Physicochemical and Chemical Variation in Neem Oils and Some Bioactivity Leads against *Spodoptera litura* F.

Jitendra Kumar and Balraj S. Parmar*

Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi 110012, India

Forty-two neem ecotypes of India have been found to show a wide variation in the content of oil, and their physicochemical characteristics (color, specific gravity, refractive index, iodine value, acid value, and saponification value), total fatty acid, fatty acid composition (oleic, stearic, palmitic, linoleic, myristic, arachidic, and behenic acids), and the key meliacins (azadirachtin, nimbin, and salannin). The azadirachtin content did not correlate with any of the physicochemical and chemical parameters, but the nimbin and salannin contents correlated significantly with each other. The refractive index (+ve correlation) and iodine value (−ve correlation) showed weak but significant correlation with the contents of nimbin and salannin. Insect growth inhibition of *Spodoptera litura* revealed a wide variation in the EC_{50} of the oils. The salannin and azadirachtin contents of the oils correlated the most with bioactivity. The iodine and acid values correlated weakly but significantly with bioactivity.

Keywords: *Neem; neem oil; physicochemical variation in neem; chemical variation in neem; neem bioactivity*

INTRODUCTION

The ever increasing emphasis on developing environmentally benign pest control agents has brought neem, *Azadirachta indica* A. Juss, to the fore. Several reports confirming the effectiveness of neem-based products against various insect pests have appeared in the literature and can be referred to in the reviews by Saxena (1989), Schmutterer (1990), Singh (1993), Singh and Kataria (1991), and others.

Neem oil is a key derivative that finds application as an important pest control agent. In India, the oil-based formulations have been prescribed to contain a minimum of 300 ppm of azadirachtin (Parmar and Ketkar, 1993). It is, however, known that besides azadirachtin, there are several other constituents of the oil that influence its bioactivity (Schmutterer, 1990).

Neem kernels contain 30−45% (w/w) oil (Koul *et al*., 1990; Parmar and Ketkar, 1993). Variation in its physicochemical characteristics such as color, specific gravity, refractive index, saponification, iodine and acid values, and chemical constituents, namely the fatty acid composition, is reported (Roy and Dutt, 1929; Child and Ramanathan, 1936; Hilditch and Murti, 1939; Dasa Rao and Seshadari, 1942; Skellon *et al*., 1962). The variation in content of the key meliacin, azadirachtin, is also well documented (Ermel *et al*., 1987; Rengasamy *et al*., 1993). Despite the variation, specifications for neem oil are already prescribed (ISI, 1975; *The Indian Pharmacopoeia*, 1966), and the samples for various uses are required to conform to these.

Even though variation in the various physicochemical and chemical parameters of the oils has been reported, it is not known if the content of the meliacins correlates with any of these parameters. This paper reports information on this aspect. Additionally, preliminary information on the effect of these parameters on bioactivity against *Spodoptera litura* F. has been generated.

MATERIALS AND METHODS

Neem Oils. Forty-two neem seed samples from different areas of India were procured through M/s Neem Mission, Pune, India. The dry, cleaned seeds were decorticated manually to obtain kernels which were crushed in a Waring blender and extracted with *n*-hexane (60−80 °C) in a Soxhlet extractor for 8 h (at this stage, a drop of hexane extract when evaporated on a filter paper left no residual oily spot). Hexane was removed in a rotavapor under reduced pressure at 60 °C to yield oil.

Chemicals. Standard azadirachtin (purity 95%, HPLC), M/s Trifolio-M GMBH, Germany, was obtained through the courtesy of M/s Neem Mission. Reference salannin (purity 85%, HPLC) was obtained from Dr. E. D. Morgan, Keele University, Staffordshire, U.K., and reference nimbin (purity 85%, HPLC) was obtained from Dr. C. Devakumar of this Institute. Reference samples of methyl linoleate, methyl palmitate, and methyl oleate (Aldrich Chemical Co., Milwaukee, WI) were obtained through the courtesy of Dr. T. R. Madan of the Biochemistry Department of this Institute. Methyl myristate and methyl stearate were prepared in the laboratory by esterification of their respective acids (Aldrich) with diazomethane in diethyl ether (AOAC, 1975). For routine laboratory work, laboratory grade chemicals and solvents were used. For HPLC analysis, the solvents were of analytical grade.

Experimental Insect. Twelve-hour-old neonate first instar larvae of *S. litura* F. were used. The culture was maintained at 27 ± 1 °C, 70−75% relative humidity, and 16:8 h light−dark (LD) and was reared on castor leaves until transferred to experimental diet (no. 9795, BioServ, Inc., French Town, NJ).

Physicochemical Properties. Specific gravity (25 °C), refractive index (27 °C, M/s Toshniwal India Ltd. refractometer), and iodine, acid, and saponification values (AOAC, 1975) of oils were determined. The color was examined using Munsell color charts (1975 edition) by holding 1 mL of the oil sample in a 3.5 cm × 1 cm diameter glass tube directly behind the aperture separating the closest chip.

Methyl Esters of Fatty Acids in Neem Oils. Accurate weights of oils (around 1 g) were saponified with alcoholic KOH. Alcohol was removed under reduced pressure in a rotavapor, and the saponified oil was hydrolyzed with dilute HCl to obtain free fatty acids. The whole content was taken in a separatory funnel and partitioned with diethyl ether (2 × 5 mL). The ether extracts were esterified with diazomethane. The methyl esters were determined by gas−liquid chromatography (Hewlett-Packard, Model 5890 A, fitted with coiled glass column, 2 m × 2 mm i.d., packed with 3% SE-30 on

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Table 1. Physicochemical Properties of the Test Neem Oils

sample no.	yield (% w/w)	saponification value [mg of KOH (g of oil)$^{-1}$]	IV (Wijs')	AV (%)	RI at 27 °C	sp gravity (g cm^{-3})	color		
							hue	value/chroma	color
1	45.0	235.0	38.9	20.6	1.4622	0.92	5.0 Y	6/8	olive yellow
2	27.8	204.0	68.1	3.0	1.4578	0.98	2.5 Y	5/6	light olive brown
3	26.4	229.0	59.8	23.3	1.4615	0.92	2.5 Y	8/8	Yellow
4	52.5	217.0	80.4	26.5	1.4650	0.93	7.5 YR	4/6	strong brown
5	35.6	208.4	73.8	15.4	1.4575	0.98	2.5 Y	6/8	olive yellow
6	42.3	212.0	70.0	8.9	1.4632	0.98	5.0 Y	6/8	olive yellow
7	44.0	224.0	65.0	8.8	1.4635	0.93	5.0 Y	4/4	olive
8	36.0	187.0	84.2	20.1	1.4600	0.93	10.0 R	3/3	dusky red
9	45.0	185.0	66.0	8.3	1.4610	0.92	5.0 Y	5/6	olive
10	40.0	194.0	70.0	14.0	1.4640	0.90	2.5 Y	6/8	olive yellow
11	40.5	130.0	75.9	6.9	1.4630	0.98	2.5 Y	5/6	light olive brown
12	27.5	232.4	86.4	22.3	1.4600	0.93	2.5 Y	7/8	yellow
13	31.4	245.0	91.8	49.0	1.4620	0.93	5.0 Y	8/6	olive
14	33.4	220.5	33.8	4.1	1.4640	0.90	2.5 Y	6/8	olive yellow
15	35.7	193.4	80.0	15.6	1.4600	0.91	7.5 YR	4/6	strong brown
16	50.6	165.0	57.7	6.6	1.4600	0.92	2.5 Y	6/8	olive yellow
17	19.0	211.0	102.0	44.6	1.4635	0.89	5.0 Y	6/8	olive yellow
18	52.5	196.0	68.8	6.6	1.4610	0.89	5.0 Y	7/8	yellow
19	22.0	216.9	88.5	18.1	1.4615	0.97	5.0 Y	6/6	olive yellow
20	42.0	188.0	66.0	10.8	1.4590	0.91	5.0 Y	6/6	olive yellow
21	39.0	222.0	64.3	14.7	1.4610	0.83	5.0 Y	7/8	yellow
22	18.5	224.8	122.6	41.0	1.4630	0.94	5.0 Y	5/6	olive
24	37.0	215.4	66.5	9.8	1.4600	0.91	5.0 Y	6/4	pale yellow
25	35.6	232.0	95.0	8.8	1.4615	0.93	5.0 Y	6/8	olive yellow
26	28.6	221.0	85.2	4.1	1.4615	0.94	5.0 Y	5/6	olive
27	52.0	212.0	60.9	6.6	1.4630	0.93	5.0 Y	7/3	pale yellow
28	41.0	217.0	85.0	48.9	1.4615	0.83	5.0 Y	6/4	olive yellow
29	32.3	224.5	79.8	3.8	1.4618	0.83	5.0 Y	7/2	pale yellow
30	35.5	212.0	81.0	7.7	1.4620	0.93	5.0 Y	6/8	olive yellow
31	39.9	218.4	61.0	17.5	1.4600	0.88	5.0 Y	6/4	pale yellow
32	35.8	240.8	66.4	34.9	1.4620	0.98	5.0 Y	7/4	pale yellow
33	39.0	212.0	86.4	4.7	1.4632	0.97	5.0 Y	5/6	olive
34	44.0	210.0	80.9	2.6	1.4632	0.98	5.0 YR	4/6	olive yellow
35	41.6	232.0	68.1	2.6	1.4635	0.97	5.0 Y	5/6	olive
36	35.0	176.8	65.0	6.7	1.4675	0.92	5.0 Y	2.5/2	dark reddish brown
37	37.5	257.0	94.3	43.0	1.4630	0.91	5.0 Y	5/6	olive
38	37.0	182.0	50.8	4.1	1.4618	0.90	5.0 Y	5/6	olive
39	31.5	190.0	92.6	7.8	1.4620	0.98	2.5 Y	8/8	yellow
40	28.7	210.9	192.0	7.8	1.4650	0.89	2.5 Y	8/6	yellow
41	43.7	210.0	65.8	5.4	1.4632	0.91	5.0 Y	6/8	olive yellow
42	26.3	233.0	57.4	31.3	1.4590	0.92	2.5 Y	6/8	olive yellow

gas chrom Q, 80−100 mesh, and flame ionization detector). Column, injection port, and detector temperatures were 180, 250, and 300 °C, respectively. Carrier gas (N_2) was run at 31.8 mL min^{-1}. Methyl arachidate (eicosanoate) and methyl behenate (docosanoate) were identified on the basis of elution patterns and retention times reported in the literature (Gunstone, 1967). Retention times for methyl myristate, methyl palmitate, methyl linoleate, methyl oleate, methyl stearate, methyl arachidate, and methyl behenate were 4.00, 8.49, 11.68, 15.73, 17.66, 19.80, and 22.00 min, respectively.

Azadirachtin, Nimbin, and Salannin Content. These were determined by reversed-phase HPLC. The method of Isman *et al*. (1990) for extracting azadirachtin from oils was both cumbersome and problematic. The diethyl ether as solvent was unmanageable under the high ambient temperatures (40−45 °C) prevalent during summers. The samples could not be left for 24 h for layer separation under these conditions, as required. There was also formation of stable emulsion in many samples on shaking. Therefore, the extraction was standardized in 45% aqueous methanol and hexane (60−80 °C). One gram of oil was fortified with known amounts of azadirachin, nimbin, or salannin and transferred to a separatory funnel containing 5 mL each of 45% aqueous methanol and hexane. The contents were shaken vigorously and allowed to stand for 0.5−2 h, until the layers separated. The aqueous layer was collected, and the hexane layer was re-extracted with 5 mL of 45% aqueous methanol. The extraction was similarly repeated the third time. The aqueous layers were collected separately and analyzed for azadirachtin, nimbin, or salannin. The aqueous phases from the first, second, and third extractions yielded, respectively, 75−80%, 10−15%, and 0% azadirachtin, 60−65%, 10−15%, and 0% nimbin, and 60−65%, 10−15%, and 0% salannin. Thus, two extractions with 45% aqueous methanol were sufficient to extract the three test isoprenoids from the oils. Each of the test oils was finally extracted three times, the volume made to 15 mL in 45% aqueous methanol, and analyzed by HPLC.

A Shimadzu HPLC system fitted with LC 9A pumps in binary mode, a Rheodyne 7161 injector with a 20 μL loop, and a SPDM6A photodiode array detector was used for detection of liminoids. Samples were resolved isocratically on a 15 cm × 6 mm i.d. Shimpack CLC phenyl stainless steel column using a methanol−water (70:30) mobile phase at 1.0 mL min^{-1}. The chromatogram was run up to 20 min. Absorbance was measured at 214 and 250 nm at sensitivity of 0.05 AUFS. The data were acquired on a PCS-DG India Ltd. work station, and quantification was done in the postanalysis session. Using this, azadirachtin, nimbin, and salannin showed retention times of 7.13, 13.43, and 15.36 min, respectively, with a limit of detection of approximately 25 ppm of each in oil.

Chronic Larval Growth Bioassay. Test diets (20 g) containing four concentrations of either of neem oil, azadirachtin, nimbin, or salannin were prepared. The dry diet ingredients were impregnated with the logarithmically spaced test concentrations of the samples in 5 mL of acetone and mixed thoroughly. Diet treated with acetone only was maintained as the control. Acetone was allowed to evaporate at room temperature (40 °C) by frequently agitating the contents with a glass rod. The dried diet was added into the hot agar solution (gelling agent). After cooling, the obtained gel was cut into five pieces, which were taken individually in 30 mL plastic cups, to each of which were added two neonate larvae (within approximately 12 h of hatching). Five cups were treated with each test concentration. The treated cups were

Table 2. Fatty Acid Composition of Test Neem Oils

	oleic acid		stearic acid		palmitic acid		linoleic acid		myristic acid		arachidic acid		behenic acid		
sample no.	g/g	% w/w	g/g	% w/w	g/g	% w/w	g/g × 10^{-3}	% w/w	g/g × 10^{-3}	% w/w	g/g × 10^{-3}	% w/w	g/g × 10^{-3}	% w/w	total acid content g/g
1	0.130	67.00	0.033	15.00	0.030	16.60	3.000	1.70	1.100	0.06	ND[a]	ND	ND	ND	0.20
2	0.270	63.00	0.067	16.50	0.063	17.00	0.640	0.16	0.200	0.05	1.100	0.27	ND	ND	0.40
3	0.350	61.50	0.110	18.60	0.100	18.68	1.010	0.03	0.482	0.06	ND	ND	ND	ND	0.57
4	0.210	63.00	0.067	16.00	0.068	18.00	ND	ND	0.190	0.04	1.900	0.49	ND	ND	0.34
5	0.290	64.90	0.078	16.60	0.080	17.70	ND	ND	0.140	0.18	ND	ND	ND	ND	0.37
6	0.110	67.00	0.029	15.00	0.031	16.20	0.230	0.48	0.066	0.04	1.300	0.68	0.300	0.15	0.20
7	0.130	67.30	0.036	16.30	0.027	15.10	0.280	0.15	7.080	0.11	ND	ND	ND	ND	0.20
8	0.290	63.80	0.087	19.20	0.077	17.00	0.269	0.05	ND	ND	ND	ND	ND	ND	0.46
9	0.170	67.00	0.036	14.80	0.043	17.30	ND	ND	ND	ND	ND	ND	ND	ND	0.25
10	0.240	60.90	0.064	16.00	0.064	16.40	0.180	0.05	25.990	6.60	ND	ND	ND	ND	0.40
11	0.110	67.00	0.026	16.00	0.026	16.40	ND	ND	ND	ND	ND	ND	ND	ND	0.16
12	0.390	65.60	0.110	17.00	0.099	16.80	0.110	0.18	0.360	0.24	ND	ND	ND	ND	0.60
13	0.490	59.90	0.230	20.40	0.210	18.00	1.100	0.10	ND	ND	13.100	1.10	2.800	0.12	0.90
14	0.070	55.00	0.030	23.00	0.023	18.90	0.620	0.50	0.035	0.03	1.900	1.60	ND	ND	0.12
15	0.230	69.00	0.047	15.50	0.075	15.20	ND	ND	0.180	0.04	1.100	0.20	ND	ND	0.46
16	0.160	59.00	0.055	20.34	0.055	20.40	0.050	0.19	ND	ND	ND	ND	ND	ND	0.27
17	0.180	52.80	0.076	22.10	0.080	23.80	0.320	0.09	0.040	0.03	3.700	1.10	Nd	ND	0.35
18	0.130	62.00	0.042	19.50	0.039	18.00	0.550	0.26	0.120	0.05	ND	ND	ND	ND	0.21
19	0.180	57.70	0.067	21.35	0.060	20.10	0.330	0.10	0.290	0.10	0.850	0.27	0.840	0.26	0.31
20	0.170	54.50	0.067	21.54	0.074	23.25	0.088	0.03	0.089	0.03	0.089	0.03	1.000	0.18	0.31
21	0.330	63.00	0.094	17.30	0.097	18.60	0.310	0.05	0.140	0.02	1.500	0.27	0.950	0.01	0.52
22	0.205	61.00	0.078	19.30	0.078	19.20	ND	ND	0.100	0.03	1.010	0.25	ND	ND	0.41
23	0.130	58.00	0.042	18.30	0.048	21.00	0.119	0.50	ND	ND	2.680	1.27	ND	ND	0.23
24	0.280	64.00	0.087	18.00	0.075	17.30	0.365	0.08	0.188	0.04	ND	ND	ND	ND	0.45
25	0.440	67.00	0.100	16.00	0.110	16.40	0.250	0.03	0.228	0.03	ND	ND	ND	ND	0.66
26	0.330	64.00	0.081	15.00	0.099	18.80	0.138	0.15	0.232	0.03	ND	ND	ND	ND	0.52
27	0.310	58.50	0.055	18.00	0.095	21.00	1.576	0.50	0.181	0.05	ND	ND	ND	ND	0.47
28	0.200	54.00	0.078	21.00	0.086	23.20	0.109	0.27	0.010	0.03	ND	ND	ND	ND	0.37
29	0.180	57.00	0.073	20.80	0.069	20.10	ND	ND	0.945	0.03	2.164	0.40	1.706	0.50	0.33
30	0.220	57.90	0.077	19.50	0.067	16.80	67.425	4.80	3.322	0.83	ND	ND	ND	ND	0.40
31	0.330	66.30	0.091	17.70	0.085	15.80	0.077	0.01	1.750	0.34	0.364	0.03	0.444	0.04	0.51
32	0.160	48.60	0.089	27.50	0.089	21.40	0.432	0.13	1.400	0.04	6.545	2.00	1.100	0.16	0.32
33	0.310	66.00	0.077	16.50	0.079	17.00	0.015	0.01	0.140	0.03	0.130	0.03	0.340	0.08	0.47
34	0.200	53.90	0.051	13.40	0.059	17.10	8.800	2.25	55.800	13.30	ND	ND	ND	ND	0.38
35	0.060	60.00	0.015	14.40	0.029	25.00	ND	ND	ND	ND	ND	ND	ND	ND	0.11
36	0.100	69.00	0.021	14.30	0.023	15.70	0.890	0.59	0.170	0.11	ND	ND	ND	ND	0.15
37	0.380	63.50	0.180	16.80	0.200	18.60	ND	ND	0.480	0.04	0.060	0.50	ND	ND	0.76
38	0.150	60.40	0.050	20.60	0.045	18.90	0.097	0.02	ND	ND	ND	ND	ND	ND	0.25
39	0.130	60.90	0.044	20.00	0.038	18.10	ND	ND	0.215	0.10	ND	ND	ND	ND	0.21
40	0.490	62.00	0.140	17.70	0.160	20.25	ND	ND	ND	ND	ND	ND	ND	ND	0.79
41	0.130	65.00	0.046	16.00	0.041	14.50	5.280	1.9	2.060	1.20	ND	ND	ND	ND	0.22
42	0.330	60.40	0.140	20.60	0.130	18.50	ND	ND	ND	ND	ND	ND	ND	ND	0.60

[a] ND, not detectable.

placed in plastic boxes lined with water-soaked paper towels to maintain high humidity, and boxes were held in an environmental chamber at 27 °C and 16:8 h LD. After 7 days, all larvae were weighed and mean weights for each treatment group were expressed as a percentage of control (percent reduction in weight).

The bioactivity information is limited in scope owing to the small population of the test insect against which the tests were conducted.

Data Analysis. The effect of various physicochemical parameters of the test oils, namely saponification value, acid value, iodine value, refractive index, specific gravity, hue value, and fatty acids (oleic, stearic, and palmitic), on the oil yield and azadirachtin, salannin, or nimbin content of oils was worked out by an all-variable model and by a stepwise multiple regression model by using the software package SPSS. In the same way, the effect of physical and chemical (including the meliacins) parameters on EC_{50} was separately worked out. The nonlinear data on azadirachtin, nimbin, and salannin contents and EC_{50} were transformed into linear form by ln transformation. EC_{50} values (50% reduction in weight) were determined by probit analysis as per Finney's (1971) linear progression program. Percent reduction in weight in different treatments was corrected for the reduction in weight in control as per Abbott (1925).

RESULTS AND DISCUSSION

Oil Content. The oil content of the samples is reported in Table 1. It ranged from 18.5% to 52.5% (w/w, kernel basis). The oil content of neem kernels has been earlier reported in the range of 30−45% (Koul *et al*., 1990; Parmar and Ketkar, 1993). Sixty-nine percent of the present test samples conformed to this range. Of the remaining, 21.5% contained oil below it and 9.5% above it.

Physicochemical Properties. The physicochemical properties of the oils are reported in Table 1.

Color. Oil color varied widely from light olive brown to dusky red with the hue ranging from 2.5 Y to 10.0 R. The percent distributions of samples in the observed four hue values of 2.5 Y, 5.0 Y, 7.5 YR, and 10.0 R were, respectively, 26.2, 66.6, 4.8, and 2.4, implying that the dark intensity of the color, though existent, was relatively less prevalent.

Specific Gravity. The values ranged from 0.83 to 0.98 g cm^{-3}. *The Indian Pharmacopoeia* (1966) prescribes a specific gravity range of 0.90−0.92 for neem oils. Only 35.7% of the samples fell within this range; 12% of the samples were below it and 52.3% above it. The Indian Standards Institution (ISI, 1975) also prescribed a value of 0.90 for both the kernel and the depulped seed oils. Only 7.1% of the samples complied with this requirement.

Refractive Index. The refractive indices ranged between 1.4575 and 1.4675 as compared to the value of

1.4615 prescribed by IS 4765 (ISI, 1975). Only 12% of the test samples corresponded to the prescribed value, with 33.3% below and 54.7% above it. In earlier studies, the refractive index values of neem oil have been reported to vary from 1.4560 to 1.4620 (Roy and Dutt, 1929; Child and Ramanathan, 1936; Dasa Rao and Seshadari, 1942). The present results also show a similar variation.

Iodine Value. The values ranged from 33.8% to 192.0%. Only 40.5% of the samples were within the ISI (1975) prescribed range of 65.0−80.0, 21.5% being below it and the remaining 38.0% above it. *The Indian Pharmacopoeia* (1966) prescribed the iodine value range of 65−70%. When compared with it, only 28.5% samples were in order, with 21.5% below and 50.0% above the range. A range of 36.9−74.3% of iodine value in neem oils has been reported earlier by several workers (Roy and Dutt, 1929; Child and Ramanathan, 1936; Hilditch and Murti, 1939; Dasa Rao and Seshadari, 1942; Skellon *et al*., 1962). A far wider range was observed in the present samples.

Acid Value. The acid value ranged from 2.6% to 49.0%, with only 12.0% of the samples conforming to the prescribed ISI (1975) range of 15−20%. About 59.5% of the samples were below this range and 28.5% of samples above it. *The Indian Pharmacopoeia* (1966) prescribes an acid value requirement of <22. As per this, only 23.8% of the samples fell outside the limit of the requirement.

Different workers (Roy and Dutt, 1929; Child and Ramanathan, 1936; Hilditch and Murti, 1939; Dasa Rao and Seshadari, 1942; Skellon *et al*., 1962) have earlier reported similar results with the acid values of neem oil ranging from 0.77% to 41.0%. The present results reveal prevalence of similar variation.

Saponification Value. The values ranged from 130.0 to 257.0 mg of KOH (g of oil)$^{-1}$. Only 21.5% of the test samples fell within the prescribed ISI (1975) range of 180.0−205.0. Seven percent of the samples were below it and 71.5% above it. The range of the saponification value (196.0−200.0) prescribed in *The Indian Pharmacopoeia* (1966) gave a sample distribution percentage as follows: within range, 2.4%; below range, 23.8%; and above range, 73.8%. Different workers (Roy and Dutt, 1929; Child and Ramanathan, 1936; Hilditch and Murti, 1939; Dasa Rao and Seshadari, 1942; Skellon *et al*., 1962) have earlier reported similar results with saponification values of neem oil ranging from 196.0 to 234.6. The present results reveal prevalence of a still wider variation in Indian neem ecotypes.

Chemical Constituents. *Total Fatty Acids.* The total fatty acid content ranged from 0.11 to 0.90 g (g of neem oil)$^{-1}$. Oleic, stearic, palmitic, linoleic, myristic, arachidic (eicosanoic), and behenic (docosanoic) acids were detected (Table 2). Between 90% and 99% of the total fatty acids was constituted by oleic (48.6−69.0%), palmitic (14.5−25.0%), and stearic acid (13.4−27.5%), ranging, respectively, from 0.060 to 0.490, from 0.023 to 0.210, and from 0.015 to 0.230 g (g of oil)$^{-1}$. The other fatty acids (myristic, arachidic, linoleic, and behenic) were present in negligible or nondetectable amounts. A wide variation in the contents of different fatty acids is, thus, evident.

Azadirachtin, Nimbin, and Salannin. The content of the three key meliacins in the test neem oils is given in Table 3. A wide variation in their content is apparent. Azadirachtin ranged from nondetectable (ND) to 2323.2 ppm, nimbin from ND to 18132.0 ppm, and salannin from ND to 47150.0 ppm. Nearly 16.7% samples contained azadirachtin alone and 2.4% nimbin alone, but none contained salannin alone. There were 26.2% samples containing both nimbin and salannin, 4.7% containing both azadirachtin and salannin, and 2.4% containing both azadirachtin and nimbin. About 26.2% samples contained all three meliacins and 21.4% contained none.

Table 3. Azadirachtin, Nimbin, and Salannin Contents of Test Neem Oils (Values in Parts per Million)

sample no.	azadirachtin	nimbin	salannin
1	417.0	294.0	189.9
2	2323.2	802.0	3137.5
3	450.6	ND[a]	ND
4	ND	ND	ND
5	ND	1436.3	15367.5
6	79.2	1557.5	3908.8
7	276.0	2037.5	ND
8	ND	4120.0	7737.5
9	330.0	1633.7	4808.8
10	ND	2373.0	5021.3
11	ND	565.0	1225.0
12	765.0	69.9	172.5
13	ND	ND	ND
14	ND	9123.8	47150.0
15	ND	3907.5	2600.0
16	253.0	ND	ND
17	ND	26.0	ND
18	ND	75.8	192.0
19	ND	ND	ND
20	ND	ND	ND
21	107.8	ND	ND
22	ND	ND	ND
23	201.0	Nd	1580.0
24	608.8	ND	ND
25	280.0	ND	2050.0
26	1873.0	4803.0	10975.5
27	599.5	147.3	356.3
28	1470.8	ND	ND
29	88.5	ND	ND
30	ND	917.5	1280.0
31	ND	ND	ND
32	Nd	ND	ND
33	ND	3335.0	6035.0
34	ND	1353.8	967.4
35	209.8	2438.6	4591.5
36	27.8	18132.0	12400.0
37	ND	5308.8	5015.0
38	209.0	4720.0	3693.0
39	780.0	ND	ND
40	ND	ND	ND
41	88.0	7215.0	2195.9
42	ND	ND	ND

[a] ND, not detectable.

The azadirachtin content of commercially available samples of neem oils from different sources in India has been reported earlier to vary from ND to 4026 ppm (Isman *et al*., 1990). However, information on salannin and nimbin contents was not available. The azadirachtin contents reported in this study are within the range reported earlier.

Effect of Physicochemical and Chemical Factors on Oil Yield and Azadirachtin, Nimbin, and Salannin Content of Oils. *Oil Yield.* The multiple regression revealed a significant negative correlation of oil yield with acid value. On the basis of the data given in Table 4, the following relationship between oil yield and acid value (AV) was obtained:

$$\text{oil yield} = 46.3911(\pm 4.1497) - 0.1304(\pm 0.0516)X_3 \quad \text{(AV)}$$

$$R^2_{(1,40)} = 0.14$$

Azadirachtin Content. The multiple regression analy-

Table 4. Regression Analysis for Prediction of Oil Yield of Test Neem Oils from Physicochemical Characters and Chemical Constituents

serial no.	explanatory variable	variable notation	all-variable model		stepwise model	
			regression coeff	SE[a] of regression coeff	regression coeff[b]	SE of regression coeff
1	saponification value	X_2	−0.0652	0.0759		
2	acid value	X_3	−0.1057	0.0673	−0.1304*	0.0516
3	iodine value	X_4	−0.1870	0.1392		
4	refractive index	X_5	4.5656	3.5448		
5	specific gravity	X_6	−7.2834	37.0895		
6	hue value	X_7	1.4388	0.9147		
7	azadirachtin	X_8	−0.0652	0.0759		
8	nimbin	X_9	−0.0580	0.7125		
9	salannin	X_{10}	−0.0813	0.6712		
10	oleic acid	X_{11}	0.8160	16.2200		
11	stearic acid	X_{12}	−3.6165	15.4429		
12	palmitic acid	X_{13}	−6.3391	8.6945		
13	intercept		55.1708	36.5930	46.3911**	4.1497
14	F value		1.12		6.39*	
15	R^2 value		0.3155	8.4638	0.14	0.81

[a] SE, standard error. [b] *, significant at 5% probability level; **, significant at 1% probability level.

Table 5. Regression Analysis for Prediction of Nimbin Content in Test Neem Oils from Physicochemical Characters and Chemical Constituents

serial no.	explanatory variable	variable notation	all-variable model		stepwise model	
			regression coeff[a]	SE of regression coeff	regression coeff[a]	SE of regression coeff
1	oil yield	X_1	0.0039	0.0484		
2	saponification value	X_2	−0.0033	0.0203		
3	acid value	X_3	−0.0057	0.0183		
4	iodine value	X_4	−0.0205	0.0372		
5	refractive index	X_5	0.9213	0.9343		
6	specific gravity	X_6	2.3517	9.6617		
7	hue value	X_7	0.1475	0.2468		
8	azadirachtin	X_8	−0.0946	0.1333		
9	salannin	X_{10}	0.7045	0.1162	0.7946**	0.0781
10	oleic acid	X_{11}	−4.9107	4.1276		
11	stearic acid	X_{12}	2.3806	4.0038		
12	palmitic acid	X_{13}	−1.1754	2.2759		
13	intercept		−0.4516	9.9021	0.5483	0.4709
14	F value		7.80**		103.52**	
15	R^2 value		0.76	2.21	0.71	2.17

[a] *, significant at 5% probability level; **, significant at 1% probability level.

Table 6. Regression Analysis for Prediction of Nimbin Content in Test Neem Oils from Physicochemical Characters

serial no.	explanatory variable	variable notation	all-variable model		stepwise model	
			regression coeff[a]	SE of regression coeff	regression coeff[a]	SE of regression coeff
1	oil yield	X_1	−0.0075	0.0703		
2	saponification	X_2	−0.0236	0.0255		
3	acid value	X_3	−0.0327	0.0228		
4	iodine value	X_4	−0.1113*	0.0469	−0.1412**	0.0412
5	refractive index	X_5	3.1643*	1.2123	2.8307*	1.1993
6	specific gravity	X_6	22.0100	13.2600		
7	hue value	X_7	0.5197	0.3468		
8	intercept		−13.9345*	14.3077	2.0093	1.8187
9	F value		3.17*		6.76**	
10	R^2 value		0.39	3.26	0.26	1.23

[a] *, significant at 5% probability level; **, significant at 1% probability level.

sis revealed no significant correlation between any of the physicochemical characters and the azadirachtin content at 5% probability level.

Nimbin Content. In a stepwise model, salannin was entered at the first step and gave a high R^2 value (0.71). Thereafter, no other parameter was included. On the basis of the data of regression analysis given in Table 5, the nimbin content based on salannin (Sal) content (X_{10}) may be predicted by the equation

$$\ln \text{nimbin} = 0.5483(\pm 0.4709) + 0.7946(\pm 0.0781) \ln X_{10}\,(\text{Sal})$$

$$R^2_{(1,40)} = 0.71$$

The influence of physicochemical characteristics, excluding the chemical ingredients, on nimbin content was also examined by regression analysis. Iodine value (X_4) (IV) was entered at the first step and gave an R^2 value of 0.16. Entry of refractive index (X_5) (RI) at the second step increased the R^2 value to 0.26. Data presented in Table 6 yielded the following regression equation for predicting nimbin content:

$$\ln \text{nimbin} = 2.0093(\pm 1.8187) - 0.1412(\pm 0.0412)X_4\,(\text{IV}) + 2.8307(\pm 1.1993)X_5\,(\text{RI})$$

$$R^2_{(2,39)} = 0.26$$

Salannin Content. A positive correlation with nimbin content ($R^2 = 0.72$) of the oils was shown (Table 7). On

Table 7. Regression Analysis for Prediction of Salannin Content in Test Neem Oils from Physicochemical Characters and Chemical Constituents

serial no.	explanatory variable	variable notation	all-variable model		stepwise model	
			regression coeff[a]	SE of regression coeff	regression coeff[a]	SE of regression coeff
1	oil yield	X_1	−0.0062	0.0514		
2	saponification value	X_2	−0.0119	0.0212		
3	acid value	X_3	−0.0119	0.0193		
4	iodine value	X_4				
5	refractive index	X_5	6.1950	4.3366		
6	specific gravity	X_6	10.3436	10.0841		
7	hue value	X_7	0.0634	0.2633		
8	azadirachtin	X_8	0.0375	0.1425		
9	nimbin	X_9	0.6937	0.1309	0.9078**	0.0892
10	oleic acid	X_{11}	6.1953	4.3366		
11	stearic acid	X_{12}	−2.6589	4.2468		
12	palmitic acid	X_{13}	−1.8445	2.4025		
13	intercept		−6.4580	10.4410	0.7530**	0.4978
14	F value		7.94**		103.52**	
15	R^2 value		0.77	3.24	0.72	2.18

[a] *, significant at 5% probability level; **, significant at 1% probability level.

Table 8. Regression Analysis for Prediction of Salannin Content in Test Neem Oils from Physicochemical Characters

serial no.	explanatory variable	variable notation	all-variable model		stepwise model	
			regression coeff[a]	SE of regression coeff	regression coeff[a]	SE of regression coeff
1	oil yield	X_1	−0.0044	0.0750		
2	saponification value	X_2	−0.0159	0.0273		
3	acid value	X_3	−0.0280	0.0243		
4	iodine value	X_4	−0.1370**	0.0501	−0.1641**	0.0431
5	refractive index	X_5	3.1522*	1.2931	2.9177*	1.2543
6	specific gravity	X_6	25.4100	14.1489		
7	hue value	X_7	0.4424	0.3699		
8	intercept		−18.0242	15.2614	2.6105	1.9023
9	F value		3.20*		7.29**	
10	R^2 value		0.40	3.466	0.29	3.53

[a] *, significant at 5% probability level; **, significant at 1% probability level.

the basis of the nimbin (Nim) content (X_9), its content may be predicted by the following regression equation:

$$\ln \text{salannin} = 0.7530(\pm 0.4978) + 0.9078(\pm 0.0892) \ln X_9 \text{ (Nim)}$$

$$R^2_{(1,40)} = 0.72$$

The influence of physicochemical parameters, excluding the chemical ingredients, was also examined. Iodine value (X_4) was entered in the first step and gave an R^2 value of 0.19. Entry of refractive index (X_5) in the second step increased the R^2 to 0.29. Thereafter, inclusion of any other variable kept its F value insignificant. Data presented in Table 8 yielded the following regression equation for predicting salannin content:

$$\ln \text{salannin} = 2.6105(\pm 1.9023) - 0.1641(0.0431)X_4 \text{ (IV)} + 2.9177(\pm 1.2543)X_5 \text{ (RI)}$$

$$R^2_{(2,39)} = 0.29$$

Bioactivity. Pure azadirachtin and salannin showed EC_{50} values of 0.29 and 72.0 ppm, respectively, against the neonate larvae of *S. litura* F. Pure nimbin, however, was inactive up to a concentration of 400 ppm. The EC_{50} values of the test oils, based on the limited bioassay study, ranged from 1.8 to 3550.3 ppm and bore no apparent correlation with the azadirachtin, salannin, nimbin, or fatty acid content of the samples, either individually or combined. In a stepwise model of multiple regression analysis, salannin was entered in the first step and gave an R^2 value of 0.37. Azadirachtin was entered in the second step and jointly with salannin gave $R^2 = 0.60$ (azadirachtin alone $R^2 = 0.23$). Further entries of the other variables kept its F value insignificant. Thus, of the multiple test factors, only salannin and azadirachtin influenced EC_{50} most favorably, being significant at 1% level. In the all-variable model also, azadirachtin and salannin showed significant correlation with EC_{50} at 1% and 5% levels, respectively.

The following regression equation for predicting EC_{50} has been obtained on the basis of the significant chemical parameters:

$$\ln EC_{50} = 7.2415(\pm 0.3527) - 0.2829(\pm 0.0684) \ln (\text{Aza}) - 0.2975(\pm 0.0497) \ln (\text{Sal})$$

$$R^2_{(2.39)} = 0.60$$

An R^2 value of 0.60 with azadirachtin and salannin indicated that besides these two meliacins, other meliacins or other factors not included in this study may also be responsible for imparting bioactivity to neem oil.

Regressing only the physicochemical factors (excluding the chemical constituents with EC_{50} as dependent variable) revealed that the EC_{50} increased linearly with iodine value and acid value. The combined correlation coefficient ($R^2 = 0.27$), though significant, was not very strong. The relationship of EC_{50} with acid value and iodine value may be depicted by the equation

$$\ln EC_{50} = 2.5125(\pm 0.8867) + 0.0230(0.0112) \text{ (AV)} + 0.0538(\pm 0.0203) \text{ (IV)}$$

$$R^2_{(2,39)} = 0.27$$

Isman *et al.* (1990) reported that the bioactivity of the oils against the variegated cutworm, *Peridroma saucia*,

and milkweed bug, *Oncopeltus fasciatus*, correlated highly with their azadirachtin content, but the same was not true against the strawberry aphid, *Chaetosiphon fragaefolii*. Rengasamy *et al.* (1993) reported that the bioactivity against *S. litura* did not correlate with the azadirachtin content of the oils. In other studies, the bioactivity has been reported to correlate with oil dose (Balandrin *et al.*, 1988) and with the volatile content of the oil (Pathak and Krishna, 1985; Pillai *et al.*, 1988). In the present study, salannin and azadirachtin appear to be the key meliacins responsible for the activity. Since the bioassay has been conducted against a small population of the test insect, the leads indicated need to be verified with larger and diverse insect populations.

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