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CHEMICAL CHARACTERIZATION OF NEEM LEAF EXTRACTS AND ITS FUNGITOXIC EFFECT ON CERTAIN PLANT PATHOGENS

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Abstract

The effect of neem leaf extracts on seed borne fungi, viz, Alternaria, Rhizopus, and Pyricularia were studied in vitro in culture medium along with the characterization of extracts. The growth of the fungal pathogens was inhibited significantly and controlled in both ethanolic and water extracts of all ages and of all concentrations used. The ethanolic extract of neem leaf was found to be the most effective for retarding the growth of fungal pathogens. Leaf extracts of neem which are cheaply available and environmentally safe are promising for protecting crop species against the fungal pathogens which may ultimately lead to crop yield and productivity.

Keywords: Characterization, extract, in vitro, pathogens, productivity.

Introduction

We always prefer to have a resistant variety in agriculture for crop production. In the absence of resistant varieties, we go in for chemical control. Although chemical control is an imperative need, due to environmental pollution and development of resistance to various organisms, we go in for biological control of plant diseases. As a source of bio control agent, neem has already emerged at the top of the list of plants with the highest potential. The Meliaceae family, especially Azadirachta indica (Indian neem tree) contains more than 30 biologically active principles of which Nimbin and Azadirachtin[1] are the most active insecticidal and fungicidal ingredients and are most predominantly present in the leaves, seeds and other parts of the neem tree[2].

In this present study, the effects of leaf extracts of various ages of neem leaves along with different extractants were studied. The invitro culture medium of Alternaria, Rhizopus and Pyricularia was studied and subsequent chemical characterization of the neem leaf extracts were tested for its antifungal activity.

Material and Methods

Extraction of leaf extracts:

The mature leaves were collected separately from neem plants growing in the university campus, Bharath University, Chennai, India. For fungitoxic and secondary metabolic studies, fresh leaves of 3-5 days old and 8-10 days old were collected during emergence time (March-April). The collected fresh leaves of neem were washed thoroughly in tap water and sterile water, air dried at 29°C, weighed (100gms) and ground in a sterile mortar. The paste was added to 100ml of sterile distilled water in 250ml beaker, stirred vigorously and allowed to stand for 1hour and then filtered through sterile cheese cloth to obtain water extract.

The percentage of inhibition of fungal growth by the leaf extract was calculated using the formula:

The percent inhibition of fungal growth = 100(DC-DR)/DC*

Where, DC = diameter of control

DR = diameter of test

In vitro tests:

Species of Alternaria, Rhizopus and Pyricularia were collected from the Department of Plant Pathology, TNAU, Coimbatore and are maintained in pure culture on potato dextrose agar (PDA) slants at 4°C. For evaluation, of invitro fungitoxic activity of the biocide (plant extract of neem), the phytoextracts were added to PDA medium in different concentrations (0.1%, 0.5%, and 1%) in separate sterilized Petriplates. Each plate was inoculated with a mycelial disk of 5mm diameter, taken from the 7 day old culture, raised on PDA. The inoculated plates were incubated at 30±1℃ and diameter of the colony of pathogen was measured in each case for successive 7 days[3]. The results are shown in table 1.

Table 1: Antifungal activities and chemical characterization of neem leaf extracts on the growth of fungal species.

Experimental Material	Nature of leaf extract	Ages (days)	Conc. Of extract (%)	Measurement of % inhibition of growth (mm) of fungi in artificial medium after hours of incubation (hrs)						
				24	48	72	96	120	144	168
Alternaria	Alcoholic Extract	3-5 Young	0.1	12.79	9.3	36.84	32.69	43.91	47.56	5.90
			0.5	27.40	19.90	47.36	36.54	49.38	48.89	41.49
			1.0	100	100	87.35	78.85	82.72	78.89	68.89

			CD	2.75	2.19	2.61	3.15	3.41	2.95	2.82
		8-10 Mature	0.1	15.95	14.20	39.30	37.00	45.14	49.15	51.12
			0.5	20.40	19.90	42.35	33.15	39.38	39.15	40.50
			1.0	33.50	37.18	49.00	39.15	52.00	51.00	49.15
			CD	2.52	2.15	2.40	2.11	2.45	2.62	2.95
	Water Extract	3-5 Young	0.1	0.00	4.00	27.45	29.35	33.71	40.71	0.00
			0.5	12.45	13.70	39.15	31.30	37.75	39.45	36.71
			1.0	23.10	21.15	37.10	30.90	36.45	37.00	38.00
			CD	3.52	3.65	3.45	3.25	3.52	3.00	3.15
		8-10 Mature	0.1	5.00	4.00	27.45	31.15	37.41	45.15	27.10
			0.5	15.75	7.00	36.84	31.40	39.15	38.10	39.15
			1.0	24.15	23.10	37.95	38.50	51.50	52.10	46.10
			CD	2.35	2.75	1.98	1.75	2.95	2.15	2.06
	Control		0.0	19	25	33	51	80	90	90
Rhizopus	Alcoholic Extract	3-5 Young	0.1	16.57	13.35	30.95	22.22	26.80	10.11	0.00
			0.5	47.10	7.00	51.85	6.00	35.71	17.05	17.10
			1.0	100	100	90.50	71.11	65.86	60.11	63.45
			CD	3.61	3.95	4.10	3.75	3.92	3.65	2.65
		8-10 Mature	0.1	37.50	4.00	41.62	40.45	30.36	8.31	10.00
			0.5	54.17	5.00	59.95	6.00	35.21	27.78	15.56
			1.0	100	100	85.45	82.10	78.57	68.05	64.41
			CD	4.05	3.85	3.75	3.92	3.85	4.05	4.15
	Water Extract	3-5 Young	0.1	20.73	1.00	14.30	11.11	14.29	2.86	0.00
			0.5	35.50	23.15	19.05	17.18	23.21	4.17	5.97
			1.0	40.67	3.00	26.20	24.44	33.93	3.78	0.00
			CD	3.27	2.95	2.75	2.62	2.95	3.50	0.01
		8-10 Mature	0.1	16.67	6.67	11.90	15.56	21.43	1.39	0.00
			0.5	25.00	16.67	21.43	2.00	25.00	8.72	0.00
			1.0	33.15	33.33	26.19	13.33	19.64	9.50	10.22
			CD	1.97	2.99	3.45	2.98	3.15	3.10	2.90
	Control		0.0	24	30	42	45	56	72	90
Pyricularia	Alcoholic Extract	3-5 Young	0.1	12.79	12.00	33.84	30.69	36.91	41.56	4.00
			0.5	31.58	24.00	47.36	35.54	49.38	48.59	40.22
			1.0	100	100	81.58	68.85	72.61	76.89	68.90

			CD	3.91	4.05	2.99	3.72	4.19	4.21	4.72
		8-10 Mature	0.1	21.05	24.00	44.74	34.92	46.38	46.78	46.10
			0.5	40.10	36.00	4.00	38.46	53.79	57.78	51.10
			1.0	100	100	74.21	84.62	86.65	84.44	77.10
			CD	4.15	4.65	4.10	3.95	3.62	3.10	3.95
	Water Extract	3-5 Young	0.1	0.00	5.00	26.95	34.62	39.51	6.67	0.00
			0.5	4.26	13.10	31.58	40.38	44.14	40.44	31.67
			1.0	23.32	2.00	44.74	5.00	58.26	46.67	38.89
			CD	2.75	4.20	3.91	2.65	2.92	2.85	2.95
		8-10 Mature	0.1	5.26	4.00	31.58	42.31	43.68	42.22	24.49
			0.5	15.79	8.00	36.84	41.31	51.85	50.33	41.14
			1.0	23.32	16.00	41.11	5.00	61.43	63.88	59.11
			CD	2.15	2.78	2.85	3.95	3.71	3.27	4.10
	Control		0.0	24	30	42	45	56	90	90

Characterisation of Neem isolates:

For extraction, isolation and identification of active ingredients, such as phenolics, terpenoides, etc., solvent extraction procedure of Harborn, 1998 was adopted. Extracts obtained were concentrated to 1ml and 20µl loaded on TLC plates (Silica gel G 0.2ml) 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), methanol:toluene(8:2). The spot was observed on the TLC plates and Rf value was calculated by using the following formula.

Rf=distance travelled by center of component/distance travelled by solvent front

Where, Rf value signifies the retention factor, ie, more the molecular weight, the more will be the distance travelled by the isolates.

Results:

***Invitro* tests:** The results of the present studies indicated that the growth of the fungal pathogens, Alternaria, Rhizopus and Pyricularia was inhibited with the crude aqueous and alcoholic extract of different aged leaves of neem (Table 1). It is evident from the results that the inhibition of growth of the fungal pathogens was more pronounced with ethanolic leaf extract, as against the aqueous leaf extracts. A significant inhibition of growth of the pathogens was observed in the artificial culture media containing older leaf extracts of neem. Among the aqueous and alcoholic

leaf extracts of aged leaves, it is surmised that the higher concentration of leaf extracts were more effective on the growth inhibition of fungal pathogens.

Characterization of Neem leaf Isolates:

The quantitative estimation and identification of active principles of the crude leaf extracts of neem were performed by TLC method (Table 2). In the present study, TLC separation of ethanolic extract of the plant material provides a large number of compounds, as revealed by the fluorescent spots, visualized through UV light (Table 2). Two of the spots (0.09 and 0.91) were found to have the same Rf value, as that of the standard Nimbin. Others could not be identified. Among the various TLC solvents tried, such as Acetic acid:ethanol(1:3), Acetic acid:water(1:10), Hexane:ethyl acetate(1:1) and methanol:toluene(8:2), solvent were the best as it were able to separate spots from the crude extract.

Table 2: Thin Layer Chromatography of leaf extract and 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	0.24	Light Green	Dark
0.91	-	Blue	0.10	-	Pink	0.51	Green	Pink	0.20	Light Green	Pink
			0.19	-	Pink				0.34	Light Green	Pink
			0.22	Light Green	Dark				0.35	Light Green	Pink
			0.28	Light Green	Pink				0.41	Light Green	Pink
			0.38	Light Green	Pink				0.83	-	Blue
			0.48	Green	Pink				0.88	-	Blue
			0.56	Light Green	Pink						
			0.91	-	Blue						

Discussion

The effect of neem extracts against the growth of Alternaria, Rhizopus, and Pyricularia was treated invitro and characterization of the neem leaf extracts were also mediated by the TLC method. The present studies indicated that the radial growth of the pathogens were inhibited invitro by water and alcoholic leaf extracts of Azadirachta indica, indicating the presence of antifungal, fungitoxic property in the plant tissues, which agrees with the findings reported by other workers and different pathogens and plants[4,5]. The ethanolic leaf extract was more effective than the water extracts of neem. It is also surmised that the mature leaf of 8-10 days old extracts were found to have more inhibitory

effect. The differences in the antifungal activity could be attributed to have the presence of active principles that could be extracted by different solvents which could be influenced by factors such as method of extraction, type of extracting solvents, and age of the plant[5]. Shekhawart and prasads, 1991 also reported on this and findings[6] are in consonance with his report also. He has also reported on the active principles of neem constitute mostly of triterpenoides, eg. Nimbin, Nimbidin, and Azadirachtin, etc.

In the present study, the TLC separation of ethanolic extract of plant material presents a large number of compounds, as revealed by the fluorescent spots, visualized through 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. Others could not be identified due to lack of standards. Hexane:ethyl acetate(1:1) and Methanol:toluene (8:2) solvents were the best and were able to separate 9 and 7 spots from the crude plant extracts.

Hence it may be concluded that the neem plant (Azadirachta indica), a common medicinal plant could be used as a source of potent biological agent that have immense fungal toxic effect to several fungal pathogens like, Alternaria, Rhizopus and Pyricularia.

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