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Pyrethrin from *Tanacetum cineriifoliun* as repellent against mosquitoes

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ABSTRACT

The present study is conducted to find the efficacy of plant extracts to act as mosquito repellents. Initially, the larvicidal activity of the extracts and the toxicity study was conducted. From the results, the extracts with maximum larvicidal activity and the minimum toxicity were used for the further analysis. The least toxicity was observed with the ethanol extracts of *Lantana camara*, *Tagetes erecta* and *Tanacetum cineriifolium*. The *Lanatana camara* and *Tanacetum cineriifolium* showed the maximum larvicidal activity. The DNA sugar damage assay is performed and read at 532 nm. The hemolytic activity of the extract is conducted and the results indicate that the extracts showed no hemolytic activity at the concentration of 100 and 200mg/L. Only the flower extracts of *Tagaetes erecta, Lantana camara* and *Tanacetum cineriifolium* showed moderate hemolytic activity at 500 and 1000 mg/L. Finally, the HPLC analysis of the extracts showed that the ethanol extracts of *Lantana camara* and *Tanacetum cineriifloium* revealed the presence of marker compound. Therefore the ethanol extracts of *Lantana camara* and *Tanacetum cineriifolium* were taken up for the membrane stabilization assay. The skin irritation potential of the cream prepared using the ethanol extract of *Tanacetum cineriifolium* was analysed, the cream showed no irritation.

***Key Words*:** Larvicidal assay, irritation, stabilization, toxicity, extracts.

INTRODUCTION

Mosquito-borne diseases have an economic impact, including loss in commercial and labor outputs, particularly in countries with tropical and subtropical climates; however, no part of the world is free from vector-borne diseases (Fradin and Day, 2002). Malaria is one of the serious scourges inflicted upon humidity. Every year, about 300 to 50 million people are estimated to be affected by malaria, and it further threatens 2,400 million (40%) of the world's population (WHO, 2005).

Mosquitoes in the larval stage are attractive targets for pesticides because mosquitoes breed in water, and it is thus easy to deal with them in this habitat. The uses of conventional pesticide in the water source, however, introduce much risk to people and/or environment. Natural pesticides, especially those derived from plants are more promising in this aspect (Amar and Mehlora, 2006). The chemical repellents are said to have a broader spectrum of activity. The synthetic repellents are manufactured using compounds such as DEET (N, N –diethyl -3 methyl –benzamide), Permethrin, Detlamethrin, Allethrin, Methoflurin, Bifenthrin or DDT. DEET is used in industry standard for insect repellents, and it works by blocking insect receptors used to detect carbon di oxide and lactic acid, which are used to find the host.

Chassalia kolly (Schumach) Hepper (Rubiaceae) is used in ethno-medical practices for the treatment of typhoid and fevers that are endemic in Nigeria as well as in the tropics and as an insect repellent. The methanolic extract of the dried whole plant *Chassalia kolly* was investigated for insecticidal, antimicrobial, phyto and cytotoxicity activities (Patricia *et al.*, 2010). Cytotoxicity was evaluated using brine shrimp lethality assay revealing its relatively non-toxicity with an LD_{50} value greater than 1000 μg/ml. Phytotoxicity using the Lemna bioassay showed a moderate growth inhibitory effect against Lemna minor. The insecticidal assay by contact toxicity method also revealed a moderate insecticidal rate of 40% against *Rhizopertha dominica* at the concentration of 1572.7 μg/cm^2. The extract contains glycosides, alkaloids and flavonoids. These results provide some scientific basis for the utilization of the plant in ethno-medicine for the treatment of typhoid fever and as an insect repellent (Patricia *et al.*, 2010). *Vitex negundo* Linn., of the family Verbenaceae possesses potent mosquito repelling activity against *Aedes aegypti*. *Tanacetum cineriifolium* is a cosmopolitan genus, comprising about 300 species of herbs and under shrubs, among which a few yield the commercial insecticide known as Pyrethrum. *Lantana camara* is considered as aggressive, and it has been regarded as one of the 10 most noxious weeds in the world. The plant is said to have carminative, antispasmodic and antirheumatic uses in traditional medicines (Grime *et al.*, 2006). The plant has antibacterial, insecticidal and nematicidal activity (Omara, 2005).

An attempt has been made in the present study to evaluate the larvicidal effect of various plant extracts that were screened and shortlisted. The plant extracts were also assessed for *in vitro* safety profile prior to the *in vivo* safety and toxicity studies. The plant extracts were tested for its hemolytic activity, cytotoxicity, membrane stabilization assay, DNA sugar damage assay. Those plant extracts that showed the larvicidal effect were only tested for the properties for assessing its safety to humans. In addition, HPLC profiles of the extracts were taken to identify any compounds that are similar to the pyrethrin of synthetic repellents.

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MATERIALS AND METHODS

Collection of plant material

The plants, including flowers of *Tanacetum cineriifolium*, *Tagetes erect* were obtained from the areas in and around Coimbatore, Tamil Nadu, India and *Lantana camara* were

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collected from Ooty, Tamil Nadu, India. The leaves of *Vitex negundo, Azadirachta indica* and *Citrus aurantifolia* were obtained from Coimbatore, and the rhizomes of *Acorus calamus* were obtained from T. Stanes-production unit. The taxonomic identification was made with the help of relevant literature.

Preparation of plant extracts
The leaves and flowers of the plant material were shade dried (28±2°C), ground and sieved to get fine powder from which the extracts were prepared. The air-dried, powdered plant materials were used for the preparation of extracts. The flowers were successively extracted by sequential extraction (low polar-high polar): hexane, petroleum ether and ethanol (Merck). Leaves and rhizome were extracted with aqueous solvent.

Essential oil extraction
Samples of fresh leaves (20g) were subjected to hydro distillation process for two hours, in a Clevenger-type apparatus (CORNING, Model #3410-1L). The essential oils collected subsequently were kept refrigerated at < 4°C until use.

Collection of mosquito larvae
Culex quinquefasciatus larvae were collected from the stagnant water area of Coimbatore district and identified by Chief Entomologist, T. Stanes. The larvae were kept in plastic and enamel trays containing tap water. They were maintained and reared under a photoperiod of 14:10 (L: D) at 27±2°C.

Preparation of extracts
From the stock solution, 100 ppm was prepared with dechlorinated tap water. The larvicidal activity was assessed by the procedure of WHO (1996) with some modification and as per the method of Rahuman *et al.* (2000).

Bio- efficacy of plant extracts
For the bioassay test, ten numbers of early fourth instar larvae were transferred to 100 ml distilled water in 120 ml disposable plastic cups. One drop of larval food was added per cup. The cups were treated with the prepared concentrations of extracts. Control was maintained using solvents such as hexane, petroleum ether and ethanol at the same concentration. The mortality of larvae was recorded at 24 hours and 48 hours post treatment. The experiments were conducted at 28±2°C and 70-80% relative humidity.

For recording the percentage mortality for each extract, the moribund and dead larvae were considered. Lethal Concentration (50) (LC_{50}) —A calculated concentration of a chemical to which exposure for a specific length of time is expected to cause death in 50% of a defined experimental animal population. These values indicated the 50% mortality. The values are calculated using probit regression analysis. When the control mortality was between 5 and 20%, the effects were corrected by using Abbott's formulae (Abbott 1925):

$$Corrected\ mortality = \frac{observed\ mortality(\%) - control\ mrtality}{100 - control\ mortality(\%)} \times 100$$

Antioxidant assay
In this assay, all solutions were prepared freshly. The reaction contained 0.5 ml of 2-deoxy-2-ribose, 0.5 ml of phosphate buffer, 1 mL solution of various concentrations of the extracts (in buffer), 2 mL of 4.8mM ferrous ammonium sulphate. After an incubation period of 1 h at 37°C the extent of deoxyribose degradation was measured by the TBA reaction. 1.0 mL of TBA (1% in 50 mM NaOH) and 1.0 mL of TCA (2.8%) were added to the reaction mixture and the tubes were heated at 100°C for 15 min. After cooling, the absorbance was read at 532 nm against a blank (containing only buffer and deoxyribose). The absorbance (A1) read at the end of the experiment was used for the calculation of the percentage inhibition of deoxyribose degradation by the test compound. The calculation was done using the formula:

$$I\% = \frac{A0 - A1}{A0} \times 100$$

A_0 = the absorbance of the control reaction (full reaction, containing no test compounds) and A_1= the absorbance in the presence of the inhibitor.

The IC_{50} value represented the concentration of the compounds that caused 50% inhibition. All experiments were carried out in triplicate.

Toxicity assay
Lethal toxicity assay
The eggs of brine shrimp *Artemia salina* were purchased from Fisheries College, Tuticorin. The eggs were hatched within 48 hours of being placed in artificial sea water (Saline). The Brine shrimp lethality assays on the extracts were carried out using initial concentrations of 10, 100 and 1000 μg/ml extracts. The test tubes were filled with 10ml of brine and ten shrimps (*Artemia salina*) were added to in each of three replicates. The tubes were maintained at room temperature. Survivors were counted after 24 hrs. The data were processed and LC_{50} values were obtained. Solvent served as negative and control.

Hemolytic activity
A glass stoppered flask is filled with 1/10 of the volume with sodium citrate to prepare erythrocyte suspension, swirling is done to ensure that the inside of the flask is done to ensure that the inside of the flask is thoroughly moistened. Introduced a sufficient volume of freshly collected blood and shaken immediately. Citrated blood prepared in this way can be stored for 8 days at 2-4°C. One millilitre citrated blood is placed in a 50ml volumetric flask with phosphate buffer (pH 7.4) and carefully diluted to volume. The diluted blood suspension 2% solution can be used as long as supernatant fluid remains clear and colourless and stored at a cool temperature. The solutions are freshly prepared. The extract of the plant material and dilution should be prepared as specified as the test procedure, using the phosphate buffer solution pH 7.4.

HPLC analysis
RP-HPLC method proved to be in good correlation with standard AOAC method and has been successfully used to separate the pyrethrin from the pyrethrum extracts (Kasaj *et al.*, 1999). The HPLC was performed under standard conditions. The mobile phase used for the separation was Acetonitrile-water (25:75). The flow rate of the mobile phase was maintained at 1ml/minute. The column used for the HPLC was C_{18} RP. The wave length of the standard was found to be 230nm. Therefore the HPLC was performed at 230 nm.

Cream preparation
A mosquito repellent cream was formulated based on the standard methods using the most effective extracts. Melt the measured quantity of bees wax in a vessel and to this polysorbate 60 and sorbitan stearate was added and

Table 1: LC_{50} value of larvicidal activity of the plant extracts.

Species name	Extract	LC_{50} Value (mg/L)
Citrus aurantifolia leaf	Aqueous	5.84
Vitex negundo leaf	Aqueous	7.07
Azadirachta indica leaf	Aqueous	7.32
Rhizome Acorus calamus	Aqueous	7.07
Lantana camara	Ethanol	4.56
Lantana camara	Hexane	3.57
Lantana camara	Petroleum ether	5.48
Tanacetum cineriifolium	Ethanol	4.11
Tanacetum cineriifolium	Hexane	3.75
Tanacetum cineriifolium	Petroleum ether	3.59
Tagetes erecta yellow	Ethanol	4.73
Tagetes erecta yellow	Hexane	4.11
Tagetes erecta yellow	Petroleum ether	3.91
Tagetes erecta orange	Ethanol	4.11
Tagetes erecta orange	Hexane	5.39
Tagetes erecta orange	Petroleum ether	7.66

heated to 65° in a water bath. Glycerine and hot water are premixed and added to the above mixture and stirred in a container on a hot plate until a cream is obtained. After the temperature of the mixture cools to 35°C, perfume and methyl paraben are added.

Skin irritation potential of repellent cream

The human 4-h patch test (Basketter *et al.*, 1997) using a commercially available patch, the Hill Top 25 chambers (Hill Top Research, Cincinnati, OH, USA), was operated to evaluate the skin irritancy of formulation in 10 healthy adult volunteers (7 females and 3 males; age range from 16-60 years). The patch test procedure involved application to the skin of 0.2 ml of test solution on a Hill Top Chamber containing a Webril pad. The patch, embedded with 25% ethanolic solution, 25% formulation, was placed on the upper inner arm of the volunteers for up to 4 h. Absolute ethanol and a 20% aqueous solution of sodium lauryl sulfate (20% SLS), used as negative and positive references, respectively, were applied in a similar manner. To avoid the production of unacceptably high reactions, test materials were applied progressively from 15 to 30 min through 1, 2, 3, and 4 h. The shorter exposure periods were omitted if the study director was satisfied that excessive reactions would not occur following longer exposure. Following the 4-h period of exposure to the test sample, the patch was removed, and the treatment sites were gently wiped with wet gauze to remove excess test material and then washed with distilled water. The sites of application were examined and scored at 24, 48, and 72 h after patch removal. Irritation responses were scored by using a four-point scale of increasing severity (Basketter *et al.*, 1997).

RESULTS

Larvicidal activity

The larvicidal activity of the extracts suggests that the flower extracts are more potential larvicidal agents. The highest larvicidal activity was exhibited by the *Tanacetum cineriifolium* and *Lantana camara* extracts. The ethanol extracts of *Tanacetum cineriifolium* are the most potent larvicidal agent among the tested extracts. The other extracts also showed relatively mild larvicidal effects. The aqueous extracts showed only moderate toxicity against the larvae. Similarly, the *Tagetes erecta* flower extracts also showed only lower larvicidal effects in comparison to other extracts of *Citrus aurantifolia*, *Vitex negundo*, *Acorus*

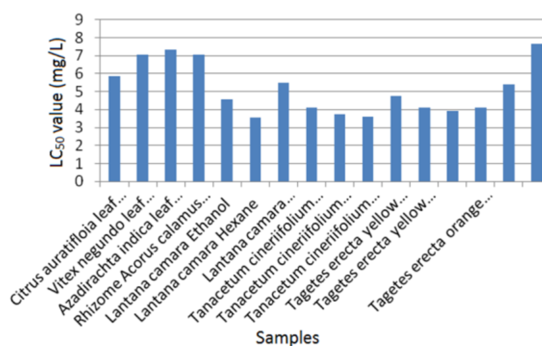


Figure 1: LC_{50} value of larvicidal activity of the plant extracts.

calamus, and Azadirachta indica are 5.84, 7.07, 7.32 and 7.07mg/L, respectively. The results are presented in table 1 and figure 1.

The ethanol extracts of *Lantana camara, Tanacetum cineriifolium* and *Tagetes erecta* (yellow and orange varieties) have exhibited larvicidal activity with LC_{50} values of 4.56 mg/L, 3.57mg/L and 5.48mg/L respectively. The hexane and petroleum ether extracts of *Lantana camara* showed an LC_{50} value of 3.75 mg /L and 3.91 mg /L respectively. The hexane and petroleum ether extracts of *Tanacetum cineriifolium* showed the values of 3.59mg/L and 4.11 mg/L respectively. The hexane and petroleum ether extracts of the yellow and orange varieties of *Tagetes erecta* exhibited only moderate larvicidal activity with the LC_{50} value of 4.73mg/L, 11mg/L, 5.39mg/L and 7.76mg/L, respectively. The *Lantana camara* and *Tanacetum cineriifolium* extracts prove to be the most potent larvicidal agents. They are therefore chosen for further analysis.

DNA Sugar damage assay

The ability of the extracts to prevent the damage to the ribose sugar moiety of the DNA is inhibited by the plant extracts. The results are presented in figure 2. The best inhibition of the ribose sugar damage is exhibited by the hexane extracts of *Lantana camara* and *Tanacetum cineriifolium* with a percentage inhibition of 17.05 and 15.89% respectively. The petroleum ether and ethanol extracts of

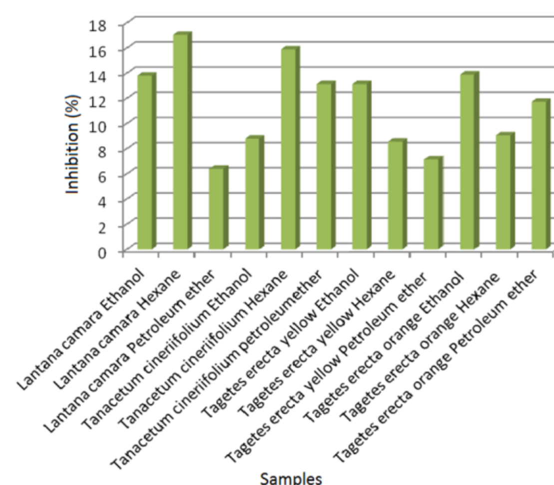


Figure 2: Percentage inhibition of DNA sugar damage.

Table 2: Cytotoxicity percentage of various plant extracts by using *Artemia salina*.

Species name	Extract	Mortality (%)	Control Mortality (%)
Tagetes erecta	Volatile oil	16.7	16.7
Citrus aurantifolia	Volatile oil	25	25
Acorus calamus	Volatile oil	0	0
Azadirachta indica	Volatile oil	0	0
Citrus aurantifolia leaf	Aqueous	10	10
Vitex negundo leaf	Aqueous	0	0
Azadirachta indica leaf	Aqueous	0	0
Rhizome Acorus calamus	Aqueous	28	28
Lantana camara	Ethanol	30	12.5
Lantana camara	Hexane	50	16.6
Lantana camara	Petroleum ether	70	40
Tanacetum cineriifolium	Ethanol	30	12.5
Tanacetum cineriifolium	Hexane	60	33.3
Tanacetum cineriifolium	Petroleum ether	70	40
Tagetes erecta yellow	Ethanol	40	25
Tagetes erecta yellow	Hexane	60	33.3
Tagetes erecta yellow		70	40
Tagetes erecta orange	Ethanol	40	25
Tagetes erecta orange	Hexane	70	50
Tagetes erecta orange	Petroleum ether	80	60

Lantana camara showed a percentage inhibition of 6.04% and 13.8%, respectively. The percentage inhibition exhibited by ethanol and petroleum ether extracts of *Tanacetum cineriifolium* was found to be 66.05 and 13.14%.The ethanol, hexane and petroleum ether extracts of the yellow and orange varieties of *Tagetes erecta* showed the inhibition levels of 13.14, 8.56 7.15, 13.89, 9.06 and 11.73% respectively. Therefore, the best DNA sugar damage inhibition is shown by the hexane extracts of *Lantana camara* and *Tanacetum cineriifolium*.

Cytotoxicity Assay

The cytotoxicity assay was performed using the various extracts at the concentration of 100mg/L. The *Artemisia salina* (Brine shrimp) larvae are taken for the cytotoxicity assay. The assay was performed, and percent mortality for twenty-four hours was calculated. The cytotoxicity assay as carried out using volatile oils of leaves of *Citrus aurantifolia, Azadirachta indica,* rhizome of *Acorus calamus* and flowers of *Tagetes erecta;* aqueous extracts of *Citrus aurantifolia, Vitex negundo, Azadirachta indica* and rhizome of *Acorus calamus.* The results are presented in figure 3 and table 2. It shows that among the four volatile oils only the oils of *Acorus calamus* and *Azadirachta indica* showed least cytotoxicity (0%).The greatest toxicity among the extracts and volatile oils tested was shown by *Citrus aurantifolia* volatile oil and *Azadirachta indica* aqueous extract. The leaf aqueous extract of *Citrus aurantifolia* shows mild toxicity of merely 10%.

The cytotoxicity assay is also performed using the ethanol, hexane and petroleum ether extracts of *Lantana camara, Tagetes erecta and Tanacetum cineriifolium*. The results indicate that the least toxicity was observed with the ethanol extracts of *Lantana camara, Tagetes erecta* and *Tanacetum cineriifolium*. The results obtained from the table 1 indicate that the highest toxicity was observed in the petroleum ether extracts of *Tagetes erecta* orange variety. The results indicate that the ethanol extracts of the flowers can be carried out for further analysis due to the low toxicity exhibited by them.

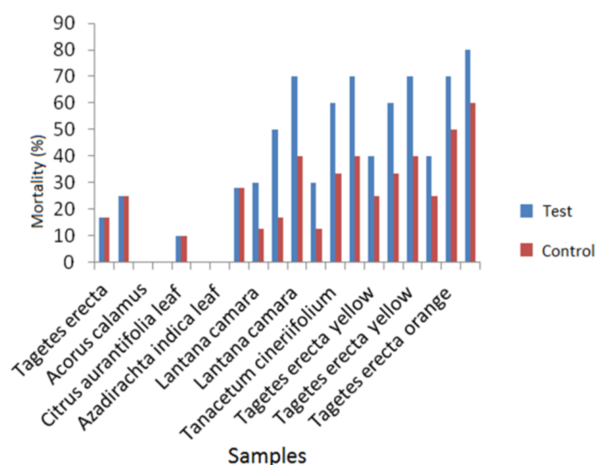


Figure 3: Cytotoxicity effect of various extracts.

Hemolytic activity

The hemolytic activities of the chosen plant extracts were performed, and it is seen that the least hemolytic activity is shown by the aqueous extract of *Citrus aurantifolia*. The tests were performed at various concentrations 100, 200, 500 and 1000mg/L. The results of hemolytic activity of the various concentrations of the extract are given in the table 3. The aqueous extract of Acorus calamus showed hemolytic activity only for the concentrations of 500 and 1000mg/L. Similarly the leaf extracts of *Azadirachta indica,* and *Vitex negundo* exhibited hemolysis only at the concentrations of 500 and 1000mg/L. It was found that the Flower extracts pose only a moderate hemolytic activity as there was hemolysis only for the high concentration of 500 and 100 mg/L.

HPLC Analysis

The HPLC analysis reveals the presence of the marker compounds in the ethanol extracts of *Lantana camara* and *Tanacetum cineriifolium*. The ethanol extract content of *Lantana camara* shows 0.89% of the active compound (Figure 4). The percentage of pyrethrin in the sample as indicated by the HPLC analysis is given in the table 4.

The analysis was carried out using the conditions that are suitable for the elution of the cypermethrin/ pyrethrin and it was observed that the fraction that was eluted at the retention time of minutes could possess the larvicidal activity. The compounds need to be fractionated and retested again to confirm the property and also to identify the fraction.

Table 3: Hemolytic activity of plant extracts.

Species name	Extracts	Concentration (mg/L)			
		100	200	500	1000
Lantana camara	Ethanol	-	-	+	+
Lantana camara	Hexane	-	-	-	-
Lantana camara	Petroleum ether	-	-	+	+
Tanacetum cineriifolium	Ethanol	-	-	+	+
Tanacetum cineriifolium	Hexane	-	-	+	+
Tanacetum cineriifolium	Petroleum ether	-	-	+	+
Tagetes erecta yellow	Ethanol	-	-	+	+
Tagetes erecta yellow	Hexane	-	-	+	+
Tagetes erecta yellow		-	-	+	+
Tagetes erecta orange	Ethanol	-	-	+	+
Tagetes erecta orange	Hexane	-	-	+	+
Tagetes erecta orange	Petroleum ether	-	-	+	+

Table 4: Percentage of pyrethrin obtained by HPLC analysis.

Sample Name	*Tanacetum cineriifolium*	*Lantana camara*
Petroleum ether	0.54%	0.034%
Ethanol	0.94%	0.89%
Hexane	0.24%	Trace

Table 5: Skin irritant potential of formulation, 20% SLS, and absolute ethanol.

Treatment	Number of volunteers	Scoring for skin irritation			
		0	+	++	+++
25% AHE	10	10	0	0	0
20% SLS	10	7	2	1	0
Ethanol	10	10	0	0	0

The individual pyrethrum esters are unavailable, thus most HPLC quantification methods use a commercial pyrethrum mixture with an estimated amount of 25% of total pyrethrins as a standard solution. The amount of total pyrethrins in the assayed sample was estimated by calculating the sum of measured peak areas of individual pyrethrins. The calibrating intervals covered the range of occurrence of all six compounds in the analyzed sample. These calibrating curves were used to determine the amounts of total pyrethrins, pyrethrins I, pyrethrins II, as well as the amounts of each pyrethrin ester in the assay and their percentage in dried flowers. The same detector response for all six esters based on their very similar chemical structure was assumed. For the first time, the results demonstrated that ethanol could be more effective in the extraction of pyrethrins than hexane or methanol. Considering its lower cost and toxicity, it is being recommended as the optimal solvent for laboratory and industrial-scale purposes.

Skin irritation potential of the prepared cream

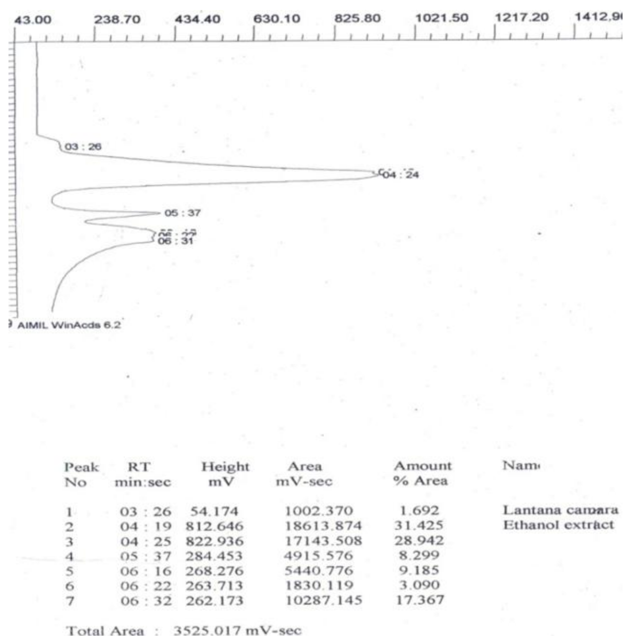
The results obtained from evaluating skin irritancy indicated that none of the 10 volunteers, who took part in the 4-h patch test, exhibited a positive skin irritant reaction to the formulation at any of the assessments. Similar results were also obtained from the application of absolute ethanol, a negative control reference.

On the contrary, three human volunteers showed positive irritant reaction to a positive control reference, 20% SLS, a widely used cosmetic ingredient. At 24 h of 20% SLS-patch removal, two adult females and one adult male showed positive irritation with slight (+) and moderate (++) reactions, respectively (Table 5).

DISCUSSION

Medicinal plants are part and parcel of human society to combat diseases, from the dawn of civilization. Plant products or plant-derived compounds are promising alternatives to synthetic insecticides in controlling insect pests of medical importance (Kelm and Nair 1996). Plants have been used since ancient times to repel or kill the bloodsucking insects in the human history and, even now, in many parts of the world people are practicing plant substances to repel or kill the mosquitoes and other bloodsucking insects. Plants can provide safer alternatives to modern deadly poisonous synthetic chemicals.

A study was performed earlier by the authors using the neutral red incorporation which detects the lysosomal functionality to determine the cytotoxicity of different extracts. After three-day treatment, all *Chrysanthemum*



Peak No	RT min:sec	Height mV	Area mV-sec	Amount % Area	Nam
1	03 : 26	54.174	1002.370	1.692	Lantana camara Ethanol extract
2	04 : 19	812.646	18613.874	31.425	
3	04 : 25	822.936	17143.508	28.942	
4	05 : 37	284.453	4915.576	8.299	
5	06 : 16	268.276	5440.776	9.185	
6	06 : 22	263.713	1830.119	3.090	
7	06 : 32	262.173	10287.145	17.367	

Total Area : 3525.017 mV-sec

Figure 4: HPLC report of *Lantana camera* ethanol extract.

extracts were generally tolerated by the Vero cells. However, the petroleum ether extract of *C. trifurcatum* roots presented a lower CC_{50} in comparison with other extracts. Concerning the cytotoxic potential of *Chrysanthemum* species, Ukiya *et al.* (2008) reported that arnidiol, a triterpene isolated from edible *Chrysanthemum* flowers, exhibited remarkable cytotoxic activity against a panel of human cancer cell lines, which suggested this compound to be useful as an anticancer agent (Ukiya *et al.*, 2008) Before conducting antiviral activity tests, the cytotoxicity of the extracts on the Vero cells was studied. Most of the extracts were well tolerated by Vero cells. The 50 per cent cytotoxic concentrations of all extracts were > 200 µg/ml, except the petroleum ether extract of *C. trifurcatum* roots (CC_{50} = 129.7 µg/ml), indicating that *Chrysanthemum* extracts were none or not much toxic on the Vero cells and *C. coronarium* extracts showed high cytotoxicity. The most active one appeared to be the petroleum ether–diethyl ether extracts (Gurkan *et al.*, 1998) The essential oil from *C. sibiricum* exhibited cytotoxic properties along with mild antioxidant activity (IC_{50} = 97.2 µg/ml) (Lee *et al.*, 2002). Kannathasan *et al.* (2007) reported that the methanol leaf extracts of *V. negundo, Vitex trifolia, Vitex peduncularis,* and *Vitex altissima* were used for larvicidal assay with LC_{50} value of 212.57, 41.41, 76.28, and 128.04 ppm, respectively, against the early fourth instar larvae of *C. quinquefasciatus*.

It was reported earlier that the isolated compound neemarin, from *Azadirachta indica* exhibited LC_{50} and LC_{90} values of 0.35 and 1.81 mg/L for *A. stephensi*. The petroleum ether (60-80°C) extracts of the leaves of *V. negundo* were evaluated with LC_{50} and LC_{90} values of 2.4883 and 5.1883 mg/L against larval stages of *C. tritaeniorhynchus* in the laboratory (Karunamoorthi *et al.*, 2008).

The benzene, chloroform, ethyl acetate, and methanol *Azadirachta indica* showed the highest effective attractancy of 90.09, 94.20, 85.43 and 95.75% were observed at 100 ppm and the lowest effective attractancy of 47.17, 61.94, 49.28 and 68.12% were observed at 25 ppm against *Anopheles stephensi*, respectively (Govindarajan *et al.*, 2008a). *Azadirachta indica* showed complete ovicidal

activity in eggs of *Culex tarsalis* and *Culex quinquefasciatus* exposed to 10 ppm concentration (Ouda *et al.*, 1998)

Fifteen peaks were detected by HPLC in the aqueous ethanol extract of *Chrysanthemum indicum* flower. 10 peaks were qualitatively and quantitatively determined, among which three were glycosides. The other five peaks were not identified owing to lack of authentic references. The total concentration of the detected flavonoids was 137.29±1.13 mg g-1. Quercitrin was the most abundant flavonoid, and myricetin was the next abundant one. The two flavonoids amounted to 65.3% of total concentration of the detected flavonoids. Vitexin and apigenin were the least abundant flavonoids, being less than 0.1 mg kg-1. Quercitrin was reported to have anti-oxidant and anti-carcinogenic activities via its inhibition of neoplastic transformation by blocking activation of the MAPK pathway and stimulation of cellular protection signaling (Ding *et al.*, 2010). Quercitrin concentration in the *Chrysanthemum indicum* flower of this study was significantly higher than that detected in *M. edule* shoot extracts reported by Hanen *et al.* (2009). Myricetin exhibited several pharmacological benefits, and its antioxidant properties were thought to contribute to its cancer-preventive effects. APE1 (apurinic/aprimidinic endonuclease) performs an essential function in DNA base-excision repair pathway, and it has become a target for researchers looking for means to prevent cancer cells from surviving chemotherapy. The knocking down APE1 could lead to tumor cell sensitivity, thus preventing cancer cells from persisting after chemotherapy (Luo *et al.*, 2008). Myricetin was confirmed to be an inhibitor of APE1, and it enhanced cellular sensitivity to the alkylating agent methyl methanesulfonate (Simeonov *et al.*, 2009). Luteolin-7-glucoside is a flavonoid derivative, ranked third abundant flavonoid compound in the tested sample which was reported to have antiasthmatic activity in an ovalbumin - induced lung inflammation via the down-regulation of T helper 2 cytokine transcripts as well as the inhibition of prostaglandin E-2 production (Jin *et al.*, 2009). It also had antioxidant and inflammatory activities (Ha *et al.*, 2006), hepatoprotective effects (Lima *et al.*, 2006) and inhibitory effect on aortic vascular smooth muscle cell proliferation (Kim *et al.*, 2006). Based on its abundant flavonoids, it is considered that *Chrysanthemum indicum* flower is a good source of natural quercitrin and myricetin.

In a study, the authors reported that the whole plant extract exhibited a minimum membrane stability of 3.65 ± 0.85% and maximum activity of 27.95 ± 1.27%. The mode of response of the erythrocyte was both monophasic and biphasic. Ethanol extract exerted a minimum membrane stability of 2.86 ± 1.58% and maximum activity of 91.76 ± 6.12%, respectively Ethyl acetate fraction (EAF) gave membrane stability of 13.52 ± 5.20 and 95.98 ± 3.15% as minimum and maximum percentage activity respectively. The response of the red blood cells was also monophasic and biphasic to the fractions. Moreover, butanol fraction also exerted minimum and maximum percentage stability activities of 4.84 ± 1.05 and 59.57 ± 4.19%, respectively. The response of the red blood cell was mainly monophasic at all the tested Concentrations The results revealed that both ethanolic and ethyl acetate fractions contained principles that protected the erythrocyte membranes effectively. Moreover, ethyl acetate fraction provided the highest protection against induced lyses. Also, it was noted that all the extracts and fractions showed dose dependent membrane stabilizing activity over all the concentration ranges. The activities of the extracts/fractions were higher than that of the standard drugs even at lower concentration ranges.

It has been reported that certain saponins and flavonoids exerted a profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro*, while tannins and saponins possess an ability to bind cations, thereby stabilizing erythrocyte membranes and other biological macromolecules (Oyedapo *et al.*, 2004) It was noted that ethanolic extract and ethyl acetate which gave positive tests for flavonoids exhibited highest membrane stabilities of 91.76 ± 6.12 and 95.98 ± 3.10%, respectively. This implied that the membrane stabilizing activities of these fractions were aided by the presence of flavonoid in them. The lowest activities observed with whole plant extract could be due to the masking of the action of the above named molecules that are associated with membrane stabilizing activities by other phyto-constituents. On the basis of these results, it could be inferred that the extracts/fractions of *L. camara* contained principles that were capable of stabilizing bovine red blood cells membranes against heat and hypotonic- induced lyses. The plan therefore could be regarded as a natural source of membrane stabilizers and was capable of providing an alternative remedy for the management and treatment of inflammatory related disorders and diseases.

The results of skin irritation potential derived from the study made by Teuten *et al.*,2008 revealed that, while nine of 27 human volunteers showed a positive skin irritant to 20% SLS, a widely used cosmetic ingredient, no irritant was observed in 25% AHE-treated ones. The foregoing results thus suggest that a single and short (4-h) topical application of AHE was not an irritant to human skin. Even though this study may not adequately reflect the effects of long-term and routine use of AHE as a topical repellent, the result obtained was important supportive evidence to establish the safety of AHE for its proposed applications to human volunteers in a mosquito repellent study. Subsequently, it was pleasing to find that AHE produced no irritation on human volunteers in laboratory repellent tests.

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