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Original Article

BIOASSAY GUIDED FRACTIONATION AND GC-MS ANALYSIS OF *EUPHORBIA LACTEA* EXTRACT FOR MOSQUITO LARVICIDAL ACTIVITY

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ABSTRACT

Objective: Plant latex is a source of various secondary metabolites useful as pharmaceuticals and pesticides. This study aims at screening *Euphorbia lactea* latex for larvicidal activity against three mosquito vectors, safety to non-target organisms, bioassay guided fractionation and GC-MS analysis of the active fraction.

Methods: Latex was collected from *E. lactea* and extracted with ethyl acetate (EA). The crude residue after removal of the solvent was screened against the larvae of *Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti* to get the median lethal concentrations (LC_{50}). The EA extract was also tested against the non-target organisms *viz*., Notonecta sp, *Nepa cincera,* Dragon fly nymph, *Dytiscus harrissi* and *Lithocerus indicus* to find out the safety to aquatic predators. The EA extract was subjected to bioassay guided fractionation and GC-MS analysis.

Results: EA extract of *E. lactea* latex was effective against the larvae of *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* with respective LC_{50} values 21.01, 25.65 and 49.69 mg/L. The *E. lactea* extract was found safe to aquatic mosquito predators Notonecta sp, *N. cincera,* Dragon fly nymph, *D. harrissi* and *L. indicus* as indicated by higher LC_{50} values 168.77, 193.09, 218.82, 294.22 and 318.87mg/L respectively. The calculated suitability index for *E. lactea* extract ranged between 3.39-15.17 showing predator safety. Bioassay guided fractionation of *E. lactea* extract resulted in an active fraction and identified the chemical constituents by GC/MS analysis as 1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1ar-(1aà,4á,4aá, 7à,7aá,7bà)]- a tricyclic sesquiterpenoid and 2,6-Octadiene, 2,4-dimethyl- an aliphatic hydrocarbon.

Conclusion: Identified the active fraction and the chemical constituents from *E. lactea* latex extract responsible for the mosquito larvicidal action.

Keywords: Mosquito; Larvicide; *Euphorbia lactea*; Plant extract; Sesquiterpene; Bti.

INTRODUCTION

Mosquito-borne diseases are in the escalating trend leading to substantial morbidity and mortality in spite of centuries of control efforts [1]. Control of the mosquito larvae is dependent on continued applications of organophosphates (chlorpyrifos, temephos, and fenthion) and insect growth regulators (diflubenzuron and methoprene) [2]. Effective, frequent use of controlling agents has disturbed natural biological control systems and led to insecticide resistance and amplified environmental and human health concerns [3]. This warrants the need for the development of new strategies for selective control of mosquito larvae. Plants are good source of alternative agents for control of mosquitoes [4, 5]. because they are rich in bioactive chemicals. Euphorbia genus is known to contain a wide variety of terpenoids, ranging from mono-, sesqui-, and diterpenes to triterpenoids and steroids known for their toxicity or potential therapeutic activity [6]. *E. lactea* is a succulent plant distributed mainly in tropical regions. All parts of the plant contain milky latex. Diterpenes isolated from *E. lactea* and *E. laurifolia* showed differential activity against HIV-1[7]. Latex from *E. lactea* contains tirucallol, a tetracyclic triterpene, which suppress ear edema in mouse [8]. The molluscicidal activity of *E. lactea* latex has been reported [9]. However, the mosquito larvicidal activity of this plant has not been studied earlier. This study aims at screening of plant latex from *E. lactea,* for larvicidal activity against three mosquito vectors *viz*., *An. stephensi, Cx quinquefasciatus* and *Ae. aegypti*, safety to non-target organisms, bioassay guided fractionation and GC-MS analysis to identify the active principle.

MATERIAL AND METHODS

General

Laboratory grade solvents such as petroleum ether, chloroform, ethyl acetate, methanol and Silica gel 60 F_{254} TLC sheets (E. Merck, Mumbai, India), ethanol (Hayman, England) and other chemicals mentioned (Sisco Research Laboratory, Mumbai, India) were used for the study. FT-IR spectrum was recorded on a Shimadzu FT-IR model 8300 (Shimadzu Corporation, Kyoto, Japan) using KBr (E. Merck, Mumbai, India). The GC/MS analysis was carried out at Indian institute of crop processing technology (IICPT), Thanjavur, Tamil Nadu, India. HPLC system (ThermoFinnigan, CA) composed of Spectra System P4000 solvent delivery system, Spectra System AS3000 autosampler and PDA detector SN4000 was utilized with ChromQuest 4.0 Chromatography workstation.

Plant latex collection and extraction

E. lactea was collected from Gingee hills, Villupuram District, Tamil Nadu, India and identified by a botanist. Voucher specimens were kept in the laboratory for future reference. Fresh latex was collected by making small incisions near the youngest bud and left to flow off into bottles with wide mouths. The latex was gently handled to maintain homogeneity during transport to the laboratory and kept at a temperature of 4^0C. Extractions were carried out with ethyl acetate. Each time 100 ml of the latex was extracted twice with 200ml of ethyl acetate solvent at room temperature. The solvent was removed at reduced pressure with the help of rotary vacuum evaporator to yield a viscous residue (8-10gm). The crude extract residues were used for bioassay against vector mosquitoes.

Bioassay

Mosquito larvicidal activity

The residues obtained after removal of the solvent were weighed and dissolved in absolute alcohol to get a 10% stock solution. Bioassay for the larvicidal activity was carried out as per ICMR common protocol [10]. Preliminary screening was done at 200ppm. Mosquito larvae were collected from the rearing and colonization laboratory of Vector Control Research Centre, Pondicherry. Twenty

five late 3rd instar larvae were introduced into 150 ml paper cup containing 100ml of water with each concentration. A total of four replicates kept for each concentration. Equal number of control cups was kept without the extract. Mortality was recorded after 24 and 48 hrs. The moribund and dead larvae in four replicates were combined and expressed as a percentage of larval mortality for each concentration. The test cups were held at 27± 2°C and 80-90 relative humidity and a photoperiod of 12 hrs light followed by 12 hrs dark. In case of any control mortality between 5-20%, the observed percentage mortality was corrected using Abbott's formula [11]. The experiment was repeated three times with proper doses to get the median lethal dose LC_{50}. Data from all replicates was pooled for analysis. A positive control *Bacillus thuringiensis israelensis* (Bti) (lyophilized powder of the biocontrol agent obtained from the Microbiology division of VCRC) and a negative control (solvent without test material) were used during the screening.

Effect on non-target organisms

The crude extract of *E. lactea* was screened against the non-target organisms as per standard procedure to find out the suitability index. Five aquatic predators of mosquito larvae (Notonecta sp, *Nepa cincera,* Dragon fly nymph, *Dytiscus harrissi, Lithocerus indicus* were released into 500ml disposable bowl containing 250 ml tap water. Only one predator was used in one bowl so as to avoid cannibalism. The predators were exposed to test concentrations ranging from 50 to 500mg/L. Four replicates were kept for each test concentration along with four replicates of untreated controls. The predators were observed for mortality and other abnormalities such as sluggishness and reduced swimming activity after 24 hrs exposure. LC_{50} and LC_{90} values were obtained by probit analysis. Suitability index (SI) or Predator safety factor (PSF) was calculated for each species of predator using the following formula [12].

$$\text{SI or PSF} = \frac{LC_{50} \text{ values of non-target organism}}{LC_{50} \text{ values of target vector species}}$$

Chi-square values and 95% fiducial confidence intervals (FI) were also calculated. Statistical evaluation was done using Statistical Package of Social Sciences (SPSS) 16.0 for windows, Significance level was set at $p<0.05$.

Bioassay guided fractionation and characterization of the fractions

Part of the ethyl acetate extract of *E. lactea* was further subjected to bioassay guided fractionation. Crude extract was fractionated by means of column chromatography using silica gel 60-120 mesh size. Twenty five gm silica gel was used for making slurry with petroleum ether (60-80°C) and loaded in a glass column of length 30cm and internal diameter 2cm. Each time 0.5gm of crude sample was loaded. Column chromatographic elutions were carried out with petroleum ether (7x10ml) followed by 10% ethyl acetate in petroleum ether (7x10ml) and 20% ethyl acetate in petroleum ether (15x10ml). Fractions were collected in separate quantified beakers and examined by thin layer chromatography (TLC). This was done on silica gel plates (Merck, $60F_{254}$) using petroleum ether/acetone in 80-20 ratio as the mobile phase. Visualization and identification of spots that indicate constituents of each fraction was done using an Ultra Violet lamp at a wave length of 254nm, by keeping in Iodine chamber and also by Anisaldehyde-suphuric acid spray followed by heating at 105°C. Finally, fractions having similar spots were pooled and concentrated. Each pool was screened for mosquito larvicidal activity at 100ppm against all the three species of mosquitoes. The effective fraction (Frs7-9 pooled) was further purified using preparative TLC using Petroleum ether /acetone (80:20) yielding further four fractions A1, A2, B1 and B2.

Phytochemical screening of the extract

Chemical tests were carried out for the presence of alkaloids, carbohydrates, saponins, tannins, phenolics, flavanoids and terpenes on the *E. lactea* extract and the most active fraction using standard procedures [13, 14].

GC-MS analysis

GCMS analysis of the active fraction B2 was carried out using a Perkin Elmer Clarus 500 Gas chromatograph equipped with a Mass detector Turbo mass gold-Perkin Elmer and a 30 x 0.25mm x 0.25µm Elite column coated with Elite-5MS (5% Diphenyl/95% Dimethyl poly siloxane). The carrier gas used was Helium at a flow rate of 1.0 ml/min at a column pressure of 42 Kpa. Component separation was achieved following a temperature program of 200°C (2°C/min). MS parameters were: Electron energy 70eV, mass scan (m/z) 45–450amu, source temperature 200°C and MS running time 36min. Peak identification was carried out by comparison of the mass spectra obtained with mass spectral data available on NIST Version-Year 2005.

HPLC analysis

An isocratic HPLC method was used for the determination of active ingredient content in *E. lactea* fraction. A Zorbaxsil normal phase analytical column (250 x 4.6 mm) and a mobile phase combination of 90:10 chloroform: methanol was used at 1ml/min flow rate. The fractions were analysed at 254nm using PDA detector.

RESULTS AND DISCUSSION

Due to the favourable safety of the microbial and botanicals based insecticides to the environment, the non-target organisms and mammals they may serve as alternate to the synthetic insecticides in the future for insect control. In the present study the latex collected from *E. lactea* was extracted with ethyl acetate and screened at different concentrations for larvicidal activity along with a negative control as ethanol and positive control as the biocontrol agent Bti against three species of mosquito larvae *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* to get the median lethal concentration (LC_{50}) values as given in Table 1.

Table 1: Larvicidal activity of ethyl acetate extract of *E. lactea* latex and Bti (+ve control) against three mosquito species

Test material	Mosquito species	LC_{50} (ppm)	LC_{90} (ppm)	95% confidence limit		χ^2
				LCL	UCL	
E. lactea latex extract	*An. stephensi*	21.01	33.78	31.43	36.96	2.847
	Cx. quinquefasciatus	25.65	47.01	42.73	53.31	0.053
	Ae. aegypti	49.69	90.58	71.38	167.31	4.167
Bti Positive control	*An. stephensi*	0.035	0.048	0.040	0.061	4.446
	Cx. quinquefasciatus	0.025	0.036	0.034	0.039	1.846
	Ae. aegypti	0.029	0.038	0.036	0.041	0.220

The LC50 values were 21.01, 25.65 and 49.69mg/L respectively against An. stephensi, Cx. quinquefasciatus and Ae. aegypti. The LC50 values of the positive control Bti were 0.035, 0.025 and 0.029mg/L respectively against An. stephensi, Cx. quinquefasciatus and Ae. aegypti. No control mortality was observed. The E. lactea extract was tested against the non-target organisms (Table 2) Notonecta sp, N. cincera, Dragon fly nymph, D. harrissi, L. indicus and the respective LC50 values were 168.77, 193.09, 218.82, 294.22 and 318.87mg/L. The calculated suitability index for E. lactea extract is given in Table 3. The values ranged between 3.39-15.17 showing predator safety. A value more than one for suitability index indicates that the chemical has better safety

The phytochemical analysis of ethyl acetate extract of *E. lactea* showed the presence of terpenes and carbohydrates while the active fraction B2 showed only the presence of terpenes. Alkaloids, tannins, saponins, flavanoids and phlobatannins were absent in the extract. The results of the bioassay guided fractionation of *E. lactea* extract resulted in a fraction B2 with R_f 0.431 effective against all the three species of mosquito larvae tested (fig.1).

The B2 fraction was subjected to GC/MS analysis at IICPT, Thanjavur, Tamil Nadu, India. The result of the GC/MS analysis of the active fraction is given in Table 4.

The graph showing the presence of two major peaks with retention times 26.07 and 28.26 minutes by GC analysis of fraction B2 is given in fig.2 and the percentage peak areas were 67 and 33% respectively.

Table 2: Effect of ethyl acetate extract of *E. lactea* latex on non target organisms

Species	LC_{50} (ppm)	LC_{90} (ppm)	95% confidence limit		χ^2
			LCL	UCL	
Notonecta sp.	168.77	285.62	261.56	320.16	1.946
N. cincera	193.09	317.75	288.79	363.32	0.869
Dragon fly nymph	218.82	369.12	321.78	453.59	1.340
D. harrissi	294.22	446.00	415.89	486.96	0.609
L. indicus	318.87	460.77	380.83	841.72	7.673

Table 3: Suitability Index/Predator safety factor of different mosquito predators with respect to mosquito larvae exposed to ethyl acetate extract of *E. lactea* latex.

Predator species	Suitability Index		
	An. stephensi	*Cx. quinquefasciatus*	*Ae. aegypti*
Notonecta sp.	8.03	6.57	3.39
N. cincera	9.19	7.52	3.88
Dragon fly nymph	10.41	8.53	4.40
D. harrissi	14.003	11.47	5.92
L. indicus	15.17	12.43	6.41

Table 4: Components identified in B2 Fraction of *E. lactea* – (Code no. 326) [GC MS study]

No.	RT	Name of the compound	Molecular Formula	MW	Peak Area %
1	26.07	2,6-Octadiene, 2,4-dimethyl-	$C_{10}H_{18}$	138	66.60
2	28.26	1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1ar-(1aà,4á,4aá,7à,7aá,7bà)]-	$C_{15}H_{26}O$	222	33.40

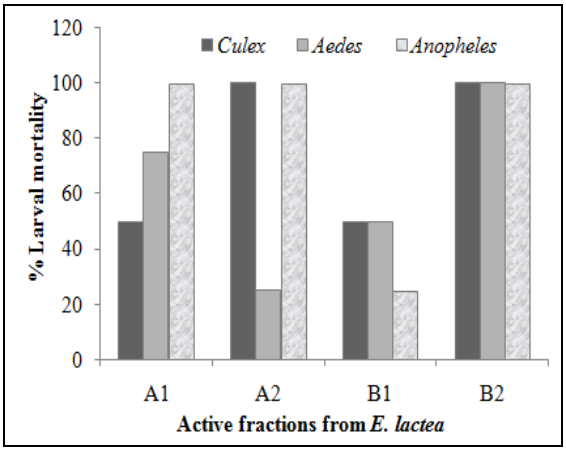
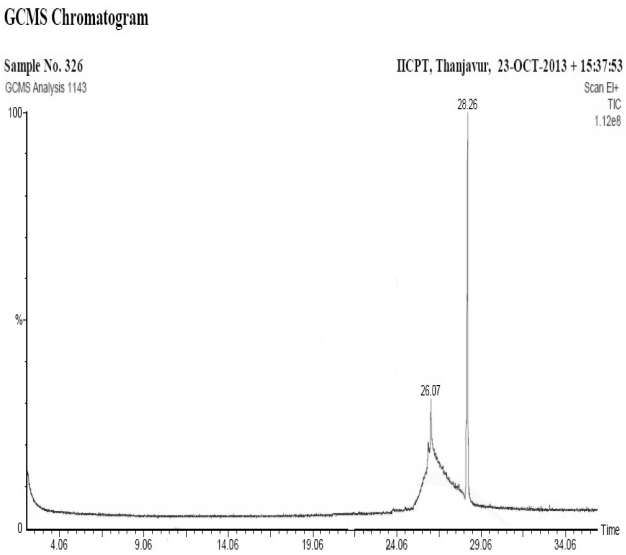


Fig. 1: Bioassay guided fractionation of *E. lactea*

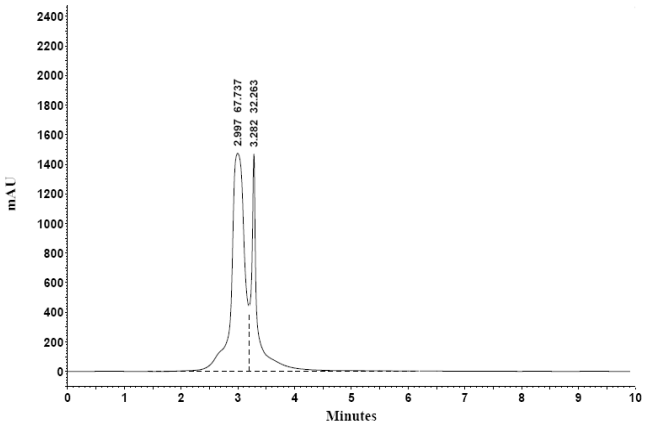
The result of HPLC analysis of B2 fraction is given in fig.3. HPLC analysis also shows the presence of two major peaks with retention times 2.9 and 3.2 minutes with percentage peak areas of 68 and 32% respectively. The IR spectrum shows an absorptions at 3414 cm-1 showing the presence of an –OH group; 2962, 2928 and 2852 cm-1 C-H (stretch) and 1450, 1417 and 1384 cm-1 C-H (bending).

The two chemical components present in fraction B2 are 2,6-Octadiene,2,4-dimethyl-(1B2) and 1H-Cycloprop[e]azulen-4-ol,decahydro-1,1,4,7-tetramethyl-,[1ar-(1aà,4á,4aá,7à, 7aá,7bà)]-(2B2) (fig. 4). NIST library search of the MS spectrum matches shows that 2B2 is an aromadendrane type sesquiterpene alcohol. Analysis of the results by comparing with the literature [15, 16] showed that the molecule 2B2 is one of the active principles responsible for the mosquito larvicidal activity.



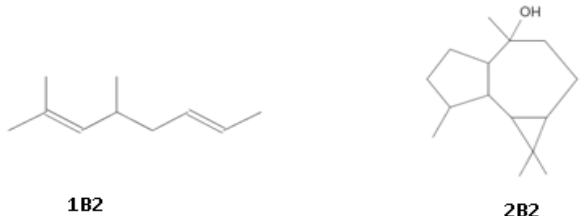
GC shows two peaks with retention times 26.07 and 28.26 minutes

Fig. 2: GC-MS analysis of B2 fraction of *E. lactea*



HPLC chromatogram shows two peaks with retention times 2.997 and 3.282 minutes and the respective % peak areas are 67.737 and 32.263.

Fig. 3: HPLC analysis of B2 fraction of *E. lactea*



1B2- 2,6-Octadiene, 2,4-dimethyl-; **2B2-**1H-Cycloprop[e]azulen-4-ol,decahydro-1,1,4,7-tetramethyl-,[1ar-(1aà,4á,4aá,7à,7aá,7bà)]-

Fig. 4: Chemical constituents identified in B2 fraction of *E. lactea*

Terpenes are natural products that often possess a carbon framework comprised of units of the five-carbon arrangement. Most terpenes possess carbon content in multiples of this five carbon C_5 arrangement. Sesquiterpenes comes with C_{15} (C_5 x3) arrangement. Sesquiterpenes are of particular biological importance. Terpenes are found in latex and resins of some plants and physiological function of these compounds is generally believed to be a chemical in defence against certain pathogens causing human and animal disease [17]. **2B2** is a tricyclic sesquiterpene containing a seven, five and three membered rings.

There is no previous report of the mosquito larvicidal activity of *E. lactea* latex extract, however, the molluscicidal activity [9] and anti-inflammatory activity [8] have been reported. The persistence of latent HIV-infected cellular reservoirs represents the major hurdle to virus eradication in patients treated with highly active antiretroviral therapy, referred to as HAART. A compound (3,12-di-O-acetyl-8-O-tigloylingol) isolated from *E. lactea* showed a potent HIV-1 reactivating effect [7]. The present study reports the mosquito larvicidal activity of *E. lactea* latex and the active fraction containing 1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1ar-(1aà,4á,4aá, 7à,7aá,7bà)]- a tricyclic sesquiterpenoid and 2,6-Octadiene, 2,4-dimethyl- an aliphatic hydrocarbon.

CONCLUSION

This study has identified the active fraction and the chemical constituents from *E. lactea* latex extract responsible for the mosquito larvicidal action. These molecules may be further used for chemical synthesis of more potent insecticidal analogues.

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