Identification of Antifungal Compounds from the Seed Oil of *Azadirachta indica*

T.R. Govindachari, G. Suresh, Geetha Gopalakrishnan, Balaganesan Banumathy and S. Masilamani[1]

Evaluation of the activity of the cold expeller neem oil (*Azadirachta indica* A. Juss.) and the fractions derived through solvent partitioning, against *Drechslera oryzae, Fusarium oxysporum* and *Alternaria tenuis* showed that the active antifungal fraction is a mixture of tetranortriterpenoids. Further, testing the triterpenoidal mixture derived from the 90% methanol (MeOH) extract of neem oil against 13 phytopathogenic fungi revealed that various species were inhibited to different degrees. Direct preparative High Performance Liquid Chromatography (HPLC) of the active fractions and subsequent bioassay of the semi-pure fractions indicated that the active fractions contained major compounds such as 6-deacetylnimbin, azadiradione, nimbin, salannin and epoxyazadiradione. Pure azadiradione, nimbin, salannin and epoxy-azadiradione did not have appreciable activity. However, when these terpenoids were mixed and bioassayed, they showed antifungal activity, indicating possible additive/synergistic effects.
KEY WORDS: Neem oil; antifungal activity; 6-deacetylnimbin; azadiradione; nimbin; salannin; nimbin; epoxyazadiradione; preparative HPLC.

INTRODUCTION

Constitutive antifungal triterpenoids of higher plants are known from *Mollugo pentaphylla* (mollugenol A and B), *Ecballium elaterium* (cucurbitacin I) and *Chisocheton paniculatus* (1,2-dihydroxy-6α-acetoxyazadirone and three related meliacins) (4). Although a number of triterpenoids of the limonoid type have been isolated from seeds/seed oil of the Indian neem tree (*Azadirachta indica* A. Juss.) (1), information is not available on their antifungal activities. Use of the crude extractives of seeds of neem for control of plant pathogenic fungi is known and has been amply documented (6,7). The only references to antifungal activities of neem constituents relate to nimbidin (supposedly a mixture of a number of triterpenoids from seed oil) against *Rhizoctonia nodulosum, Alternaria tenuis, Fusarium oxysporum, Helminthosporium nodulosum* and *Curvularia tuberculata* (5), and isomeldenin and nimonol against groundnut leaf rust (12). Using some phytopathogenic fungi as test organisms, Steinhauer (10) isolated a compound with antifungal activity from neem kernel extracts, the identity of which is not known. Herein, we have attempted fractionation, isolation and identification of antifungal triterpenoids from neem oil, the results of which are presented.

Received March 10, 1997; revised version Oct. 17, 1997; final version Jan. 4, 1998; web site posting March 4, 1998.
[1]Centre for Agrochemical Research, SPIC Science Foundation, Madras 600 032, India [Fax: +44-2352163; e-mail: aparajit@giasmda.vsnl.net.in].

Extraction and isolation of neem compounds

Neem oil (1 *l*), obtained by using a cold mechanical expeller, was partitioned between *n*-hexane (1 *l*) and 90% methanol (MeOH) (1.5 *l*) and the MeOH extract was concentrated to dryness *in vacuo* at 45°C (62.8 g). The extract was subjected to preparative HPLC for the isolation of triterpenoids as illustrated in Figure 1. Details of the isolation and purification of major compounds from neem oil, *i.e.*, deacetylnimbin, azadiradione, nimbin, salannin and epoxyazadiradione, were described by us previously (3). Pure compounds were identified by comparison of ^{1}H, ^{13}C and 2D NMR data, HR-Mass spectra, IR, $[\alpha]_D$, C, H analysis and HPLC analysis. Standard pure compounds were routinely purified in our laboratory through preparative HPLC which forms the source.

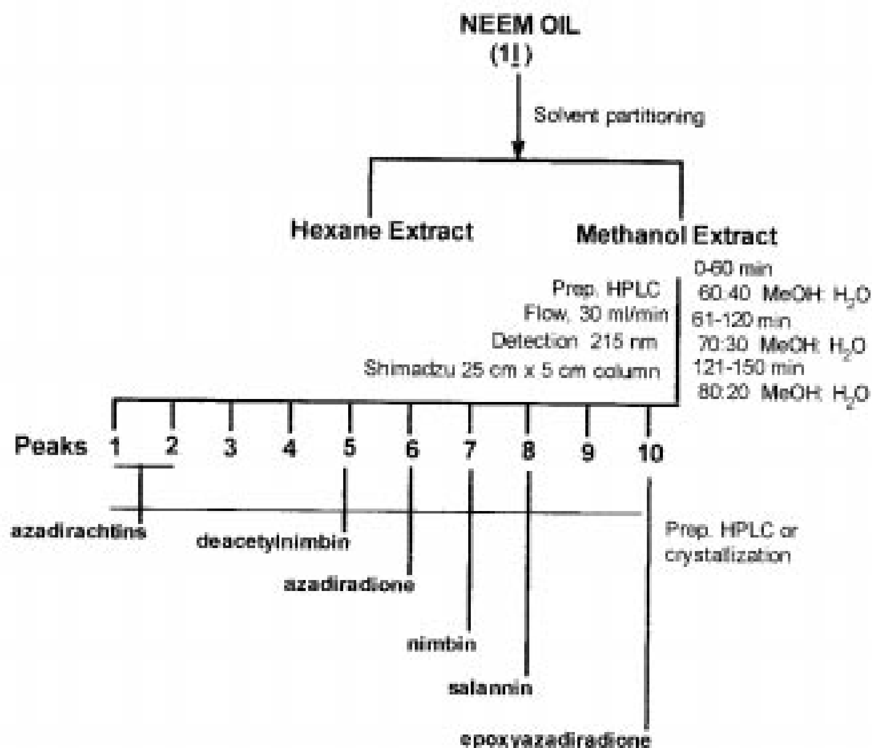

Fig. 1. Extraction and isolation of antifungal compounds from neem oil (8).

Antifungal bioassays

Drechslera oryzae (Breda de Hann) Subram. & Jain (causes leaf spot disease in rice), *Alternaria tenuis* Nrrd. (causes leaf spot in vegetables), and *Fusarium oxysporum* f.sp. *vasinfectum* (Atk.) Snyder & Hansen (wilt pathogen of cotton) were used as test organisms

in all the experiments. The 90% MeOH extract of neem oil was also tested against 13 phytopathogenic fungi other than the above mentioned (see Table 2).

Czapek-Dox medium was used for maintaining the cultures on sterile petri dishes. Weighed quantities of neem oil components to be tested were dissolved in acetone (1 ml), and incorporated into the molten medium (at *ca* 45°C), to reach the desired concentration, 1000 ppm. Addition of an aliquot of acetone alone to the medium served as the control (final acetone percentage = 2%). Fifteen ml of the medium was poured into each sterile petri dish under aseptic conditions and left to settle. Circular plugs (5 mm diam) of the test mycelial mats (punched in mycelial mats grown on Czapek-Dox medium in sterile petri dishes) were inoculated centrally and left to grow. In all cases triplicates were maintained. Neem oil and the extracts were maintained at a concentration of 1000 ppm in the media for studying radial growth inhibition of *D. oryzae, A. tenuis* and *F.o. vasinfectum.* The 90% MeOH extract of neem oil was tested also against 13 further fungal species. Rate of radial growth of the fungus is expressed as diameter in mm at the end of a specific time period after inoculation for each fungus. Data on the rate of radial growth were noted after 72 h and subsequently every 24 h for a total period of 240 h. These data were subjected to analysis of variance using completely randomized blocks and the mean growth values were calculated using the SNK test in a COSTAT program. From the Newman-Keuls mean growth values, the percentage inhibition over control was calculated.

RESULTS AND DISCUSSION

Although neem oil has been used for control of phytopathogenic fungi, such as *Fusarium oxysporum* f.sp. *ciceris, Rhizoctonia solani, Sclerotium rolfsii, Sclerotinia sclerotiorum, Botrytis cinerea, Penicillium expansum, Glomerella cingulata, Alternaria alternata, Aspergilllus niger, Curvularia lunata, Sphaerotheca fuliginea, Plasmopara viticola, Diplocarpon rosae* and several rust pathogens (6), concentrations needed for complete field control were shown to be as high as 2% to 10% (6). High concentrations of neem oil are known to induce phytotoxicity (6). In our experiments the concentration for the antifungal activity assays was kept at 1000 ppm. Cold expeller neem oil at 1000 ppm either brought about no inhibition (against *F.o. vasinfectum*) or minimal inhibition (11.4% against *D. oryzae* and 31.1% against *A. tenuis*) against the test fungi (Table 1). While this confirms earlier reports (6), it may, in part, explain the reason for the use of higher concentrations of neem oil for field control.

In an effort to identify the component(s) that actually impart antifungal activities, we resorted to solvent partitioning of the neem oil between *n*-hexane and 90% MeOH. The *n*-hexane extract showed only marginal inhibitory activity against *A. tenuis* (13.6%) and no activity against *D. oryzae,* while it accelerated the growth of *F.o. vasinfectum* marginally (Table 1). Hexane extracts contain the non-polar substances like fats and fatty acids (9), which, as carbon sources, could stimulate fungal growth. The 90% MeOH extract, on the other hand, inhibited growth of the test fungi to varying degrees. At 1000 ppm, the inhibition percentages for *F.o. vasinfectum, A. tenuis* and *D. oryzae* were 26.5, 35.1 and 72.4, respectively (Table 1). Even with synthetic fungicides, a minimum inhibition concentration (MIC) of 1000–5000 mg/*l* (= 1000–5000 ppm) is considered good activity (8). Based on the inhibition profiles of the 90% MeOH extract, it is evident that *D. oryzae* was the most susceptible among the three fungi, while *F.o. vasinfectum* and *A. tenuis* were less inhibited. Steinhauer (11) investigated the inhibition of phytopathogenic fungi by a

TABLE 1. Inhibition of fungi, in percentage *vs* the control, by neem oil components

Fraction/Compound	*Drechslera oryzae*$^{+}$		*Alternaria tenuis*$^{+}$		*Fusarium oxysporum* f.sp. *vasinfectum*$^{++}$	
	Control	1000 ppm	Control	1000 ppm	Control	1000 ppm
Neem oil	(88±1.4)	11.4 (78±1.4)	(88±0)	31.0 (60.7±0)	(88±0)	0 (88±0)
Hexane extract	(88±0)	0 (88±0)	(85.7±0.8)	13.6 (74±0.8)	(79.3±0.5)	−11.0 (88±0.5)
MeOH extract	(88±0.5)	70.8 (25.7±0.5)	(85.7±0.8)	35.8 (55±0.8)	(79.3±0.3)	26.5 (58.3±0.3)
HPLC peak 1	(88±1.2)	5.7 (83±1.3)	(76.3±1.2)	3.9 (73.3±1.2)	(76.7±0.5)	2.2 (75±0.5)
HPLC peak 2	(88±1.7)	0 (88±1.7)	(76.3±0.8)	-15.3 (88±0.76)	(76.7±0.4)	-1.7 (78±0.4)
HPLC peak 3	(88±3.9)	65.1 (30.7±3.9)	(73.3±0.6)	11.3 (65±0.6)	(68±2.5)	15.7 (57.3±2.5)
HPLC peak 4	(88±3.1)	77.6 (19.7±3.1)	(73.3±0.9)	11.3 (65±0.9)	(68±2.4)	13.7 (58.7±2.4)
HPLC peak 5	(88±3.5)	59.9 (35.3±3.5)	(76.3±0.6)	30.5 (53±0.6)	(76.7±0.5)	49.2 (39±0.5)
HPLC peak 6	(88±2.7)	70.5 (26±2.7)	(76.3±1.3)	30.5 (53±1.3)	(76.7±0.8)	38.7 (47±0.8)
HPLC peak 7	(88±3.0)	69.0 (27.3±3.04)	(76.3±0.8)	31.8 (52±0.8)	(76.7±0.1)	39.6 (46.3±0.1)
HPLC peak 8	(88±5.9)	72.7 (24±5.9)	(76.3±0.6)	32.2 (51.7±0.63)	(76.7±0.5)	35.7 (49.3±0.5)
HPLC peak 9	(88±2.5)	70.1 (26.3±2.5)	(76.3±0.6)	38.4 (47±0.6)	(76.7±0.6)	39.6 (46.3±0.6)
HPLC peak 10	(88±2.3)	82.6 (15.3±2.3)	(73.3±0.9)	30.6 (51.3±0.9)	(68±2.8)	8.4 (62.3±2.8)
6-deacetylnimbin	(88±4.0)	63.3 (32.3±4.0)	(57±1.2)	15.3 (48.3±1.2)	(77.3±0.2)	37.5 (48.3±0.2)
Azadiradione	(88±0)	0 (88±0)	(57±1.1)	-4.7 (59.7±1.1)	(77.3±0.2)	2.1 (75.7±0.2)
Nimbin	(88±0.8)	64.8 (31±0.8)	(57±0.6)	5.8 (53.7±0.6)	(77.3±0.2)	-1.8 (78.7±0.2)
Salannin	(88±3.0)	26.5 (64.7±3.0)	(57±1.2)	-9.3 (62.3±1.2)	(77.3±0.9)	16.3 (64.7±0.9)
Epoxyazadiradione	(88±2.7)	15.1 (74.7±2.7)	(57±1.5)	3.5 (55±1.5)	(77.3±0.1)	0.8 (76.7±0.1)

% Inhibition values derived from Newman-Keuls means of growth (i.d., in mm) of the fungus in treated *vs* control.

Values in parentheses indicate the fungal radial growth (mm) ± SE.

$^{+}$After 240 h. $^{++}$After 168 h.

series of commercial neem products, through linear growth by a method similar to that employed in the present study. Again a *Drechslera* species, *D. teres*, was the one out of ten phytopathogenic fungi that was the most inhibited by all products. The 90% MeOH extract was also tested (at 1000 ppm) against a number of other phytopathogenic fungi

(Table 2). The results confirmed the growth inhibition of different fungi to varying degrees. Among the 13 fungi tested, 90% MeOH extract was most effective against *Pythium aphanidermatum* (71.8%) and *Pestalotiopsis mangiferae* (46.5%), while *Bipolaris indica, Botryodiplodia theobromae, Pyricularia oryzae* and *Fusarium equiseti* were inhibited least (< 20%). Analysis of the 90% MeOH extract by analytical HPLC revealed the presence of major triterpenoids (3). Fungal inhibitory activity may hence be attributed to the triterpenoidal fraction. An attempt was made, therefore, to evaluate the different preparative HPLC fractions of the 90% MeOH extract in order to identify the compound(s) that impart antifungal activity.

TABLE 2. Antifungal activity of 90% MeOH extract (at 1000 ppm) of neem oil against phytopathogenic fungi

Fungus	Source of the fungus	Radial growth (mm ±SE)[z]		% Inhibition[z]
		Control[y]	1000 ppm	
Bipolaris indica Rai, Wadhwani &Tewari	leaves of *Helianthus annuus*	56.4±8.4[b]	48.5±7.1	14.0
Botryodiplodia theobromae Pat.	petiole of *Carica papaya*	72.3±6.0[g]	64.9±9.5	10.2
Colletotrichum lindemuthianum (Sacc. & Magnus) Brioso & Cavara	leaves of *Piper nigrum*	51.6±10.4[a]	41.2±8.4	20.7
Colletotrichum dematium (Pers.:Fr.) Grove	leaves of *Capsicum indicum*	48.3±9.0[a]	36.5±6.8	24.4
Curvularia lunata (Wakker) Boedijn	leaves of *Arachis hypogaea*	61.2±9.4[c]	45.3±7.2	26.0
Fusarium equiseti (Corda) Sacc.	leaves of *Solanum tuberosum*	55.4±10.6[f]	44.5±7.1	19.6
Fusarium solani (Mart.) Sacc.	leaves of *Solanum tuberosum*	41.6±11.8[f]	32.4±1.1	22.1
Koleroga noxia Donk	twigs of *Coffea arabica*	69.2±7.5[g]	51.7±6.4	25.3
Nectria galligena Bresad.	twigs of *Rosa* sp.	53.0±10.0[e]	34.06±6.0	35.8
Pestalotiopsis mangiferae (Henn.) Stey.	fruits of *Mangifera indica*	49.6±6.5[c]	26.6±2.9	46.5
Phoma betae Frank.	fruits of *Capsicum indicum*	56.5±9.6[c]	41.1±6.0	27.2
Pyricularia oryzae Cavara	leaves of *Oryza sativa*	35.4±6.4[d]	29.3±4.6	17.2
Pythium aphanidermatum (Edson) Fitzp.	seedlings of *Solanum tuberosum*	27.4±5.9[b]	7.7±2.8	71.8

[z] % Inhibition values and the fungal radial growth in diam (mm) ±SE derived from Newman-Keuls means of growth (i.d., mm) of the fungi at 1000 ppm *vs* control at maximum growth.
[y] After 252 (a), 238 (b), 214 (c), 190 (d), 166 (e), 142 (f), 94 (g) hours of growth.

Preparative HPLC (on a 25 cm × 5 cm, C18 column with MeOH:H_2O as an eluent in a stepwise gradient) resolved the 90% MeOH extract into ten peaks (3), after the elution of which the column was washed with 100% methanol in order to remove the non-polar components. Analysis of all the ten peaks employing analytical HPLC using a C18 column revealed that peaks 1 and 2 contained mainly azadirachtins A, B, D, H and I (2). Peak 1 contained azadirachtins A, B, D, H and I at 6%, 10%, 10%, 9% and 5% (isolated yields),

respectively. Peak 2 contained azadirachtins A, B, D, H and I at 3%, 5%, 3%, 5% and 2% (isolated yields), respectively. Mixtures of peaks 1 and 2 did not show any appreciable inhibition (Table 1). From the foregoing, it may be surmised that azadirachtins do not possess any antifungal activity. Peaks 3* and 4* yielded small amounts of material, with little or no activity against *F.o. vasinfectum* and *A. tenuis,* but with considerable activity against *D. oryzae* (65.2% and 77.7%, respectively, at 1000 ppm). Attempts are being made to collect these fractions in sizeable quantities to investigate further the individual fractions. Peak 5 was identified as 6-deacetylnimbin of 96% purity (by analytical HPLC) and showed appreciable inhibition against *D. oryzae* (59.9%), *A. tenuis* (30.6%) and *F.o. vasinfectum* (49.2%) at 1000 ppm (Table 1). Pure 6-deacetylnimbin (purified by HPLC) (2) retained antifungal activity against *F.o. vasinfectum* and *D. oryzae,* but showed drastic reduction in activity against *A. tenuis*. Peak 6 (80% azadiradione as the major component), peak 7 (85% nimbin as the major constituent) and peak 8 (90% salannin as the major constituent) evinced excellent inhibitory activity against *D. oryzae* and moderate activity against *A. tenuis* and *F.o. vasinfectum* at 1000 ppm (Table 1). When purified, azadiradione and salannin manifested a drastic reduction in activity against all the test fungi. Nimbin in pure form had reduced activity against *F.o. vasinfectum* and *A. tenuis*, but retained its activity against *D. oryzae.* Only a small quantity of solid could be recovered from peak 9, which resolved into at least three major components in analytical HPLC. Peak 9 showed excellent inhibitory activity against *D. oryzae* (70.1%) and was moderately active against *F.o. vasinfectum* (39.6%) and *A. tenuis* (38.4%) at 1000 ppm (Table 1). Peak 9 is being investigated further. Peak 10 (85% epoxyazadiradione as the major constituent) was most effective against *D. oryzae* (82.6%) and much less effective against *A. tenuis* (28.6%) and *F.o. vasinfectum* (8.3%). Epoxyazadiradione in pure form did not exhibit inhibitory activity against any of the test fungi. It is possible that in pure form the major triterpenoids from oil have very low or no antifungal activity, whereas in combination they show excellent activity against all the three test fungi, suggesting additive/synergistic effects.

Our studies have shown that the 90% MeOH fraction contained mainly terpenoids. Hence, it was suspected that the triterpenoids may be antifungal. Loss of activity after purification prompted us to recreate a mixture by combining pure compounds. Retention of activity of such mixtures of triterpenoids may indicate that there could be additive/synergistic effects of terpenoids in 90% MeOH extracts.

In order to evaluate the finding that the major terpenoids act additively/synergistically, five pure terpenoids were mixed in the following proportion, based on our studies of the concentrations of various triterpenoids as they occur naturally in neem oil from different sources: epoxyazadiradione (1 part): salannin (5 parts): nimbin (4 parts): azadiradione (2 parts): 6-deacetylnimbin (4 parts). This mixture was dissolved in acetone, incorporated into the molten media to obtain concentrations of 1, 10, 100 and 1000 ppm. Assays of the antifungal activities of the mixture against the three test fungi revealed again that maximum inhibition was observed with *D. oryzae* (70.1%) at 1000 ppm (Fig. 2). It is also not surprising that differences existed in inhibition percentages among the test fungi. Hence, the concentration needed to inhibit each fungal species effectively has to be worked out independently. Both the natural triterpenoidal mixture from neem oil, as well as a mixture

* HPLC peaks 3, 4 and 9 were analyzed by preparative HPLC and found to be complex mixtures; the quantities of material collected were also very small. Hence, it was not possible to fractionate these peaks further at the present juncture. This has been illustrated in an earlier paper (2).

made up from pure salannin, nimbin, azadiradione, deacetyl nimbin and epoxyazadiradione were similar in their antifungal activity against the test fungi.

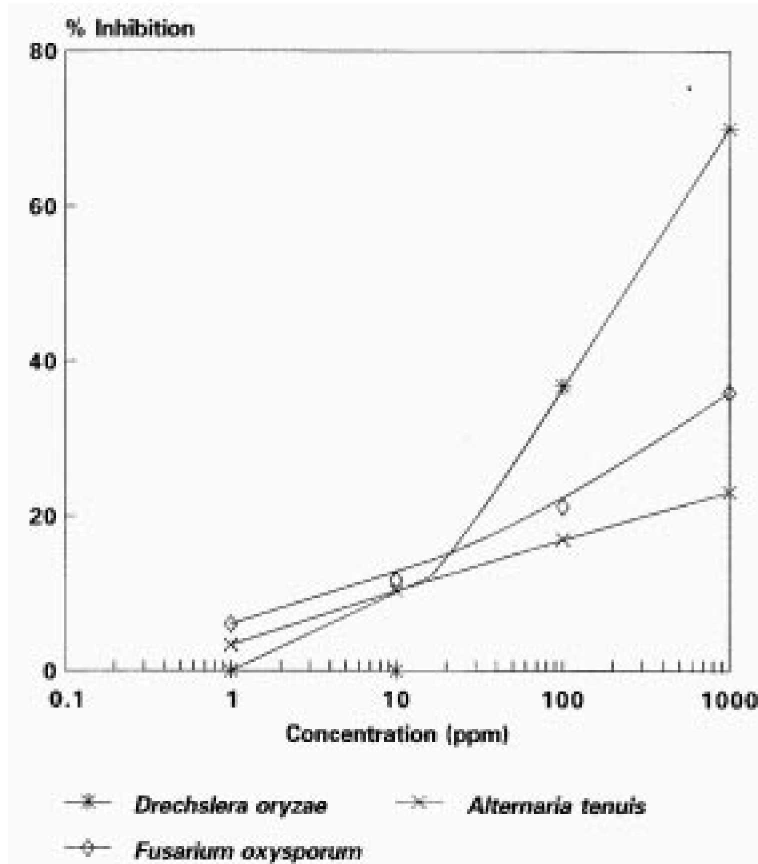

Fig. 2. Antifungal activity of mixture of neem oil triterpenoids, expressed as percent inhibition.

ACKNOWLEDGMENTS

The authors thank the Department of Biotechnology, New Delhi, for financing the research project on antifungal compounds from Meliaceae and Simaroubaceae. B.B. is grateful to CSIR, New Delhi, for a Research Fellowship.

REFERENCES

1. Devkumar, C. and Sukh Dev. (1993) Chemistry. *in:* Randhawa, N.S. and Parmar, B.S. [Eds.] Neem Research and Development. Society of Pesticide Science, New Delhi, India. pp. 63-96.
2. Govindachari, T.R., Gopalakrishnan, G. and Suresh, G. (1996) Isolation of various azadirachtins from neem oil by preparative high performance liquid chromatography. *J. Liq. Chromtogr. & Rel. Technol.* 19:1729-1733.
3. Govindachari, T.R., Suresh, G. and Gopalakrishnan, G. (1995) A direct preparative high performance liquid chromatography procedure for the isolation of major triterpenoids and their quantitative determination in neem oil. *J. Liq. Chromatogr.* 18:3465-3471.

4. Grayer, R.J. and Harborne, J.B. (1994) A survey of antifungal compounds from higher plants, 1982-1993. *Phytochemistry* 37:19-42.
5. Khan, M.W., Alam, M.M., Khan, A.M. and Saxena, S.K. (1974) Effect of water soluble fractions of oil cakes and bitter principles of neem on some fungi and nematodes. *Acta Bot. Indica* 2:120-128.
6. Locke, J.C. (1995) Fungi. *in:* Schmutterer, H. [Ed.] The Neem Tree, Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes. VCH, Weinheim, Germany. pp. 118-125.
7. Parveen, G. and Alam, M.M. (1993) Bioactivity against plant pathogens. *in:* Randhawa, N.S. and Parmar, B.S. [Eds.] Neem Research and Development. Society of Pesticide Science, New Delhi, India. pp. 144-153.
8. Paulus, W. (1993) Microbicides for the Protection of Materials. A Handbook. Chapman and Hall, London, UK.
9. Rukmini, C. (1987) Chemical and nutritional evaluation of neem oil. *Food Chem.* 42:119-124.
10. Steinhauer, B. (1994) Antifungal compounds from *Azadirachta indica. in:* Kleeberg, H. [Ed.] Practice Oriented Results on Use and Production of Neem Ingredients and Pheromones. 3rd Workshop (Wetzlar, Germany), pp. 93-97. Druck & Graphic, Giessen, Germany.
11. Steinhauer, B. (1996) Fungicidal activity of commercial neem products. *in:* Kleeberg, H. and Micheletti, V. [Eds.] Practice Oriented Results on Use and Production of Neem Ingredients and Pheromones. IVth Workshop (Bordighera, Italy), pp. 105-116. Druck & Graphic, Giessen, Germany.
12. Suresh, G., Narasimhan, N.S., Masilamani, S., Partho, P.D. and Gopalakrishnan, G. (1997) Antifungal fractions and compounds from uncrushed green leaves of *Azadirachta indica. Phytoparasitica* 25:33-39.