Effect of Emulsion Size and Shelf Life of Azadirachtin A on the Bioefficacy of Neem (*Azadirachta indica* A. Juss) Emulsifiable Concentrates

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In a study of 33 recipes of neem oil based emulsifiable concentrates, the specific surface area of the emulsions and cream plus oil layer separation in emulsions at 24 h revealed a correlation of −0.6874 between them and correlations of −0.8940 and 0.6972, respectively, with bioefficacy (LC_{50}) against the 3-day-old second-instar larvae of the Bihar hairy caterpillar, *Spilosoma obliqua* Walker. Nearly 96−99% of azadirachtin A in emulsifiable concentrates (aza-A content = 617.93−1149.65 ppm) degraded during the heat stability test at 54 ± 1 °C for 14 days with half-lives ranging between 1.84 and 4.53 days. The LC_{50} values against *S. obliqua* were, however, statistically at par in both the pre- and the post-heat-treated samples, suggesting a similar effect of azadirachtin A and its degradation products on the bioactivity. The half-life of azadirachtin A could be enhanced by storing the concentrates at lower temperatures. A low pH of the formulation solvent did not check the degradation of azadirachtin A, as reported with aqueous solutions in the literature.

Keywords: *Azadirachta indica A. Juss; emulsifiable concentrate; azadirachtin A; particle size; shelf life; bioefficacy*

INTRODUCTION

Numerous papers have highlighted the insecticidal, antifeedant, growth inhibitory, oviposition deterrent, antihormonal, antifertility, and other effects of neem (*Azadirachta indica* A. Juss) against various insects (Singh and Kataria, 1991; National Research Council, 1992; Schmutterer and Singh, 1995; Kumar and Parmar, 1998). The bioefficacy results have attracted the attention of the pesticide industry in India and abroad. Nearly four dozen products are either marketed or awaiting commercialization in India alone (Parmar and Ketkar, 1996). Several others (namely Margosan-O, Align, Turplex, Azatin, Benefit, Neemix, Safer's Bioneem) have been reported in the United States. In addition, several other countries are in the process of commercializing neem-based pesticides.

Neem is commonly formulated as an emulsifiable concentrate (EC) based on either its seed/kernel oil or the alcohol extract of the seed/kernel. The various products reportedly vary in their composition and field performance (Parmar, 1995). Besides the recipe composition and the target pest, the ecotype variation could affect bioactivity (Rengasamy et al., 1993; Rengasamy and Parmar, 1995; Kumar and Parmar, 1996, 1997). One of the possible effects of recipe variation could be on the size of the resultant emulsion obtained on dilution of EC in water, which could be macro or micro. The recipe variation could also influence the shelf life of azadirachtin A (aza-A), the principal standard ingredient in these formulations. These two aspects have been investigated in this study.

MATERIALS AND METHODS

Neem Oil. Commercial grade oil was procured from Neem Mission, Pune, India.

Azadirachtin A. Reference azadirachtin A (purity = 95% HPLC, Trifolio-M GmbH) was obtained through the courtesy of Neem Mission, Pune, India.

Laboratory Solvent and Chemicals. For routine laboratory work, laboratory grade, and for HPLC analysis, analytical grade, chemicals and/or solvents were employed.

Formulation Solvents and Surfactants. Common formulation solvents and surfactants employed in formulating emulsifiable concentrates were studied. Commercial grade aromax and solvent naphtha (Bharat Refineries, Mathura, India), cyclohexanone (Sarabhai-Merck Chemicals, Bombay, India), and xylene (BDH Laboratories, Bombay, India) were used as solvents. Sixteen surfactants were used, namely, calcium dodecyl benzene sulfonate (DBS-Ca; Ahura Chemical Products Pvt. Ltd., Bombay, India), sodium petroleum sulfonate (Savita Chemicals, Bombay, India), Agrimul N_4R and Agrimul N_4S [blends of DBS-Ca and polyoxyethylene (POE), derivatives of fatty alcohol; Henkel Industries, Bombay, India], Emulsol EL, Emulsol CFA, Emulsol MA, and Emulsol MAS (respectively, a nonionic alkyl ether, a fatty ethoxylate, a nonionic ethylene oxide condensate, and blended ethers; HICO Products, Bombay, India), Atlox 3400 B, Tween-20, Tween-80, and Span 40 [respectively, blend of DBS-Ca and POE (*n*) nonylphenol, POE (20) sorbitan monolaurate, POE (20) sorbitan monooleate, and sorbitan monopalmitate; Atlas Chemical Industries, N.V.), Hyoxid-X-45, Hyoxid AAO (both alkyl aryl ethers; Aries Agrovet Industries, Bombay, India), and Triton X-100 [POE (10) *tert*-octyl phenol; BDH Laboratories, Bombay, India). The test recipes are given in Table 1.

Test Insect. Three-day-old second-instar larvae of the Bihar hairy caterpillar *Spilosoma obliqua* Walker reared on castor leaves at 27 ± 1 °C, 70−75% relative humidity, and 16/8 h light/dark cycle were used.

Preparation of Emulsifiable Concentrates. On the basis of an initial screen, 33 recipes were chosen to prepare neem oil ECs (25%, w/w/5% emulsifier, Table 1).

Physicochemical Properties of ECs. Emulsion characteristics, cold test, acidity/alkalinity, and flash point were studied as per CIPAC (MT36-1) (CIPAC, 1970) and IS: 14300 (ISI, 1995). Conformity of requirements was assessed as per

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Table 1. Recipes of Neem Oil Emulsifiable Concentrates Prepared and Evaluated in the Laboratory

recipe	active material	components of recipe: surfactant	components of recipe: solvent
1	neem oil	Agrimul N_4S	cyclohexanone
2	neem oil	Atlox-3400B	cyclohexanone
3	neem oil	DBS-Ca	cyclohexanone
4	neem oil	sodium petroleum sulfonate	cyclohexanone
5	neem oil	Triton X-100	cyclohexanone
6	neem oil	Tween-20	cyclohexanone
7	neem oil	Tween-80	cyclohexanone
8	neem oil	Span-40	cyclohexanone
9	neem oil	Emulsol MA	cyclohexanone
10	neem oil	Emulsol MAS	cyclohexanone
11	neem oil	Emulsol EL	cyclohexanone
12	neem oil	Emulsol CFA	cyclohexanone
13	neem oil	Hyoxid-X-45	cyclohexanone
14	neem oil	Hyoxid AAO	cyclohexanone
15	neem oil	Agrimul N_4R	xylene
16	neem oil	Tween-20	xylene
17	neem oil	Tween-80	xylene
18	neem oil	Emulsol EL	xylene
19	neem oil	Hyoxid-X-45	xylene
20	neem oil	Hyoxid AAO	xylene
21	neem oil	Agrimul 52B	aromax
22	neem oil	Atlox-3400B	aromax
23	neem oil	Tween 20	aromax
24	neem oil	Tween 80	aromax
25	neem oil	Emulsol EL	aromax
26	neem oil	Hyoxid-X-45	aromax
27	neem oil	Hyoxid AAO	aromax
28	neem oil	Tween-20	solvent naphtha
29	neem oil	Tween-80	solvent naphtha
30	neem oil	Emulsol EL	solvent naphtha
31	neem oil	Emulsol CFA	solvent naphtha
32	neem oil	Hyoxid-X-45	solvent naphtha
33	neem oil	Hyoxid AAO	solvent naphtha

IS: 14300 (ISI, 1995). In samples showing a creamed layer of ≥1 mL at a 30 min interval, heat stability was not studied. To obtain a wide range of particle size of the emulsions, the oily separation, wherever observed up to 1 h, was considered a part of the creamed layer in the evaluation of the conformity of the emulsion characters as per IS: 14300 (ISI, 1995). The prescribed emulsion stability criteria describe that any separation including creaming at the top and sedimentation at the bottom of the 100 mL emulsion prepared in standard hard water of 342 ppm hardness by diluting 2 mL of EC shall not exceed 2 mL.

Particle Size Distribution of Emulsions. The distribution was determined by using a Malvern Instrument model system 2600 droplet and particle size analyzer fitted with a He−Ne laser light source, a PS 14B cell system, and a work station loaded disk Malvern series 2600 software in easy mode.

Shelf Life Based on Azadirachtin A. For this study 16 recipes were selected on the basis of their superior physicochemical and bioactivity performances. Fifty milliliters of each EC was incubated in stoppered borosilicate glass bottles at 54 ± 1 °C for 14 days in an air oven. Initial azadirachtin A contents of these samples ranged between 600 and 1150 ppm, attained through approximate addition of azadirachtin A in methanol. Samples were drawn at 0, 1, 2, 3, 6, 10, and 14 day after incubation. These were subjected to a cleanup step to separate azadirachtin A from the interfering solvent and surfactant components (Azam et al., 1995).

Estimation of Azadirachtin A. Azadirachtin A was analyzed by high-performance liquid chromatography (HPLC) employing a Shimadzu HPLC fitted with LC-9A pumps in isocratic mode, a Rheodyne 7161 injector with a 20 μL loop, a Shimpack CLC-phenyl stainless steel column (6 mm diameter × 15 cm), and a SPDM 6A photodiode array detector. The operating conditions were as follows: mobile phase, MeOH/water (65:35) at 1.0 mL min^{-1}; detector wavelength, 214 and 250 nm; sensitivity, 0.05 AUFS. The data were acquired on a PCS-DG India Ltd. workstation, and quantification was done in the postanalysis session at 214 nm. Suitable aliquots (5−20 μL) of the test solutions were injected by employing a Hamilton syringe. The retention time of azadirachtin A was 5.00 min. After every four to five injections, the column was cleaned by an MeOH/water gradient elution to avoid erratic column behavior.

Bioassay. Each bioassay was performed with six concentrations in three replications. The concentrations were distributed around the approximate LC_{50} (percent oil basis), which was determined initially with each recipe. A stock emulsion of the required strength was prepared, and the subsequent dilutions were made by taking its calculated quantity and diluting with emulsion water. Test emulsions were sprayed using a Potter Precision Laboratory spray tower connected with a Humer air receiver (Potter, 1941). Both the larvae and the leaves used as their food were sprayed. Ten larvae each were taken in Petri dishes, and 1 mL of emulsion was sprayed at 0.35 kg cm^{-2} pressure. Likewise, 7- or 8-day-old castor leaves (∼25 cm^2 surface area) were separately sprayed with 1 mL each of the spray emulsion on each side. The sprayed larvae/leaves were dried under a fan and transferred to a glass jar (15 × 10 cm), covered with muslin cloth, and kept at 27 ± 1 °C for post-treatment observation. An emulsion water sprayed control was simultaneously maintained. Percent larval mortality (considering moribund as dead) was recorded after 48 h. No mortality occurred in the control.

Data Analyses. These were done as per Finney (1971). The mortality data were subjected to probit analysis, and LC_{50} values were determined from regression equations using a Basic LD_{50} program, version 1.1 (Trevors, 1986). Significance of differences between the LC_{50} values was assessed by the Fisher *t* test, and standard error of the difference between the two treatments was determined in the case of significant values. Correlation coefficient was employed to find out the relationship among the paired variates (particle size versus emulsion character or bioactivity, etc.) (Microstat, 1984, Ecosoft Inc.).

RESULTS AND DISCUSSION

Physiochemical Characteristics of ECs. The physiochemical properties of emulsifiable concentrates are reported in Tables 2 and 3.

Emulsion Characteristics. Of the 33 recipes (Table 1), only 22 (1−15, 17, 20, 22−24, 27, and 30) passed the 1 h emulsion stability test as required per IS: 14300 (ISI, 1995) (Table 2). All of the recipes employing cyclohexanone as solvent passed the test, whereas only three recipes in xylene (15, 17, and 20), four in aromax (22−24 and 27), and one in solvent naphtha (30) passed it. The emulsion characteristics changed with time, and at 2, 4, and 24 h of emulsification varied significantly from those at 1 h. The volume of creamed layer that separated from the obtained emulsions either increased or decreased with time. The decrease was accompanied by a corresponding increase in the volume of separated oil in the latter case.

According to the nature of layer separation, the test recipes could be grouped into three categories: (i) those exhibiting a creamed layer that increased with time, for example, 16 recipes numbered 5−7, 12, 15−19, 23−25, 28−30, and 32; (ii) those showing increasing oil separation with time, for example, recipes 1−4, 8, and 14; and (iii) those showing both the creamed and oil layers with time, for example, 9−11, 13, 20−22, 26, 27, 31, and 33.

The pre- and post-heat-treated samples revealed similar emulsion behaviors. The variation in emulsion characteristics provided samples with a range of emulsion size variation needed for this study.

Acidity. The emulsifiable concentrates prepared in cyclohexanone (recipes 1−14) showed acidity values

Table 2. Emulsion Characteristics of Test Neem ECs at Different Time Intervals[a]

	emulsion characteristics (mL) at											
	30 min		1 h		2 h		4 h		24 h		30 min after re-emulsification	
recipe	CL	OL	CL	OL	CL	OL	CL	OL	CL	OL	CL	OL
1	nil	nil	nil	0.5	nil	1.0	nil	1.5	nil	2.0	nil	0.5
	(nil)	(nil)	(nil)	(0.5)	(nil)	(1.5)	(nil)	(1.75)	(nil)	(2.25)	(nil)	(0.5)
2	nil	nil	nil	0.5	nil	1.0	nil	1.5	nil	2.0	nil	0.25
	(nil)	(0.25)	(nil)	(0.5)	(nil)	(1.5)	(nil)	(1.5)	(nil)	(2.0)	(nil)	(0.5)
3	nil	0.25	nil	0.5	nil	1.0	nil	1.5	nil	2.0	nil	0.5
	(nil)	(0.5)	(nil)	(0.1)	(nil)	(1.25)	(nil)	(2.0)	(nil)	(2.5)	(nil)	(0.5)
4	nil	0.25	nil	0.5	nil	1.5	nil	2.0	nil	2.5	nil	0.75
	(nil)	(0.5)	(nil)	(1.0)	(nil)	(1.5)	(nil)	(2.0)	(nil)	(2.75)	(nil)	(1.0)
5	1.0	nil	1.5	nil	2.75	nil	3.5	nil	4.5	nil	1.5	nil
6	1.5	nil	2.0	nil	3.0	nil	3.5	nil	4.5	nil	1.5	nil
7	0.5	nil	2.0	nil	2.5	nil	3.0	nil	3.5	nil	2.0	nil
8	nil	0.5	nil	1.0	nil	1.5	nil	2.0	nil	2.5	nil	0.5
	(nil)	(0.5)	(nil)	(1.0)	(nil)	(1.5)	(nil)	(2.0)	(nil)	(2.5)	(nil)	(1.0)
9	0.25	nil	1.0	nil	2.0	nil	1.0	1.5	1.0	2.0	1.0	nil
	(0.5)	(nil)	(1.25)	(nil)	(2.0)	(nil)	(1.0)	(1.5)	(1.5)	(1.5)	(1.5)	(nil)
10	0.5	nil	1.5	nil	2.0	nil	1.5	1.0	1.0	2.5	1.0	nil
	(0.5)	(nil)	(1.25)	(nil)	(2.0)	(nil)	(1.25)	(1.0)	(1.0)	(2.5)	(1.0)	(nil)
11	0.25	nil	0.5	nil	1.0	nil	1.0	0.5	0.5	1.5	1.0	nil
	(0.5)	(nil)	(1.25)	(nil)	(1.0)	(0.25)	(1.25)	(0.5)	(1.0)	(2.0)	(1.0)	(nil)
12	0.5	nil	0.75	nil	1.0	nil	1.5	nil	2.0	nil	0.5	nil
	(0.5)	(nil)	(0.5)	(nil)	(1.0)	(nil)	(1.5)	(nil)	(2.0)	(nil)	(1.0)	(nil)
13	0.5	nil	1.0	nil	1.5	nil	1.75	nil	1.5	1.0	1.0	nil
	(0.5)	(nil)	(0.75)	(nil)	(1.5)	(nil)	(1.5)	(1.0)	(1.0)	(2.0)	(1.0)	(nil)
14	nil	0.5	nil	nil	nil	2.0	nil	2.5	nil	3.0	nil	0.5
15	1.0	nil	2.0	nil	2.5	nil	3.5	nil	4.0	nil	2.0	nil
16	5.0	nil	7.0	nil	8.0	nil	9.0	nil	11.0	nil	5.0	nil
17	0.5	nil	1.0	nil	1.5	nil	1.5	nil	2.5	nil	2.0	nil
	(0.5)	(nil)	(1.25)	(nil)	(1.5)	(nil)	(2.0)	(nil)	(2.5)	(nil)	(2.5)	(nil)
18	3.0	nil	5.0	nil	6.0	nil	7.5	nil	8.0	nil	3.0	nil
19	4.0	nil	6.0	nil	7.5	nil	8.0	nil	9.0	nil	4.0	nil
20	nil	1.0	nil	nil	2.0	3.0	2.0	3.5	2.0	4.0	1.5	nil
	(nil)	(1.0)	(nil)	(nil)	(2.5)	(3.0)	(2.5)	(3.5)	(2.0)	(4.0)	(1.5)	(nil)
21	5.0	nil	6.0	nil	6.0	1.0	4.0	3.0	3.0	5.0	5.0	nil
22	0.5	nil	1.0	nil	1.5	0.5	1.5	1.0	1.0	2.0	2.0	nil
	(0.5)	(nil)	(1.0)	(nil)	(1.0)	(1.5)	(1.5)	(1.5)	(1.5)	(2.0)	(2.0)	(nil)
23	0.5	nil	1.0	nil	1.5	nil	2.0	nil	2.5	nil	1.0	nil
	(0.5)	(nil)	(1.0)	(nil)	(2.0)	(nil)	(2.5)	(nil)	(3.0)	(nil)	(1.0)	(nil)
24	1.0	nil	2.0	nil	2.5	nil	3.0	nil	4.0	nil	2.0	nil
25	1.0	nil	3.0	nil	5.0	nil	7.0	nil	9.0	nil	2.0	nil
26	3.0	nil	5.0	nil	6.0	nil	6.0	2.0	5.0	4.0	2.5	nil
27	0.5	nil	1.0	nil	1.5	nil	2.0	nil	2.5	nil	1.0	nil
	(0.5)	(nil)	(1.5)	(nil)	(1.5)	(nil)	(2.0)	(nil)	(2.5)	(nil)	(1.0)	(nil)
28	4.0	nil	5.0	nil	7.0	nil	8.0	nil	9.0	nil	4.0	nil
29	3.5	nil	4.5	nil	5.0	nil	6.5	nil	8.5	nil	5.0	nil
30	0.5	nil	1.0	nil	1.5	nil	1.5	nil	2.0	nil	0.5	nil
	(1.0)	(nil)	(1.0)	(nil)	(1.5)	(nil)	(2.0)	(nil)	(2.5)	(nil)	(0.5)	(nil)
31	2.0	nil	3.0	nil	2.5	1.0	2.5	2.0	2.0	3.5	1.5	nil
32	2.5	nil	4.0	nil	4.5	nil	5.0	nil	6.5	nil	3.0	nil
33	4.0	nil	5.0	nil	5.0	nil	5.0	1.0	4.5	3.0	3.5	nil

[a] CL, creamy layer; OL, oily layer; solid separation was nil in all cases; values in parentheses are for post-heat-treated samples.

ranging from 0.51 to 1.42% (m/m, equiv H_2SO_4), which were in excess of the prescribed limit of 0.5% equiv H_2SO_4. This was found to be due to the cyclohexanone sample employed in the study, which had considerable acidity of its own (1.22% equiv H_2SO_4). All of the other formulations passed the acidity requirement. The acidities of the pre- and post-heat-treated samples were generally similar in all cases (Table 3).

Low-Temperature Stability. All 33 recipes passed the low-temperature stability requirement at 10 °C [IS: 14300 (ISI, 1995); Table 3]. When the recipes were subjected to a temperature of 0 °C, some of them revealed turbidity or slight solid separation at the bottom. Recipes 1, 12−14, 16, 18, 19, 23, 24, and 31−33 showed a slight turbidity at the bottom, whereas eight recipes (6, 9, 20, 21, 25, and 27−29) revealed a slight deposit of solid material at the bottom. The remaining 13 recipes (2−5, 7, 8, 10, 11, 15, 17, 22, 26, and 30) were devoid of any turbidity or solid settling at bottom or oily matter separation. The behaviors of the pre- and post-heat-treated samples were similar.

Flash Point. The flash points of the recipes (Table 3) varied with the solvent used in their preparation. High flash points were observed in recipes employing aromax and solvent naphtha (74.5−85.5 °C), followed by the recipes employing cyclohexanone (37.5−46.5 °C) and xylene (31.0−33.5 °C) as solvent. All of the recipes had flash points above the prescribed minimum limit of 24.5 °C. The flash points of the pre- and post-heat-treated samples were similar.

Particle Size Distribution. Particle size distribution of neem oil emulsions obtained from the test emulsifiable concentrates is reported in Table 4. The specific surface area of the different recipes ranged from 0.3047 to 1.8049 $m^2\ cm^{-3}$. Emulsions of three recipes (3, 4, and 12) contained the finest of the particles. The specific surface areas and particle size ranges (micrometers) of these were 1.7918 (2.5−6.5), 1.8049 (1.5−3.5), and 1.6581 (2.5−10.0), respectively.

Relationship of Specific Surface Area with Cream plus Oil Separation in Emulsions. The specific surface area of the emulsion particles (Table 4)

Table 3. Low-Temperature Stability, Acidity, and Flash Point of Test Neem ECs[a]

	low-temperature stability							
	10 °C			0 °C			acidity	flash point
recipe	SS	OL	turbidity	SS	OL	turbidity	(% equiv H_2SO_4, m/m)	(°C)
1	nil	nil	nil	nil	nil	slight	1.29	45.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(slight)	(1.28)	(45.5)
2	nil	nil	nil	nil	nil	nil	1.42	42.0
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(1.45)	(42.5)
3	nil	nil	nil	nil	nil	nil	0.83	44.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(0.85)	(44.6)
4	nil	nil	nil	nil	nil	nil	0.51	43.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(0.66)	(43.5)
5	nil	nil	nil	nil	nil	nil	1.06	44.5
6	nil	nil	nil	slight	nil	nil	2.06	46.0
7	nil	nil	nil	nil	nil	nil	1.04	40.5
8	nil	nil	nil	nil	nil	nil	0.74	42.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(0.74)	(43.0)
9	nil	nil	nil	slight	nil	nil	0.66	40.5
	(nil)	(nil)	(nil)	(slight)	(nil)	(nil)	(0.75)	(41.5)
10	nil	nil	nil	nil	nil	nil	0.55	46.0
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(0.57)	(44.5)
11	nil	nil	nil	nil	nil	nil	0.71	46.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(0.65)	(45.5)
12	nil	nil	nil	nil	nil	slight	1.42	40.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(slight)	(1.36)	(41.0)
13	nil	nil	nil	nil	nil	slight	0.97	43.0
	(nil)	(nil)	(nil)	(nil)	(nil)	(slight)	(1.05)	(42.5)
14	nil	nil	nil	nil	nil	slight	1.18	37.5
15	nil	nil	nil	nil	nil	nil	0.14	32.5
16	nil	nil	nil	nil	nil	slight	0.02	33.5
17	nil	nil	nil	nil	nil	nil	0.02	33.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(0.02)	(33.5)
18	nil	nil	nil	nil	nil	slight	0.02	31.5
19	nil	nil	nil	nil	nil	slight	0.02	33.5
20	nil	nil	nil	slight	nil	nil	0.03	31.0
	(nil)	(nil)	(nil)	(slight)	(nil)	(nil)	(0.04)	(31.5)
21	nil	nil	nil	slight	nil	nil	0.19	64.0
22	nil	nil	nil	nil	nil	nil	0.16	61.0
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(0.15)	(61.5)
23	nil	nil	nil	nil	nil	slight	0.09	67.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(slight)	(0.09)	(68.0)
24	nil	nil	nil	nil	nil	slight	0.05	68.0
25	nil	nil	nil	slight	nil	nil	0.09	69.0
26	nil	nil	nil	nil	nil	nil	0.09	81.0
27	nil	nil	nil	slight	nil	nil	0.11	76.0
	(nil)	(nil)	(nil)	(slight)	(nil)	(nil)	(0.11)	(76.0)
28	nil	nil	nil	slight	nil	nil	0.07	74.5
29	nil	nil	nil	slight	nil	nil	0.09	76.5
30	nil	nil	nil	nil	nil	nil	0.07	81.5
	(nil)	(nil)	(nil)	(nil)	(nil)	(nil)	(0.07)	(81.5)
31	nil	nil	nil	nil	nil	slight	0.05	84.5
32	nil	nil	nil	nil	nil	slight	0.08	85.5
33	nil	nil	nil	nil	nil	slight	0.06	82.5

[a] Values in parentheses are for post-heat-treated samples. SS, solid separation; OL, oily layer; slight, negligibly small amount.

showed a significant correlation ($r = -0.6874$) with cream plus oil layer separation in emulsions at 24 h (Table 2), implying that mere observation of the layer separation of emulsions provided a fair idea about the particle size distribution of the product. Generally, the bigger particle sized emulsions broke down more quickly than the smaller ones. However, there were instances, for example, recipes 20 and 22, when the particle size failed to fully explain the excessive cream plus oil layer separation. Both of these recipes yielded fine emulsions with specific surface areas of 1.1160 and 1.0901 m^2 cm^{-3}, respectively; the separation of cream plus oily layer (6.0 and 3.0 mL, respectively) was unexpectedly more. Similarly, recipes 18, 19, and 21 yielded emulsions with relatively finer particles than recipe 24 but showed more layer separation than it. Such a behavior may be due to some specific ingredient(s) of the EC recipe.

Bioactivity against *S. obliqua* Walker. The calculated LC_{50} values along with the related parameters are reported in Table 5. The LC_{50} values of the different recipes varied widely, ranging from a minimum of 0.016 to as high as 0.278% (oil basis). These were grouped into three categories: <0.10%, between 0.1 and 0.15%, and >0.15%. The LC_{50} values of <0.10% with the neem materials were rated as good. Within each group, the lower the LC_{50} value, the greater the bioactivity.

Among the recipes employing cyclohexanone along with different surfactants (recipes 1−14), the samples 2−4 and 8−10 showed comparatively superior bioefficacy than the others. Recipes 1 and 11−14 ranked next in order followed by recipes 5−7. In cyclohexanone, the recipes employing sodium petroleum sulfonate, Span-40, DBS-Ca, and Atlox 3400B, as surfactants showed generally good bioactivity. The highest activity was observed in the recipe employing sodium petroleum sulfonate (4). In the case of the recipes in xylene (15−20), only two (17 and 20) employing Tween-80 and Hyoxid-AAO, respectively, showed good activity. Of the seven recipes (21−27) employing aromax, only three (22, 23, and 27), formulated by employing Atlox-3400B, Tween-20, and Hyoxid AAO, showed good activity. In the case of recipes (28−33) formulated in solvent naphtha, only one (30) employing Emulsol-EL had good activity. The data highlight the role of formulation

Table 4. Particle Size Distribution in Emulsions Obtained from Test Neem ECs

recipe	specific surface area (m^2 cm^{-3})	particle range (μm)	diameter (μm) at[a]		
			D[V,0.1]	*D*[V,0.5]	*D*[V,0.9]
1	0.9551	2.5−50	3.25	12.26	24.09
2	1.0953	2.5−50	3.19	3.75	24.72
3	1.7918	2.5−6.5	3.01	3.36	3.76
4	1.8049	1.5−3.5	2.98	3.35	3.74
5	0.3760	3.5−150	3.61	52.61	123.07
6	0.6304	5.0−150	6.14	22.67	73.81
7	0.6224	3.5−175	3.30	26.50	152.17
8	1.3301	2.0−40	3.54	15.14	13.80
9	1.3527	2.0−50	3.33	6.25	12.56
10	1.3685	2.5−40	3.13	13.38	13.72
11	1.2695	2.0−50	4.45	15.27	14.40
12	1.6581	2.5−10	3.19	3.54	3.59
13	1.0560	2.0−50	3.63	6.55	16.71
14	0.6997	3.5−100	3.33	19.60	113.16
15	0.4187	3.5−100	6.47	21.17	62.30
16	0.4220	5.0−175	6.68	20.58	99.02
17	0.9522	2.5−50	3.30	4.29	21.65
18	0.6101	6.0−175	5.30	23.08	128.17
19	0.6621	10.0−175	6.97	24.12	115.25
20	1.1160	2.0−50	3.23	7.56	15.79
21	0.6126	6.0−150	6.22	9.65	143.75
22	1.0901	2.5−50	3.19	3.75	24.78
23	1.1190	2.5−50	3.27	13.90	20.19
24	0.3168	2.5−180	3.64	15.59	127.14
25	0.3982	2.5−180	3.39	15.52	156.22
26	0.3341	2.5−180	4.05	18.76	151.91
27	0.9980	2.0−40	3.41	11.40	15.97
28	0.3249	10.0−90	4.90	28.34	62.74
29	0.5881	2.5−60	3.36	23.90	54.53
30	0.9893	2.0−50	3.24	9.57	18.84
31	0.6465	2.0−150	3.65	20.77	137.80
32	0.3047	2.5−150	6.31	27.63	131.45
33	0.3123	2.0−180	6.43	26.21	132.49

[a] *D*[V,0.1], *D*[V,0.5], and *D*[V,0.9] are standard "percentile" readings from the analysis; *D*[V,0.5] is the size at which 50% of the sample is smaller and 50% larger than it (syn. mass median diameter, MMD); *D*[V,0.1] is the size at which 10% of the same is below it; *D*[V,0.9] is the size at which 90% of the sample is below it.

Table 5. Bioassay of Neem Oil ECs against the Larvae of *S. obliqua* Walker

recipe	heterogeneity κ^2 (4 df)[a]	LC_{50} (%, oil basis)	fiducial limits
1	0.8449	0.096	0.0804−0.1156
2	2.8926	0.035	0.0257−0.0481
3	2.9621	0.033	0.0252−0.0425
4	2.9621	0.016	0.0126−0.0212
5	1.3913	0.186	0.1564−0.2222
6	0.7919	0.121	0.1038−0.1416
7	2.9009	0.125	0.1005−0.1571
8	4.6201	0.020	0.0151−0.0266
9	2.2012	0.043	0.0322−0.0566
10	4.6131	0.045	0.0331−0.0604
11	4.6084	0.072	0.0534−0.0984
12	4.3525	0.095	0.0761−0.1179
13	3.5026	0.086	0.6524−0.1139
14	3.8886	0.098	0.0775−0.1280
15	1.3288	0.179	0.1544−0.2076
16	2.0759	0.209	0.1558−0.2811
17	4.8151	0.039	0.0237−0.0589
18	2.0440	0.145	0.1164−0.1803
19	2.1866	0.114	0.0903−0.1446
20	2.8840	0.084	0.0643−0.1108
21	0.8090	0.144	0.1221−0.1688
22	2.3737	0.089	0.0663−0.1194
23	1.4640	0.083	0.0669−0.1037
24	3.1897	0.207	0.1585−0.2706
25	0.4562	0.184	0.1612−0.2096
26	1.3194	0.191	0.1474−0.2488
27	5.1643	0.091	0.0751−0.1144
28	3.7618	0.204	0.1493−0.2789
29	3.7618	0.162	0.1314−0.2000
30	4.4915	0.095	0.0781−0.1271
31	2.0540	0.117	0.0994−0.1394
32	4.3380	0.278	0.2268−0.3406
33	2.8253	0.244	0.1747−0.3407

[a] The κ^2 values of all the data were less than the tabulated value (13.277 at 1% level of significance) at 4 df. Thus, all of the data were homogeneous.

auxiliaries in influencing the activity of neem oil based emulsifiable concentrates.

Particle Size and Emulsion Characteristics in Relation to Bioactivity. The specific surface area of the emulsions (Table 4) and the emulsion characteristics (cream plus oil layer at 24 h, Table 2) revealed correlation values of, respectively, −0.8940 and 0.6972 with bioactivity (LC_{50}, Table 5). Thus, for a superior activity, microemulsions need to be formed. The extent of their formation is reflected by specific surface area values as well as the simple measurement of the separated (cream plus oil) layer. The latter can be employed as a simple index of the bioactivity of a recipe.

Shelf Life of Azadirachtin A in Neem Formulations. Periodic azadirachtin A content of the 16 formulations used in the study is reported in Table 6. The degradation of azadirachtin A was found to follow first-order kinetics. $t_{1/2}$ values are also reported in Table 6. Assuming that the degradation of azadirachtin A increased 3 times for every 10 K rise in temperature, the Arrhenius equation was used to calculate the rate constant (k). The equation was utilized to calculate theoretical $t_{1/2}$ values of azadirachtin A at different temperatures lower than 54 ± 1 °C (Table 6).

The data revealed that the azadirachtin A content, which initially ranged between 617.93 and 1149.65 ppm in different samples at 0 day, fell to 10.11 to 29.61 ppm after 14 days of heat treatment. Generally, most of the degradation occurred up to the third day of incubation. Some of the recipes (1, 3, 4, 8−12, 22, 23, 27, and 30) showed an initially faster rate of degradation (60.41−76.02%, third day) than the others (recipes 2, 13, 17, and 20, 37.61−54.45%). Nearly 96−99% of the initial azadirachtin A was degraded in all of the recipes by the 14th day of the incubation. At lower temperatures, $t_{1/2}$ values were higher, suggesting a low-temperature storage of the neem ECs to reduce azadirachtin A degradation. Except in recipe 2 with a $t_{1/2}$ of 4.53 days, these values ranged between 1.84 and 2.97 days at 54 °C. The inter-recipe variation in the $t_{1/2}$ values underscores the effect of recipe composition on azadirachtin A degradation. It is mentioned that a slight acidity of the aqueous medium has been reported to be conducive in improving the stability of azadirachtin A (Jarvis et al., 1998). In the present study, despite the slight acidity observed in cyclohexanone-based ECs, apparently no effect on azadirachtin A stabilization has been noticed.

Bioactivity of Pre- and Post-Heat-Treated Concentrates. To ascertain the effect of shelf life on the bioactivity of the formulations, the pre- and post-heat-treated samples of the 16 recipes (1−4, 8−13, 17, 20, 22, 23, 27, and 30) were bioassayed simultaneously to minimize the biological and environmental variations. The bioefficacy results are reported in Table 7. The data revealed that LC_{50} values of the post-heat-treated samples increased slightly except in recipe 10, for which a slight decrease in LC_{50} was noted. The LC_{50} values of pre- and post-heat-treated samples were statistically at par (Fisher t test). The given levels of azadirachtin A and its transformation products had apparently similar effects on the bioactivity of the oil-based concentrates. Similar observations were reported earlier (Meisner et al., 1976, 1981; Rengasamy et al., 1993; Rengasamy and

Table 6. Azadirachtin A Content of Neem Emulsifiable Concentrate at 54 ± 1 °C for 14 Days and $t_{1/2}$ Values[a]

	azadirachtin content (ppm) at							calcd $t_{1/2}$ (days) at					
recipe	0 days	1 day	2 days	3 days	6 days	10 days	14 days	54 ± 1 °C	45 °C	35 °C	25 °C	15 °C	5 °C
1	925.30	659.42 (28.73)	470.84 (49.11)	239.37 (74.13)	139.23 (84.95)	30.53 (96.70)	23.81 (97.42)	1.84	5.54	16.63	49.89	149.68	449.06
2	617.93	607.34 (1.71)	540.56 (12.52)	385.48 (37.61)	186.74 (69.77)	52.76 (91.46)	22.76 (96.31)	4.53	13.59	40.76	122.29	366.88	1100.64
3	739.69	693.20 (6.28)	408.51 (44.77)	276.29 (62.64)	127.19 (82.80)	54.89 (92.57)	13.89 (98.12)	2.73	7.02	21.07	63.21	189.63	568.91
4	685.64	661.88 (3.46)	426.50 (37.79)	217.02 (68.34)	154.30 (77.49)	56.96 (91.69)	15.51 (97.75)	2.94	8.81	26.42	79.29	237.85	713.53
8	1149.65	923.09 (19.70)	580.90 (49.47)	394.68 (65.66)	135.13 (88.24)	73.33 (93.12)	25.33 (97.79)	2.28	6.86	20.58	61.75	185.25	555.97
9	636.88	521.35 (18.13)	289.63 (54.52)	188.11 (70.46)	65.58 (89.70)	29.09 (95.43)	15.79 (97.52)	2.17	6.51	19.55	58.65	175.96	527.84
10	752.09	670.2 (10.88)	383.77 (48.97)	262.50 (65.09)	133.04 (82.31)	27.72 (96.31)	13.16 (98.25)	2.14	6.43	19.30	57.93	173.78	521.36
11	1072.29	819.13 (23.60)	626.68 (41.55)	335.79 (68.68)	129.96 (87.88)	54.60 (94.90)	29.61 (97.23)	2.28	6.84	20.51	61.55	184.64	553.94
12	1010.56	820.80 (18.77)	653.11 (35.37)	384.81 (61.92)	178.51 (82.33)	54.64 (94.59)	12.81 (98.73)	2.23	6.68	20.05	60.16	180.49	541.47
13	772.65	671.32 (13.11)	541.25 (29.94)	370.73 (52.01)	147.05 (80.96)	57.54 (92.55)	15.05 (98.05)	2.77	8.32	24.98	74.96	224.89	674.67
17	641.90	461.88 (28.04)	326.88 (49.07)	292.53 (54.42)	142.56 (77.79)	53.35 (91.68)	15.50 (97.58)	2.46	7.39	22.19	66.58	199.76	599.26
20	690.73	572.88 (17.06)	457.97 (33.69)	314.58 (54.45)	142.40 (79.38)	24.47 (96.45)	21.99 (96.81)	2.45	7.35	22.03	66.11	198.34	595.04
22	741.38	685.69 (7.51)	362.94 (51.04)	242.46 (67.29)	85.88 (88.41)	26.10 (96.47)	13.56 (98.17)	2.34	7.02	21.07	63.21	189.63	568.91
23	962.20	882.60 (8.27)	636.81 (33.81)	380.91 (60.41)	186.21 (80.64)	56.50 (94.12)	25.75 (97.32)	2.97	8.42	26.76	80.30	240.91	722.74
27	932.89	830.29 (10.99)	529.46 (43.29)	223.70 (76.02)	56.39 (93.95)	35.49 (96.19)	10.11 (98.91)	1.89	5.67	17.04	51.12	153.36	460.10
30	834.18	681.93 (18.25)	503.34 (39.67)	297.17 (64.37)	73.06 (91.24)	22.35 (97.32)	13.80 (98.34)	2.42	7.27	21.81	65.43	196.29	588.87

[a] Values in parentheses denote cumulative percent azadirachtin A degradation.

Table 7. Azadirachtin A Content and Bioactivity of Pre- and Post-Heat-Treated Neem ECs against *S. obliqua* Larvae[a]

recipe	azadirachtin content (ppm)	heterogeneity (κ^2 4 df)[b]	LC_{50} (%, oil basis)	fiducial limits
1	925.30	0.8449	0.095	0.0845−0.1146
	(23.81)	(1.5182)	(0.134)	(0.1245−0.1746)
2	617.93	2.8926	0.049	0.0413−0.0575
	(22.76)	(2.3804)	(0.050)	(0.0414−0.0603)
3	739.69	1.6530	0.050	0.0429−0.0591
	(13.89)	(2.0550)	(0.059)	(0.0461−0.0757)
4	685.64	0.3816	0.023	0.0198−0.0262
	(15.51)	(1.0506)	(0.028)	(0.0229−0.0202)
8	1149.65	2.2675	0.016	0.0134−0.0202
	(25.33)	(3.8848)	(0.018)	(0.0158−0.0219)
9	636.83	1.5086	0.033	0.0264−0.0406
	(25.79)	(2.0307)	(0.039)	(0.0311−0.0406)
10	752.09	3.2457	0.047	0.3736−0.5913)
	(13.16)	(3.2260)	(0.040)	(0.0107−0.0534)
11	1072.09	1.5243	0.075	0.0562−0.0979
	(29.61)	(0.8996)	(0.097)	(0.0882−0.1251)
12	1010.56	0.9889	0.117	0.1012−0.1365
	(12.81)	(1.1655)	(0.121)	(0.1072−0.1496)
13	772.65	3.5209	0.094	0.0804−0.1165
	(15.05)	(4.9086)	(0.113)	(0.1023−0.1513)
17	641.90	3.4229	0.045	0.0341−0.0614
	(15.50)	(2.0246)	(0.048)	(0.0385−0.0693)
20	690.73	0.8715	0.094	0.0837−0.1179
	(21.99)	(3.9326)	(0.113)	(0.1012−0.1493)
22	741.38	0.8935	0.112	0.0935−0.1343
	(13.56)	(1.3943)	(0.129)	(0.1074−0.1627)
23	962.20	2.4506	0.122	0.1016−0.1477
	(25.75)	(1.6346)	(0.128)	(0.1056−0.1572)
27	932.89	1.9889	0.107	0.0807−0.1424
	(10.11)	(1.2744)	(0.117)	(0.1052−0.1679)
30	834.18	2.5303	0.115	0.1036−0.1524
	(13.80)	(2.4812)	(0.121)	(0.0891−0.1651)

[a] Values given in parantheses are for the post-heat-treated samples. [b] The κ^2 values of all the data are less than the tabulated value (13.277 at 1% level of significance) at 4 df. Thus, all of the data were homogeneous.

Parmar, 1995; Kraus, 1995; Lyons et al., 1996; Srivastava et al., 1997; Shankar et al., 1998; Shankar and Parmar, 1998).

Conclusions. A significant negative correlation between surface area and cream plus oil separation in emulsions and the subsequent significant correlation of each of these parameters with bioactivity suggest that a simple observation on cream plus oil layer separation could serve as a quick guide to ascertain the nature of emulsion (macro or micro) formed on dilution of neem ECs in water and the subsequent bioactivity of such concentrates. The formulants employed in ECs influenced the stability of azadirachtin A, the shelf life of which could be improved by storing the concentrates at lower temperatures. A similar bioactivity of the pre- and post-heat-stored samples (96−99% azadirachtin A loss) against *S. obliqua* indicated that at the test azadirachtin A levels perhaps the total meliacins and/or other bioactive constituents/transformation products, rather than azadirachtin A alone, were responsible for it.

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