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Effect of Neem (*Azardirachta indica* A. Juss) Seeds and Leaves Extract on Some Plant Pathogenic Fungi

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Abstract: In this study plant pathogenic fungi *Alternaria solani*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* were chosen to study the effect of ethanolic, hexane and methanolic extracts of neem seeds and leaves. Antifungal effects of neem leave and seed extracts obtained by ethanol, hexane and ptrolium ether were examined separately *in vitro* against *Fusarium oxysporum*, *Rhizoctonia solani*, *Alternaria solani* and *Sclerotinia sclerotiorum*. Results indicated that seeds and leaves extracts could cause growth inhibition of tested fungi, although the rate of inhibition of tested fungi varied with different extracts and concentrations. But all these extracts and concentrations of extract inhibited the growth of pathogenic fungi at a significant level. Azadirachtin, nimonol and expoxyazdirodione were detected from neem extract by using High Performance Liquid Chromatography (HPLC). We can conclude that neem leave and seed extracts were effective as antifungal against all tested fungi but *F. oxysporum* and *R. solani* were the most sensitive fungi.

Key words: Neem extracts, ethonolic, methanolic, hexane, pathogenic fungi

INTRODUCTION

Alternaria solani, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* are well known as plant pathogens. *Alternaria solani* causes early blight in tomatoes and potatoes (Haware, 1971; Conrath *et al*., 2003; Reni and Voorrips, 2006) *Fusarium oxysporum* causes Fusarium wilt disease in different plants (Shanmugan *et al*., 2007; Ploetz, 2000). *Rhizoctonia solani* and *F. solani* causes damping off (Yangar *et al*., 2008), while *Sclerotinia sclerotiorum* causes stem rot (Mueller *et al*., 2002; Young *et al*., 2004). The neem tree is a tropical evergreen plant and is a native plant of India and Burma. Recently it gains importance in the research because of potential of using neem derivatives such as leaf, oil and seed extracts for preparation of environmental friendly herbicides (Verma and Kharwar, 2006).

Leaf extract of neem can inhibit the aflatoxin production as well as *Aspergillus parasiticus* growth (Ghorbanian, 2007; Allameh *et al*., 2002). Antifungal effects of neem leaf extract also reported from south America against *Crinipellis perniciosa* and *Phytophthora* species causing Witches broom and Pot Not of cocoa (Ramos *et al*., 2007).

Antifungal, antibacterial and anti insecticidal component Azadirachtin, limonoid and terpenoids have been extracted from seeds and leaves of neem (Dai *et al*., 2001; Nathan *et al*., 2005; Jarvis and Morgan, 2000). These neem extracts have also been reported to be effective against malaria vector *Anopheles stephensi* (Nathan *et al*., 2005; Koul *et al*., 2004). About ten years back neem trees were imported from India and planted in the Arafat area of Makkah, Saudi Arabia. The aim of this study was to analyzed the antifungal properties of the extracts of leaves and seeds of neem against some plant pathogenic fungi.

MATERIALS AND METHODS

Plant materials: Neem leaves and seeds were obtained from Arafat area of Saudi Arabia.

Plant parts were cleaned with deionized water and dried at 50°C for 24 h. The dried plant parts were ground and then sieved with 80 mesh sieve.

Extraction: The method of Phasuda and Varipat (2004) was adopted for extraction with little modification. Briefly, 20 g portions of the powdered plant materials were soaked separately in solvents (80 mL of each ethanol, hexane and petroleum ether) at ambient temperature for 24 h under shaking condition at 130 rpm. The extract was then filtered using Whatman filter paper No. 1 and re-filtered using 0.22 micro filter paper (Sartorius, Germany). The filtrate was kept in the freezer at -20°C for further study.

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Microorganisms: The microorganisms used included: *Fusarium oxysporum*, *Rhizoctonia solani*, *Alternaria solani* and *Sclerotinia sclerotiorum* were obtained from Microbiological Resource Center MIRCIN, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Assay for the antifungal effects of the neem leaves and seeds organic extract: To assay the antifungal effects of the organic extracts of neem leaves and seeds using tested microorganisms, measurement of radial growth of the used organisms were made following the technique of Phasuda and Varipat (2007) and Nwachukwu and Umechuruba (2001). The *in vitro* tests were carried out to measure the effects of the leaf extracts on radial growth of the seed-borne fungi. Potato Dextrose Agar (PDA) medium was used in this study. To every 15 mL of sterile potato dextrose agar medium in Petri dishes, 5 mL of either crude or aqueous extract of each plant sample were added. The solution in each Petri dish was gently swirled and allowed to solidify. The extract-amended medium in the Petri dishes were inoculated each alone at the centre with 5 mm inoculum- disc of each test fungus and incubated at 25±2°C for 14 days. The medium with inoculums disc but without any extract served as control. Percentage inhibition of mycelial growth by the leaf extracts was calculated using the formula:

$$\%\text{inhibition of mycelialgrowth}\,(MG_I) = \frac{D_c - D_T}{D_c} \times 100$$

Where:
D_C = diameter of control
D_T = diameter of test

Isolation and identification of neem compounds: Neem oil obtained by using a cold mechanical expeller was partitioned between *n*-hexane, ethanolic and the methanolic extract. These were then concentrated to dryness *in vacuo* (vacuum evaporator Beckman USA) at 45°C. The extract was subjected to preparative HPLC for the isolation of triterpenoids. Details of the isolation and purification of major compounds from neem oil, (i.e., deacetylnimbin, azadiradione, nimbin, salannin and expoxyazdirodione,) were described previously (Phasuda and Varipat, 2004). Pure compounds were identified by HPLC analysis. Standard pure compounds were routinely purified in our laboratory through preparative HPLC which forms the source according to the method described by Govindachari *et al.* (1995).

All experiments were carried out in Plant and Microbiology Department Science Collage, King Saud University during the period from 2007-2009.

RESULTS AND DISCUSSION

In general ethanolic and methanolic leaf extract causing more inhibition of all the fungi as compared to hexane leaf extract except in the case of *Sclerotinia sclerotiorum* where hexane extract causes more inhibition than methanolic extract but ethanolic extract remains causes the highest inhibition of growth (Sanjeet *et al.*, 2005; Mossini, 2004). At 10% concentration of extracts, *R. solani* showed the highest inhibition (55.1%) in the case of ethanolic extract and the lowest inhibition of growth was shown by *S. sclerotiorum* (21.4%) in hexane extract and this trend remain the same at other concentrations of extracts . A complete inhibition (100%) of growth was shown by *Fusarium oxysporum* and *R. solani* at 40% level of ethanolic and methanolic extracts. *Alternaria solani* exhibited the highest percent of inhibition (84.0%) followed by methanolic (78.3%) and hexane (73.1%) at 40% concentrations of leave extract, while *S. sclerotiorum* showed the highest inhibition by methnolic (86.1%) followed by hexane (76.5%) and methanolic (67.1%) (Table 1). Results of this study in general, indicates that even at 10% concentration of all types of extract could cause significant inhibition of growth (Gupta and Bansal, 2003; Amadioha, 2004).

Generally similar trends of inhibition were also observed in the case of ethanolic hexane and methanolic neem seed extracts (Table 2) as in the case of leaf extracts. All type of seeds extracts causes higher percentage of inhibition of pathogenic fungi at all concentration used as compared to leaf extracts. *Fusarim oxysporum* and *R. solani* showed 100% inhibition at 30% concentrations of ethanolic and methanolic seeds extracts. This is in contrast with leaf extract results where these fungi showed 100% inhibition at 40% concentration of these extracts. Results clearly indicate that *F. oxysporum* and *R. solani* were the most sensitive fungi to neem leaf and seed extracts followed by *A. solani* and *S. sclerotiorum*. This might be due to production of sclerotia by *S. sclerotiorum* which obviously more resistant to these extracts. Earlier reports indicates that both leaves and seeds extracts of neem have significant antifungal activities (Sanjeet *et al.*, 2005; Mossini *et al.*, 2004; Amadioha, 2004).

Azadirachtin, Azadiradione, nimonol and epoxy azadradione were yielded from the organic extract of seeds and leaves of neem (Table 3, 4). Nimonol (82%) look to be a major active component of neem organic extract. All these component extracted and also have been reported as antifungal, antibacterial, anti insecticidal (Dai *et al.*, 2001; Jarvis and Morgan, 2000; Nathan *et al.*, 2005) and could affect the production of aflatoxin by

Table 1: Effect of ethanolic, hexane and methanolic leaf extracts on the growth of pathogenic fungi (Percent inhibition)

	Percent concentrations of extracts (% inhibition)											
	10			20			30			40		
Fungi	A	B	C	A	B	C	A	B	C	A	B	C
Alternaria solani	32.0	28.2	26.2	54.8	37.0	35.0	83.0	45.2	50.8	84.0	73.1	78.3
Fusarium oxysporum	36.2	43.9	30.9	69.1	47.5	36.2	87.9	68.5	64.1	100.0	89.4	100.0
Rhizoctonia solani	55.1	28.3	40.1	71.1	46.2	45.4	90.9	54.1	72.3	100.0	77.2	100.0
Sclerotinia sclerotiorum	45.7	36.3	21.4	55.9	40.3	27.3	83.9	61.5	44.1	86.1	76.5	67.1

A: Ethanolic, B: Hexane, C: Methanolic

Table 2: Effects of ethanolic, hexane and methanolic seed extracts on the growth of pathogenic fungi (Percent inhibition)

	Percent concentrations of extracts (% inhibition)											
	10			20			30			40		
Fungi	A	B	C	A	B	C	A	B	C	A	B	C
Alternaria solani	36.1	31.7	42.9	57.2	40.8	47.8	56.7	53.4	58.0	89.5	80.1	80.7
Fusarium oxysporum	49.8	52.2	43.5	73.6	57.6	64.1	100.0	68.1	100.0	100.0	97.9	100.0
Rhizoctonia solani	58.9	46.8	48.8	75.3	49.5	69.2	100.0	64.8	100.0	100.0	82.4	100.0
Sclerotinia sclerotiorum	49.2	39.7	24.6	59.7	44.1	29.9	87.8	48.7	52.2	92.5	80.5	71.2

A: Ethanolic, B: Hexane, C: Methanolic

Table 3: High performance liquid chromatographic peaks of ethanolic neem leaf extract

Band No.	Rt (min)	Total peak area detected (%)	Compound (ID)
1	8	67.40	Azadirachtin A (8), B (4), C (9)
2	15	22.10	NI
3	23	57.50	Azadirachtin A (12), B (13), D (3), H (8)6De-acetylnimbin (37)
4	33	30.50	NI
5	37	41.40	Azadiradion (50)
6	52	65.30	Nimonol (79.0)
7	61	75.00	Expoxyazdirodione (6)
8	70	31.50	Expoxyazdirodione (45)
9	75	33.00	NI
10	88	26.50	NI

NI: Not Identified, These peaks were detected by HPLC using ethanolic solvent system and found to be complex mixtures. Values in brackets are percentage

Table 4: High performance liquid chromatographic peaks of ethanolic neem seed extract

Band No.	Rt (min)	Total peak area detected (%)	Compound (ID)
1	8	75.50	Azadirachtin A (11), B (9), C (12)
2	15	30.00	NI
3	23	66.00	Azadirachtin A (15), B (16), D (11), H (12)6De-acetylnimbin (41)
4	33	35.00	NI
5	37	49.00	Azadiradion (58)
6	52	74.00	Nimonol (85.5)
7	61	83.00	Expoxyazdirodione (14)
8	70	39.00	Expoxyazdirodione (48)
9	75	42.00	NI
10	88	38.00	NI

NI: Not Identified, These peaks were detected by HPLC using ethanolic solvent system and found to be complex mixtures. Values in brackets are percentage

Aspergillus species (Allameh *et al*., 2002). Inhibitions of seed-born infection by neem leaf extract have been reported earlier (Massum *et al*., 2009).

In conclusion the results of this study showed that locally growing neem plants which were imported to be planted have retained the antifungal activities although growing under a very different environment compared to their original native land.

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