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Nematicidal Principles from Neem (*Azadirachta indica* A. Juss) Part III Isolation and Bioassay of Some Neem Meliacins

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Eight pure C-secomeliacins including azadirachtin, a mixture of isomeric azadirachtins and two fractions of diametrically opposite polarity were derived from neem (*Azadirachta indica* A. Juss) seed kernels both by isolation and partial synthesis. Different concentrations of the C-seco meliacins between 50 and 250 ppm along with carbofuran at 20 ppm as the reference nematicide were evaluated *in vitro* against root- knot nematode (*Meloidogyne incognita* Kofoid and White) Chitwood. The bioassay results expressed as per cent mortality and LC_{50} values, respectively after 24 and 48 h treatments revealed that all the 8 meliacins were highly nematicidal with LC_{50} values ranging between 55 and 157 ppm. The non-polar fraction which was tentatively identified as a long chain ketone was inactive and the other two fractions were moderately active. A highly significant positive correlation was obtained between LC_{50} and R_f of test meliacins, thereby implicating the influence of hydrophilicity of the molecule on nematotoxicity. Another salient observation was the delayed nematicidal action of some meliacins such as nimbin and salannin, which has a striking parallelism to the typical slow mode of action in arthropods.

Neem (*Azadirachta indica* A. Juss) cake and its water extracts are known to exert *anti*-nemic action[1-3]. Though neem seeds elaborate over 70 bitter principles also known as meliacins[4], excepting the nematicidal action of azadirachtin[5], no report is available on the evaluation of other congeners of azadirachtin for possible nematicidal activity. In our earlier communication[6], we have, therefore, initiated systematic investigations towards identifying other nematicidal neem constituents and succeeded in fractionating the ethanolic extractives of neem seed kernels into glyceride fraction free from bitters, lipid soluble limonoids and polar limonoids. The nematicidal assay results pointed out that the biological activity was restricted to limonoids only. Consequently, several column chromatographic fractions of the total limonoids were screened *in vitro* against root-knot nematode (*Meloidogyne incognita* Kofoid and White) Chitwood and the preliminary results were reported earlier[7]. In this communication, we report the details of isolation of six C-*seco*- meliacins and 3 unidentified components from the total limonoid fraction and *in vitro* nematicidal screening of these compounds and two pentanortriterpenoids[14] against root-knot nematode, *M. incognita*.

MATERIALS AND METHODS

General Techniques: Melting points were uncorrected. The IR, UV and mass spectra were recorded respectively on a Perkin-Elmer

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IR spectrometer model 405, Varian UV-VIS spectrophotometer model 634 and JEOL D-300 mass spectrometer with electron impact. ^1H and ^{13}C NMR spectrometer were run on JEOL FX-200 MHz FT NMR spectrometer in $CDCl_3$ with TMS as an internal standard.

Isolation of meliacins: Freshly collected neem fruits from the Indian Agricultural Research Institute campus were depulped, shade-dried and crushed seeds were extracted with hexane in cold and the hexane extract on concentration *in vacuo* furnished a yellowish brown viscous oil (1.65 kg). The oil (1 kg) in hexane (1 L) was repeatedly partitioned with MeOH-water (19:1,1 L each) till the methanolic layer remained colourless. The methanolic portion on concentration under reduced pressure left a brownish viscous residue (105 g). A portion of the residue (35 g) was chromatographed over Florisil (60-100) mesh, 350 g) column with benzene-ethyl acetate (graded proportions) elution to give 4 fractions.

Fraction **1** (14.8 g) obtained by benzene elution contained lipid components. It was rechromatographed over silica gel (400 g) column by eluting with hexane followed by hexane-benzene (1:1) to give pure fats (10.6 g), odorous viscous liquid (1.12 g) and a white solid (3.0 g) which was coded earlier[7] as **NP-2.**

Fraction **2** (2.6 g) was obtained by benzene-ethyl acetate (9:1) elution. It contained two compounds as shown by TLC and repeated crystallisation from methanol yielded epinimbin[8] **(I)** as white needles. The residues from mother liquour (2.1 g) on column chromatogrpahy over silica gel (100 g) with gradual hexane-ethyl acetate (4:1 and 3:1) elution separated into **I** (700 mg) and nimbin[9] **(II,** 1.1 g) respectively.

Fraction **3** (7.4 g) obtained from benzene - ethyl acetate (3:1) elution was re-chromatographed on silica gel (200 g) by eluting with hexane-ethylacetate (4:1, 3:1 and 3:2 proportions in sequence) to give **II** (3.5 g) desacetyl nimbin[10] **(III,** 600 mg), salannin[11] **(IV,** 500 mg) and desacetyl salannin[12] **(V,** 300 mg).

Fraction **4** (9.5 g) was obtained by elution with the above binary mixture (3:2). It was carefully column chromatographed over Florosil^R (300 g) and eluted with diethyl ether-acetone (19:1) to obtain more of **V** (200 mg), azadirachtin[13] **(VI,** 2 g) and a fraction coded as **P-2** (3 g). Further elution with the solvent mixutre (4:1) left a residue coded as **P-3** (2.5 g).

All the pure and known compounds isolated above were rigorously characterised and gave correct spectral and physicochemical data consistent with literature. Their melting points are listed in Table 1.

Partial characterisation of NP-2: White solid m.p. 58-59° C. IR ($CHCl_3$): cm^1 2900 (str.), 2840(str.) and 1700(str.). MS (EI):m/z 312(5%), 284(50), 241(20), 129(40), 73(100), 60(87) and 57(89). PMR (60 MHz): 0.95 (m,1H), 1.3 (s,12H) and 2.1-2.4 (m,1H).

Partial characterisation of P-2 and P-3: High performance liquid chromatograph (HPLC) analyses were carried out employing Spectra Physics model SP 8000B HPLC under followed operating conditions:

column	:	Lichrosorb RP18 (250 mm x 4.6 mm)
mobile phase	:	Acetonitrile-water (1:1)
flow rate	:	1 mL. min^{-1}
detector	:	UV-VIS
wave length	:	220 nm

Azadirachtin was used as the reference compound. ^1H NMR spectra were also obtained for these two fractions.

Pentanortriterpenoids[14] VII and VIII: They were obtained by the partial synthesis from **I-III** as reported elsewhere[15].

R_f values of 11 neem constituents were determined by tlc using silica gel as stationary

phase and ethylacetate-benzene (3:7) as the developing medium in 3 replicates and the mean values were calculated.

Nematicidal screening: It was carried out against the root knot nematode (*M. incognita*) by the method reported earlier[6]. Larvae used for the screening were obtained by keeping the egg sacs (collected from a stock culture maintained on tomato under glass house condition) on a wire gauge support having two layers of tissue papers in a petri dish containing water incubated at 30+1°C.

Test chemicals: Eight C-secomeliacins (**I-VIII,** Fig.1) and three column chromatographic fractions *viz.* **NP-2, P-2 and P-3** were used along with carbofuran (Furadan 3G) as the standard nematicide for comparison. Emulsifiable concentrate (2 EC) of each test chemical was freshly prepared by dissolving 50 mg each in 2.5 mL distilled xylene containing 2 drops of Tween 80 (emulsifier) and test emulsions of 500, 400, 300, 200 and 100 ppm were derived by suitable dilution of each EC with distilled water at the time of testing. An emulsion containing 40 ppm carbofuran was prepared from Furadan 3G. One mL of nematode suspension containing ca. 100 freshly hatched second stage larvae of *M. incognita* was taken in vials (12 mL cap.) and an equal volume of test as well as standard emulsions each of different concentrations was added to each vial. An emulsion blank and distilled water control were included for the bioassay and all the treatments were replicated thrice. The loosely capped vials were incubated at 30+1°C for 48h and the number of larvae dead or moribund after 24h and

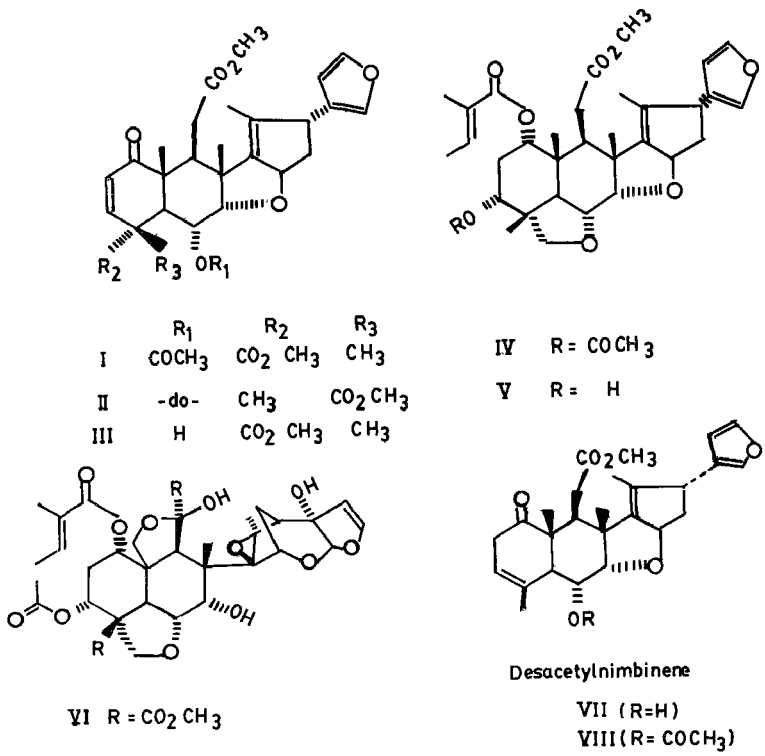

Fig.1. Structures of test meliacins

48h intervals were counted under stereoscopic binocular. in the case of moribund larvae, their irreversible immobility was confirmed by transferring them to tap water. The per cent mortality was worked out as the mean of three replications and corrected for natural mortality using Abbot's formula. The lethal median concentration (LC_{50}) values in ppm of test chemicals after 48 h exposure were computed using probit analysis.

Statistical analysis for deriving correlation and regression equation between variables R_f and LC_{50} was carried out in a PC using MSTAT software.

RESULTS AND DISCUSSION

Structure elucidation of neem constituents

The isolation of 4-epinimbin (I) as a novel constituent[8] and the partial synthesis of two pentanortriterpenoids[14-15] VII and VIII along with the isolation of known constituents II-VI from neem seeds provided 8 pure meliacins for nematicidal assay. In a preliminary report[7], three column chromatographic fractions were also included for bioassay and for the sake of continuity of communication, these fractions are referred here by the same codes *viz.* NP-2, P-2 and P-3.

On the basis of nmr, ir and mass spectral data, it is evident that NP-2 is a non-terpenoid whose structure could be tentatively assigned as a long chain ketone. Premilinary investigation of P-2 indicated its close resemblance to azadirachtin (VI). HPLC analysis revealed it as a quarternary mixture consisting of compounds in the ratio of 1: 2.1: 1.65: 1.2 with respective R_t values of 4.81, 5.54, 5.99 and 6.77 mins., when VI eluted at 7.59 mins. under identical conditions. A careful scrutiny of its high resolution PMR indicated the presence of diagnostic peaks for isomeric azadirachtins, B and F[12]. Similarly, the hplc analysis of P-3 had shown it to be a ternary mixture in the ratio of 1.2 :1: 1 with respective R_t values of 3.34, 4.05 and 4.41 mins. under the same conditions set for the analysis of P-2. Their chemical identity needs further investigation.

Nematicidal activity

The salient results of *in vitro* nematicidal assay of eleven neem constituents and the standard nematicide, carbofuran are given in Table 1. A perusal of LC_{50} values of test chemicals at 48 h after treatment would reveal that all test chemicals except NP-2 were active. The most active compound was the much touted azadirachtin (VI) while the fraction P-3, the least. The real nematicidal value of the two polar fractions can be assessed only after they are separated into pure compounds. A careful examination of the data would highlight that all the pure test compounds belong to a group called C-secomeliacins and they showed more or less the same level of nematotoxicity implying thereby that it could be a group characteristic.

A simple correlation regression analysis of log LC_{50} values of eight individual C-secomeliacins (I-VIII) with their R_f data yielded the followed equation:

$\log LC_{50} = 0.656\ R_f + 1.76$ ($n = 8$, $s = 0.168$, $r = 0.847$, $P = 0.007$), where n = number of test meliacins, s = standard error of the equation, r = correlation coefficient and P = probability level of non-significance of the equation. It is evident from the equation that the nematotoxicity of a test meliacin is significantly and highly correlated with hydrophilicity parameter R_f.

As far as the physical significance of the above equation is concerned, the observed nematotoxicity of this group of compounds could be explained largely on the basis of only parameter *viz.* R_f which is a measure of the polarity of the molecule. A smaller

Table 1. Some physico-chemical data and *in vitro* nematicidal activities of neem constituents and carbofuran (as standard)

Sl. No.	Constituents/Standard	m.p. (°C)	R_f	Mean mortality @ 100 ppm/24 h	LC_{50} (ppm) after 48 h
1	4-Epinimbin	196-7	0.65	18.8	117.0
2	Nimbin	204-5	0.55	6.9	137.5
3	Desacetylnimbin	209-10	0.45	29.4	124.0
4	Salannin	167-8	0.52	15.8	157.0
5	Desacetylsalannin	214	0.30	44.6	105.0
6	Azadirachtin	160-2	0.12	53.3	55.0
7	Desacetylnimbinene	141	0.49	21.9	130.0
8	Nimbinene	134	0.69	15.8	150.0
9	Analogue of 6 (P-2)	-	0.10	44.6	102.0
10	Polar fraction (P-3)	-	0.05	15.6	205.7
11	Non-polar fraction (NP-2)		0.85	6.9	—
12	Carbofuran (standard) @ 20 ppm			32.0	43.9 (%) Kill

CD (P=0.01): 2.5% (treatment); 0.4% (period); 0.2% (concentration)

value of R_f means that the compound is more polar and the polarity is again directly related to water solubility of the compound. It is a well established fact that a good nematicide must be more water soluble than an insecticide of the same chemical group, primarily because the nematodes are soil-water borne. In a recent publication[17], a similar conclusion was made in case of synthetic nematicides of organo-phosphorus group.

While the details of per cent mortality of test chemicals at different concentrations at 24 h after treatment can be found elsewhere[4], representative data at 100 ppm which is close to LC_{50} values at 48 h of exposure are given in Table 1. A comparison of per cent mortality at two time-intervals would reveal the delayed nematotoxicity behaviour of some meliacins. It was more pronounced in the case of nimbin as compared to its novel epimer, 4-epinimbin. It is by now well-known that neem meliacins especially azadirachtin cause growth regulation and feeding inhibition in arthropods, which are essentially slow processes. However, it is rather premature to draw any such parallel mode of action in nematodes. It can be concluded that the nematicidal property of neem stems from the C-secomeliacin constituents which occur abundantly in its seeds.

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