J. Appl. Ent. 124, 287–291 (2000)

ISSN 0931-2048

Insect antifeedant and growth regulating activities of neem seed oil – the role of major tetranortriterpenoids

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Abstract: An attempt was made to correlate insect antifeedant and growth regulatory activities of neem (*Azadirachta indica*) seed oil with the major tetranortriterpenoids. Selective elimination of triterpenoids by preparative high-performance liquid chromatography, incorporation of the eliminated compounds in defined concentrations and bioassaying the resultant fractions against *Spodoptera litura* indicated the necessity to quantify major triterpenoids for correlation of bioactivity of neem oil.

1 Introduction

Seed kernels of the Neem tree, *Azadirachta indica* A. Juss., are a rich source of biologically active tetranortriterpenoids. Subsequent to the demonstration of insect antifeedant activity of neem seed kernel extracts against desert locusts (PRADHAN et al., 1962) and the isolation of azadirachtin from them (BUTTERWORTH and MORGAN, 1968), extensive research has been carried out on the chemistry and biological activities of tetranortriterpenoids from various parts of the neem tree (KRAUS, 1995). Among them, azadirachtin-A has been shown to be a potent insect antifeedant and ecdysis inhibitor. A number of congeners of azadirachtin have also been isolated from the neem seed kernels (YAMASAKI et al., 1986; KRAUS et al., 1989; REMBOLD, 1989; GOVINDACHARI et al., 1992; ROJATKAR and NAGASAMPAGI, 1993, 1994, 1995; GOVINDACHARI and GEETHA GOPALAKRISHNAN, 1997) and semisynthetic modifications of azadirachtin-A were attempted (LEY et al., 1989). Insect antifeedant and growth regulatory activity of both natural congeners of azadirachtin (REMBOLD, 1989; GOVINDACHARI et al., 1994, 1995a) and the synthetically modified azadirachtins were also studied to arrive at possible structure–activity relationships (LEY et al., 1989).

It is generally believed that bioactivity of neem kernel extracts/neem oil is due to the azadirachtin content in them. This conclusion is borne out of correlation of azadirachtin-A content in seed extracts/oil to the insect antifeedant and ecdysis inhibition activity. On the basis of extracts of neem seeds collected from various regions of Asia and Africa, ERMEL et al. (1984, 1987) indicated strong correlation of EC_{50} values with azadirachtin content while claiming statistically significant correlation in only one extract. Subsequently, based on their studies with behaviour-disrupting and growth-disrupting activity of *Peridroma saucia*, ISMAN et al. (1990) showed a strong correlation of bioactivity with the azadirachtin content (azadirachtin-A) in neem seed oil.

While studying the problems connected with the antifeedant bioassays, SCHWINGER et al. (1984) and KRAUS et al. (1987) screened several other neem triterpenoids, especially the C-seco limonoids, and showed that salannin was as equally effective as azadirachtin as an insect antifeedant against *Epilachna varivestis*. Against *Pieris brassicae* larvae salannin was found to be more active than azadirachtin-A (LUO LIN-er et al., 1995) Azadiradione, epoxy azadiradione, nimbin and desacetylnimbin were reported to be feeding deterrents, although to a lesser extent than azadirachtin-A. (SCHWINGER et al., 1984; KRAUS et al., 1987; GOVINDACHARI et al., 1995a, 1996a). Similar results were also reported by AERTS and MORDUE (LUNTZ) (1997).

In order to ascertain whether quantification of azadirachtin-A alone would suffice to correlate bioactivity of neem extracts or whether the quantitation of other major triterpenoids are also necessary, we attempted the following: (a) selective elimination of terpenoids from a defined neem seed kernel extract by preparative high-performance liquid chromatography (HPLC); (b) incorporation of the eliminated compounds in specific concentrations; and (c) testing mixtures obtained by (a) (b) and the pure compounds for insect antifeedant and growth-regulating activities.

2 Material and methods

2.1 Preparation of neem oil fractions by preparative HPLC and quantitative estimation of major triterpenoids

Neem oil (1 l) obtained from neem seed kernels by a cold mechanical expeller was dissolved in *n*-hexane (1.5 l) and partitioned with 90% methanol (3×500 ml). The methanol layer was concentrated *in vacuo* to yield the residue E1 (57 g). The residue was subjected to preparative HPLC. For each run 5 g of the residue E1 was dissolved in 20 ml of methanol, filtered through a 0.25 μ Millipore filter (Whatman Group, Maidstone, UK) and the filtrate was subjected

 0931–2048/2000/2407–0287 $ 15.00/0

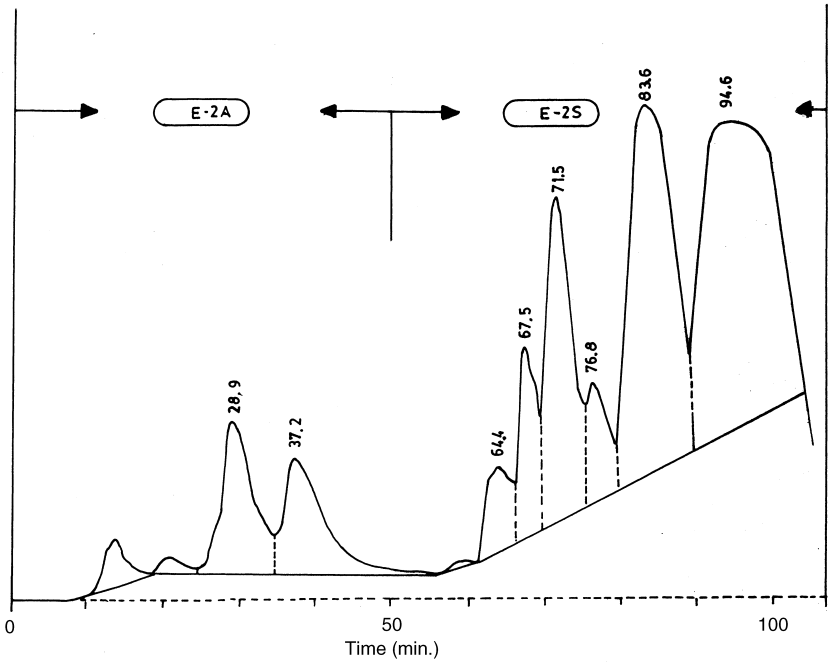

Fig. 1. Preparative high performance liquid chromatograph of the residue E1

to preparative HPLC using a Shimadzu LC 8 A system linked to CR 4 A data processor. Shimpack ODS reverse phase (C_{18}, 50 mm i.d. × 250 mm) preparative column was used. The eluent flow was 30 ml/min throughout the run. During the first 60 min methanol : water in the ratio 60 : 40 was used, thereafter the methanol : water ratio was changed to 70 : 30 until the end of the run. The fraction eluted from 10 to 50 min (E-2 A) and 51–105 min (E-2S) were collected and concentrated in vacuum to yield 12.3 g of E-2 A and 33.6 g of E-2S, respectively (fig. 1). An aliquot of the residue E-2S (4 g) was subjected to further preparative HPLC maintaining the same conditions indicated earlier and fractions from 61 to 85 min were collected and concentrated *in vacuo* to yield 2.2 g of E-2S-S. The entire procedure of the preparation of various neem fractions are indicated in fig. 2.

For the quantitative analyses of major triterpenoids 20 μl of residue (E-1, E-2 A, E-2S and E-2S-S) (10 mg/ml) was injected onto a Shimadzu (LC 8 A) HPLC fitted with a DuPont analytical ODS (10 μ) column (MAC-MOD Analytical Inc., PA, USA). Acetonitrile : water 50 : 50 was used as eluent at 1 ml/min. flow rate and detected at 215 nm. For the analyses of azadirachtins, acetonitrile : water 30 : 70 at 1 ml/min was used. Pure samples of azadirachtin-A, azadirachtin-B, desacetylnimbin, nimbin, salannin (for details of purification refer to GOVINDACHARI et al., 1995b, 1996b, 1997) were used as standards. Compounds were identified by their retention times and area measurements (valley to valley) were used for quantitation.

2.2 Insect bioassays

Extracts E-1, E-2 A, E-2S, E-2S-S and E-2S-S spiked with azadirachtin-A/azadirachtin-B/salannin (at 25, 50 and 75% concentration) were used for insect bioassays. Pure azadirachtin-A, azadirachtin-B and salannin (> 99%) were used for comparison.

Third instar larvae of *Spodoptera litura* reared on *Ricinus communis* leaves were used and a dual choice antifeedant bioassay was performed with extracts/compounds at 1 μg/cm^2 of *R. communis* leaf area (for details refer GOVINDACHARI et al., 1995a).

For each extract/compound five replicates were maintained. The percentage feeding index (PFI) was calculated (cf. LUCO et al., 1994) using the formula:

$$\text{PFI} = [\text{Area fed in treated}/(\text{Area fed in treated} + \text{Area fed in solvent control})] \times 100$$

where treated is the area treated with the solution of compound in 1 ml acetone and solvent control is the area treated with 1 ml acetone.

The effect of triterpenoids on the duration of development was also studied using third instar *S. litura* larvae (GOVINDACHARI et al., 1996a). Third instar larvae were force-fed on *R. communis* leaf discs (180 cm^2) treated with

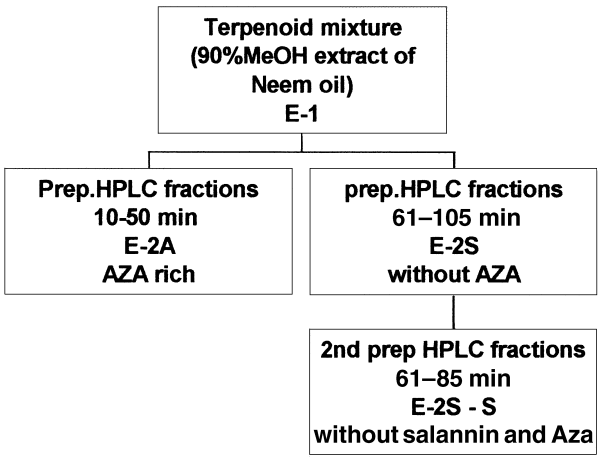

Fig. 2. Preparation of various fractions from neem oil

0.5 μg of compound/extracts per cm^2 leaf area, until they moulted. From the fourth instar, normal diet was provided until the end of the experiment. Larval durations, mortality, pupal weight and pupal mortality are presented.

3 Results and discussion

Direct preparative HPLC separation of major triterpenoids from neem kernel extracts and neem oil and their quantitative estimation by analytical HPLC are known (GOVINDACHARI et al., 1995b). On the basis of the HPLC chromatographic profile of neem oil extractives, it is possible to selectively collect azadirachtin-rich fractions and fractions devoid of azadirachtin by preparative HPLC. Accordingly, peaks appearing from 10 to 50 min in the preparative run (E-2 A) were collected. Analytical HPLC of E-2 A revealed the presence of azadirachtins A and B as the major constituents and azadirachtins D, H and I as minor constituents. The quantifications of the latter are not included in this study. E-2 A was devoid of salannin, nimbin and desacetylnimbin. The peaks appearing between 61 and 105 min were collected (E-2S) and found to be devoid of any of the five azadirachtins mentioned. E-2S had salannin, nimbin, azadiradione and desacetylnimbin as major constituents. The E-2S was subjected to another preparative HPLC and peaks appearing from 61 to 85 min (E-2S-S) and a single peak appearing between 86 and 104 (salannin) were separately collected.

E-2S-S was devoid of azadirachtins as well as salannin. Analytical HPLC of E-2S-S revealed the presence of nimbin and desacetylnimbin as the major constituents This fraction also contained azadiradione and desacetylsalannin among others as minor constituents and the analysis of the latter are not included in this study. Quantitation of major limonoids from these fractions are presented in table 1.

E-2 A had approximately 2.5- and 1.5-fold increase of azadirachtin-A and azadirachtin-B, respectively, compared with E-1. Still, there is no appreciable difference in the antifeedant activity of the methanolic extract of neem oil (E-1) and the azadirachtins-rich HPLC fraction (E-2 A). Interestingly, E-2 A did not have the other major C-seco limonoids such as desacetylnimbin, nimbin and salannin. E-2S, which was devoid of any azadirachtins, was three times less active than either the crude methanolic extract or the azadirachtin-rich fraction (E-2 A). Elimination of salannin from E-2S resulted in the fraction E-2S-S, which showed only a marginal increase in antifeedant activity.

When E-2S-S was spiked with azadirachtin-A, it showed excellent antifeedant activity even at 25% of the extract (0.25 μg azadirachtin-A/cm^2 leaf area). In the case that E-2S-S was spiked with azadirachtin-B, only a marginal improvement in antifeedant activity was noticed. There was no appreciable increase in antifeedant activity due to addition of salannin to E-2S-S and pure salannin was comparably better than E-2S-S. Hence, quantification of azadirachtin-A may be taken as a marker for correlating antifeedant activity of neem extracts against *S. litura*, although mixtures of triterpenoids also impart excellent antifeedant activity as indicated by the extract E-1 (table 2).

Duration of development of larvae and larval and pupal mortalities have been taken as indicators of growth regulation of *S. litura* for testing the neem extracts. There was no appreciable differences either in larval durations or mortality among the fractions E-1, E-2 A, E-2S and E-2S-S. Salannin and azadirachtin-A in pure form increased the larval durations more than control. The incorporation of salannin into E-2S-S resulted in significant prolongation of total larval durations of *S. litura*. Azadirachtin-B, not only increased the larval durations both in pure form as well as when incorporated into E-2S-S but also induced larval mortalities to a very significant level. In fact, by the fifth instar, 100% mortality was noticed with both pure azadirachtin-B as well as when incorporated into E-2S-S. There was no signficant

Table 1. *Details of HPLC analyses of major compounds in neem oil fractions. All values are percentages*

Compound	Aza-A	Aza-B	Deacetyl nimbin	Nimbin	Salannin	Others
E-1	1.9	8.3	6.9	5.0	21.25	56.65[1]
E-2 A	5.1	13.6	–	–	–	81.3[1]
E-2S	–	–	5.2	5.4	20.36	69.04
E-2S-S	–	–	12.8	24.1	–	63.1
25 A	25	–	9.62	18.01	–	47.37
50 A	50		6.42	12.01	–	31.57
75 A	75	–	3.21	6.03	–	15.76
Aza-A	> 99	–	–	–	–	–
25 B	–	25	9.62	18.01	–	47.37
50 B	–	50	6.42	12.01	–	31.57
75 B	–	75	3.21	6.03	–	15.76
Aza B	–	> 99				–
25 S	–	–	9.62	18.01	25	47.37
50 S	–	–	6.42	12.01	50	31.57
75 S	–	–	3.21	6.03	75	15.76
Salannin	–	–	–	–	> 99	–

[1] others include other azadirachtins such as aza D, H, I and K.

Table 2. *Percentage feeding index (PFI) of* Spodoptera litura[1] *fed on neem components-treated castor leaves*

Compound	PFI [2]
E-1	19.4 ± 3.3
E-2 A	21.7 ± 8.3
E-2S	57.4 ± 4.6
E-2S-S	44.7 ± 2.3
25 A	9.2 ± 4.7
50 A	3.9 ± 5.5
75 A	21.9 ± 7.1
Aza-A	0
25 B	39.3 ± 5.3
50 B	37.8 ± 1.8
75 B	35.4 ± 7.6
Aza B	0
25 S	48.9 ± 3.7
50 S	47.0 ± 3.1
75 S	55.2 ± 5.0
Salannin	36.3 ± 2.7

Values are means ± SD.
[1]Third instar larvae.
[2]1 μg of compound/cm^2 castor leaf.

change in larval durations due to incorporation of azadirachtin-A to E-2S-S, but considerable larval mortality was noticed (table 3).

Based on the foregoing, we conclude that quantification of any one component to correlate bioactivity of neem formulations/extracts may lead to erroneous conclusions. It is hence, necessary to quantitate the major limonoids, especially azadirachtin-A, azadirachtin-B and salannin, in order to correlate the varied biological activities of neem kernel extracts/formulations against pest insects.

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Table 3. *Effect of Neem kernel components on the different instars of* Spodoptera litura

Compound	Third instar		Fourth instar		Fifth instar		Total larval duration[2]	Pupa	
	Duration [1]	Mortality	Duration [1]	Mortality	Duration [1]	Mortality		Weight (mg)	Mortality
Control	2.96 ± 0.5^a	0	3.20 ± 0.5^e	0	5.45 ± 1.1^f	0	11.72 ± 0.9^a	294.9 ± 77.1^f	0
E-1	3.00 ± 0.5^a	0	3.48 ± 0.5^e	0	5.08 ± 0.5^d	4.0	11.65 ± 0.8^a	321.2 ± 25.8^g	21.7
E-2 A	3.33 ± 0.5^a	0	3.13 ± 0.3^d	0	6.28 ± 0.8^f	4.7	12.66 ± 1.0^a	293.4 ± 30.5^f	30.4
E-2S	3.33 ± 0.5^a	0	3.50 ± 0.7^e	0	6.04 ± 1.0^d	0	13.04 ± 1.3^a	219.8 ± 25.3^a	19.1
E-2S-S	3.19 ± 0.4^a	0	4.00 ± 1.1^f	4	5.64 ± 1.2^d	0	12.86 ± 1.3^a	290.1 ± 58.9^f	28.6
25 A	3.20 ± 0.4^a	0	3.37 ± 0.5^e	0	5.54 ± 0.7^d	10	12.17 ± 0.86^a	285.8 ± 40.6^f	23.5
50 A	3.34 ± 0.6^a	8	3.61 ± 0.5^e	0	5.94 ± 1.0^d	28	12.50 ± 1.01^a	266.2 ± 28.4^d	27.8
75 A	3.88 ± 0.7^a	0	3.40 ± 0.8^d	0	6.50 ± 0.6^b	66	13.16 ± 0.37^a	244.8 ± 57.7^b	33.3
Aza A	4.16 ± 0.2^a	28	3.33 ± 0.7^f	0	6.00 ± 1.0^c	60	13.30 ± 0.90^a	254.2 ± 61.8^c	50.0
25 B	5.44 ± 1.0^b	0	4.22 ± 0.9^b	64.1	–	100	–	–	–
50 B	3.95 ± 1.0^a	0	4.75 ± 0.5^a	61.1	–	100	–	–	–
75 B	3.20 ± 0.5^a	20	4.50 ± 1.2^c	47.4	–	100	–	–	–
Aza B	3.45 ± 0.9^a	16	5.66 ± 1.5^a	85.7	–	100	–	–	–
25 Sala	4.10 ± 1.5^a	9.5	4.60 ± 0.7^d	10.5	5.18 ± 1.3^d	26.7	15.07 ± 1.0^b	225.4 ± 19.7^a	27
50 Sala	3.72 ± 0.6^a	0	6.31 ± 2.2^f	21.7	5.19 ± 0.7^b	12.5	15.28 ± 1.0^b	225.8 ± 25.7^a	33
75 Sala	3.82 ± 0.9^a	5.3	5.22 ± 1.1^e	5.9	5.06 ± 0.8^b	25	15.20 ± 0.7^b	244.2 ± 27.5^b	23
Salannin	3.88 ± 0.9^a	0	3.36 ± 0.5^e	0	6.12 ± 1.4^c	0	13.37 ± 1.2^a	282.9 ± 28.3^e	17.4

Values are means ± SD. Values followed by different letters are statistically significant $P < 0.05$.
[1] duration in days.
[2] indicates duration of third to fifth instar larvae.
Experiment initiated with 25 individuals (Five replicates with five individuals each). Concentration of compounds used = 0.5 μg/cm^2 area of *Ricinus communis* leaf.

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