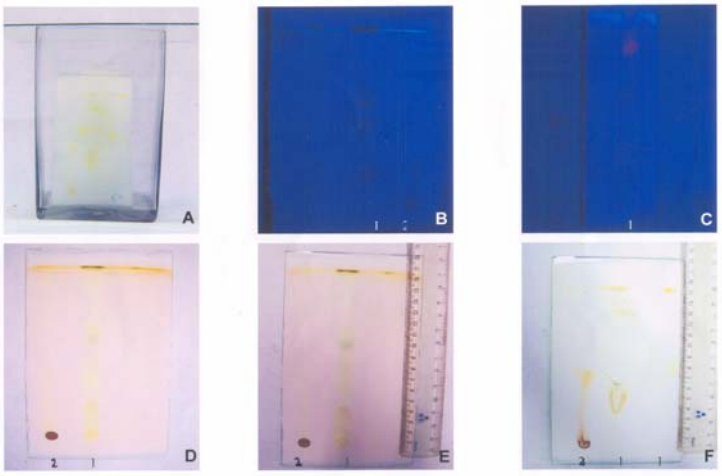

Plate 2 : Thin Layer chromatography (Photographs)

a. TLC procedure.
b. Photograph of TLC plate under UV light, solvent – Hexane : Ethyl acetate (1 : 1).
c. Photograph of TLC plate under UV light, solvent – Methanol : Toluene (8 : 2).
d. Photograph of TLC plate under visible light, solvent – hexane : Ethyl acetate (1 : 1).
e. Photograph of TLC plate under visible light, solvent – Hexane : Ethyl acetate (1 : 1).
f. Photograph of TLC plate under UV light, solvent – Methanol : Toluene (8 : 2).
1. Material
2. Standard

Chemical characterization of Neem leaf isolates: Semi quantitative estimation and identification of active principles of the crude leaf extracts of *Azadirachta indica* were performed by TLC method (Table: 2, Plate: 2). In the present study TLC separation of ethanolic extract of the plant material present a large number of compounds as revealed by fluorescents spots when visualized under UV light (Table: 2). Two of the spots (0.09 and 0.91) were found to have similar Rf values as that of the standard Nimbin. Other spots could not be identified due to lack of standards. Among the various TLC solvent tries such as Acetic acid: Ethanol (1:3); Acetic acid: Water (1:10); Ethyl acetate: Ethanol (1:3); Hexane: Ethyl acetate (1:1); Methanol: Toluene (8:2). Hexane: Ethyl acetate (1:1) was the best as it was able to separate 9 spots from the crude extract and Methanol: Toluene (8:2) solvent exhibits the second best as it was able to separate 7 spots from the crude extract.

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Table 2: Thin Layer Chromatography of leaf extracts and their Rf values.

Solvent: Hexane:Ethyl acetate (1: 1)						Solvent: Methanol : Toluene (8:2)					
Nimbin			Material			Nimbin			Material		
Rf	Visible light	UV Light	Rf	Visible Light	UV Light	Rf	Visible Light	UV Light	Rf	Visible Light	UV Light
0.09	–	Blue	0.09	–	Blue	0.45	Light green	Pink	0.27	Light green	Dark
0.91	–	Blue	0.10	–	Pink	0.51	Green	Pink	0.30	Light green	Pink
			0.19	–	Pink				0.34	Light green	Pink
			0.22	Light green	Dark				0.38	Light green	Pink
			0.38	Light green	Pink				0.45	Light green	Pink
			0.48	Light green	Pink				0.83	–	Blue
			0.58	Green	Pink				0.88	–	Blue
			0.66	Light green	Pink						
			0.91	–	Blue						

DISCUSSION

The efficacy of different extracts of neem against the growth of *Aspergillus* and *Rhizopus* was treated in vitro and chemical characterization of the neem leaf extracts were mediated by TLC method. The present results of this investigation exhibits the radial growth of *Aspergillus* and *Rhizopus* was inhibited *in vitro* By water and ethanolic leaf extracts of *Azadirachta indica,* suggesting the presence of antifungal substances in the plant tissue, which agreed with the results reported by other workers on different pathogens and plants (Tewari and Nayek, 1991; Al-Abed *et* a/., 1993, Qasem *et al,* 1996; Amadioha 1998 and Amadioha 2003). The alcoholic leaf extract was more effective than the water extracts of neem. It was also observed that the mature leaf (7-9 days old) extracts have more inhibitory effect than that of young (2-4 days old) one. The differences in the toxicity of different extracts could be attributed to the presence of the active principles that are extracted by different solvents, which may be influenced by several factors such as age of plant, method of extraction and type of extracting solvent (Nicolls, 1969 and Qasem *et al,* 1996). The greater effectiveness of ethanolic as compared with water extract of the neem leaf may be due to differences in constituent extraction (Shekhawrat and Prasads, 1991). It has been previously reported that the active ingredients of neem constitute mostly of triterpenoides, eg, Nimbin, Nimbidine, Azadirachtin etc. (Brahmachari, 2004). In the present study TLC separation of ethanolic extract of the plant material present a large number of compounds as revealed by fluorescents spots when visualized under UV light (Table: 2). Two of the spots (0.09 and 0.91) were found to have similar Rf values as that of the standard Nimbin. Other spots could not be identified due to lack of standards. Among the various TLC solvent tries such as Acetic acid: Ethanol (1:3); Acetic acid: Water (1:10); Ethyl acetate: Ethanol (1:3); Hexane: Ethyl acetate (1:1); Methanol: Toluene (8:2). Hexane: Ethyl acetate (1:1) was the best as it was able to separate 9 spots from the crude extract and Methanol: Toluene (8:2) solvent exhibits the second best as it was able to separate 7 spots from the crude extract.

Therefore, from the foregoing discussion it may be concluded that *Azadirachta indica,* a common medicinal plant could be exploited as the source of a potent biocide that have immense fungi toxic effect to several fungal pathogens like *Aspergilhts* and *Rhizopus.*

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