

Antifungal Fractions and Compounds from Uncrushed Green Leaves of *Azadirachta indica*

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Using the groundnut rust disease (causal agent *Puccinia arachidis* Speg.) as the bioassay system, two limonoids from the neem tree (*Azadirachta indica* A. Juss.) which evinced antifungal activity, were isolated through extraction, solvent fractionation and HPLC. A polar extract derived through solvent partitioning reduced the disease intensity considerably. The polar extract and the impure HPLC fractions were more effective than the pure compounds in reducing the pustule numbers and, consequently, the disease severity.

KEY WORDS: *Azadirachta indica*; neem; nimonol; isomeldenin; antifungal activity; *Puccinia arachidis*; groundnut rust disease.

INTRODUCTION

Subsequent to the isolation of azadirachtin from neem seed kernels in 1968 (2), extensive work has been done on the chemistry and pesticidal properties of compounds from the neem tree, *Azadirachta indica* A. Juss. (11). Information relating to the antifungal activities of compounds from neem is limited (7,10). Neem leaves have been shown to possess antifungal activity either by direct soil amendment or as extracts of them (7), active against a number of phytopathogens (7,8). They reduced radial growth and spore germination of *Curvularia lunata* (1), successfully controlled fruit rots of cucurbitaceous plants caused by *Fusarium equiseti* and *F. semitectum* (6), and significantly reduced fruit rot of tomatoes caused by *Aspergillus flavus* and *A. niger* (12). Aqueous neem leaf extracts controlled foliar diseases of groundnut, viz., *Puccinia arachidis* and *Mycosphaerella berkeleyi* (4).

A number of compounds have been isolated from neem leaves, including protomeliacins (triterpenoids) and limonoids (for complete review, see refs. 3, 5). Cyclic tri- and tetrasulfides have been obtained (9) from the steam distillates. Whereas insect antifeedant properties of some of the triterpenoids and limonoids are known, no information is available on the antifungal activities of these compounds or the fractions containing them. In the present investigation, two antifungal compounds have been isolated from the uncrushed green leaves of *Azadirachta indica* A. Juss.; the groundnut rust fungus *Puccinia arachidis* was used as the bioassay organism. This disease is very important in southern India; it is susceptible to fungicides and to control by such as Bordeaux mixture, fentin hydroxide and chlorothalonil.

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MATERIALS AND METHODS

Extract and isolation of fractions and compounds

Fresh, green neem leaves (5.1 kg f.wt.) were left overnight (*ca* 12 h) undisturbed in a bucket containing 25 l of *n*-hexane and the decanted hexane wash (H₁) was concentrated to a residue. The residue was resuspended in 1 l of *n*-hexane, partitioned with 95% methanol (3 × 500 ml) to give the hexane extract (H₂) and the methanol extract. The extracts were stripped of the solvent to obtain residues. The methanol extract was subjected to reversed phase semi-preparative HPLC (high performance liquid chromatography) (8 × 900 mg) on a RP-18 column (20 mm × 250 mm, Shimpack C₁₈). MeOH : H₂O, 70:30, at 15 ml/min, was used as eluant from 0 to 50 min and changed to MeOH : H₂O, 80:30, from 51 min to the end of the run. Detection was at 215 nm (for details, see Figs. 1 and 2).

Fractions containing peaks 1–6 (550 mg), peak 7 (2,874 mg) and peak 8 (720 mg) were collected. Peak 7 was shown by analytical HPLC (70:30, MeOH : H₂O, 1 ml/min at 215 nm, Shimpack ODS column, 4.6 mm × 25 cm) to have a major component (91.2%) with small impurities of peaks 1–6 (8.8%). Peak 7 on crystallization yielded pure nimonol having structure **1** (13). Peak 8 yielded a major component (93%), small polar impurities from peaks 1–6 (3.1%), and nimonol (3.9%). Peak 8 was subjected to Lobar column chromatography (Merck) (silica gel 60) using 25% EtOAc in *n*-hexane (30 ml fractions) to yield another limonoid, isomeldenin (**2**), in Lobar chromatography fractions (13). CH analysis and the NMR spectra of compounds **1** and **2** indicated that the compounds were pure.

Fig. 1. Fractionation of uncrushed neem leaf extract.

Fig. 2. The structure of nimonol (1) and isomeldenin (2).

Infection of plants by groundnut rust

Arachis hypogaea L. var. TMV-2 was raised and maintained in a number of 3 m² plots. Field-collected uredospores of *Puccinia arachidis* Speg. were suspended in water (50,000 spores/ml). Spore suspension (0.1 ml) was spread on the abaxial surface of the leaflets, detached from 60-day-old plants and kept in petri plates covered inside with wetted filter papers to maintain moisture, to facilitate infection. The fungus was subsequently maintained for further experiments on detached leaflets through uredospores collected from the infected leaves.

Fig. 3. The antifungal effect of extracts, fractions and pure compounds obtained from fresh uncrushed neem leaves, expressed as reduction of number of pustules of *Puccinia arachidis* on groundnut leaves. 3a - H₁; 3b - H₂; 3c - methanol fraction; 3d - mixture of peaks 1–6;

3e - peak 7; 3f - peak 8; 3g - pure nimonol (1); 3h - isomeldenin (2). The figures in the curves are Newman-Keul means. For comparison between concentrations on a given day, the \pm S.E. values have been recorded (see top left corner of each figure).

For the antifungal bioassay, surface area of each groundnut leaflet was measured using an electronic area meter (ΔT systems). Neem leaf fractions/compounds were dissolved in acetone to get concentrations of 0.005, 0.05, 0.5 and 5.0 $\mu\text{g}/\text{cm}^2$ of leaflet area. The test solution was spread on the abaxial surface of the leaflet. Acetone only served as the control. After air-drying, 0.1 ml of spore suspension was spread evenly on the surface of the leaflet. The leaflets were transferred into the moist petri plates, kept in a dark chamber for 48 h, and subsequently transferred to a light chamber (3,000 lux). The number of rust pustules appearing on the lower surface was counted at 24-h intervals from the 7th day after inoculation until the 13th day. The data were then subjected to analysis of variance (ANOVA) using a COSTAT program in an IBM PC/AT computer. The Newman-Keul means are presented in Figures 3 a-h. For comparison between concentrations on a given day, the \pm S.E. has been indicated in each figure.

RESULTS AND DISCUSSION

Groundnut leaves treated with the *n*-hexane wash (H_1) of the fresh uncrushed leaves had a reduced number of rust pustules (Fig. 3a). By the 13th day slightly more than 50% reduction in the number of pustules was noticed even at a concentration of 0.005 $\mu\text{g}/\text{cm}^2$ leaf area (38.2 ± 3.6 pustules, compared with 80.8 ± 4.9 pustules in the control). With 5.0 $\mu\text{g}/\text{cm}^2$ leaf area, > 80% reduction in pustules was noted on the 13th day (14.8 ± 2.7 pustules). This indicated that the *n*-hexane wash (H_1) contained compounds with antifungal activity.

The leaves treated with 0.005 μg and 0.05 $\mu\text{g}/\text{cm}^2$ *n*-hexane extract (H_2) did not show any reduction in the number of pustules on the 13th day as compared with control. In fact, from day 7 to day 11, pustule formation was accelerated at these two concentrations compared with the control (Fig. 3b). It is possible that partitioning of the *n*-hexane residue (H_1) between *n*-hexane and 95% methanol resulted in the concentration of the antifungal polar material into the methanol portion and enrichment of the fatty material and waxy material in the *n*-hexane extract (H_2). The linear fatty material and waxes in the H_2 , with the antifungal compounds now being concentrated in methanol extract, could be responsible for the acceleration of pustule formation by groundnut rust in the H_2 -treated leaves. Partitioning thrice with 95% methanol presumably did not remove the polar antifungal compounds completely, and some retention of activity was observed in the *n*-hexane (H_2) fraction at high concentrations (0.5 and 5.0 $\mu\text{g}/\text{cm}^2$ leaf area) (Fig. 3b).

The methanol fraction showed a marked increase in antifungal activity which was comparable to that of the *n*-hexane (H_1) extract (Fig. 3c). This indicated that the polar methanol fraction contained the antifungal activity. Fractionation of the polar fraction by semi-preparative HPLC resulted in at least eight distinct peaks, of which peak 7 (rt. 39.7 min) and peak 8 (rt. 52.9 min) were the major ones (Fig. 2). Peaks 1–6 were present in much lower quantities (550 mg). Individual analysis of peaks 1 to 6 by HPLC revealed that each peak was a complex mixture. Isolation of individual components was not attempted. The mixture of peaks 1–6, as eluted from HPLC, was assayed for antifungal activity. At 0.005 $\mu\text{g}/\text{cm}^2$ leaflet, mixture of peaks 1–6 brought about a 65% reduction in pustule number on day 13. At the higher concentrations of 0.05, 0.5 and 5.0 $\mu\text{g}/\text{cm}^2$, between 80% and 90% reduction in pustule number was recorded.

Peak 7 (Fig. 3e) and peak 8 (Fig. 3f), which were the major constituents of the methanol extract, brought about a marked reduction in pustule number. With both peak 7 and peak 8, even at the lowest concentration, approximately 80% reduction in pustules was noticed (18 ± 1.7 and 6.05 ± 1.7 pustules/leaflet, respectively, on day 13). Peak 8 was the most effective among the three HPLC fractions, followed by peak 7 and peaks 1–6. Peak 7, which contained negligible impurities of peaks 1–6, was distinctly more active (Fig. 3e) than pure nimonol (Fig. 3g). Similarly peak 8, which contained impurities of peaks 1–6 and peak 7, was more active (Fig. 3f) than isomeldenin (Fig. 3h). It would appear that although these pure limonoids are active in decreasing the number of pustules, in mixtures with impurities they reduced the disease intensity even more.

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