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Isolation and characterisation of triterpenoids from bioactive fraction of latex of *Euphorbia caducifolia* Haines (Family Euphorbiaceae)

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Abstract: Latex is a rich source of structurally diverse secondary metabolites with intriguing biological activities. Bio efficacy of latex from *Euphorbia caducifolia* Haines was tested against three larval species of mosquitoes, viz. *Aedes aegypti* (vector of Dengue/DHF), *Anopheles stephensi* (vector of Malaria) and *Culex quinquefasciatus* (vector of Filariasis) following the standard WHO protocol. On the basis of chromatographic fractionation, GC-MS, FTIR and NMR studies, we report two triterpenoids that were isolated from the bioactive fraction of methanol extract of latex from *E. caducifolia* Haines. The study will prove to be of immense chemotaxonomic importance.

Keywords: Latex; *Euphorbia caducifolia* Haines; bioactivity; triterpene

1. Introduction

Latex, typically a white milky fluid found in laticiferous plants is a rich source for structurally diverse secondary metabolites, including novel phytosterols and triterpenes with intriguing biological activities. The primary role of latex is self-defence of plants against grazing,

however, the healing properties of latex viz. emetic, anti-inflammatory, antibiotic, anti-haemorrhagic, antiviral, antitumor and abortive are known since ancient times. Reports on latex of *Calotropis procera*, *Carica papaya*, *Nerium indicum*, *Euphorbia royleana*, *Euphorbia tirucalli* and *Pedilanthus tithymaloides* [1-5] appear quite promising in displaying mosquito

larvicidal activity. *Euphorbia caducifolia* Haines (family: Euphorbiaceae, common name: Thour), a xerophytic shrub, grows wild in the dry and rocky region of India and Pakistan [6,7] have copious latex in the entire plant having purgative, nematocidal [8] wound healing [9] and antimicrobial [10] properties. Apart from Euphol, tirucallol, cycloartenol as major constituents, 31-norcycloartane type triterpene, cyclocadinol, 3-epi cyclolaudenol and β -sitosterol have been reported as chemical constituents of latex of *E. Caducifolia* [11-13]. The GCMS analysis of [10] fraction isolated from latex *E. caducifolia* showed presence of ethylpalmitate, 5,9- heptadecadienoate, methyl-11-octadecenoate, methyl octadecenoate and 3,7,11,15-tetramethyl- 2-hexadecene-1-ol. Ahemad et al. have reported diterpenoid, caudicifolin and jilkinolide A in ethanolic root extract of *E. Caducifolia* [14]. Bhattacharya et al. [15] have reported physico-chemical characteristics of both whole and toluene soluble portions of latex of *E. caducifolia* and suggest these as promising process aid for future rubber industry.

Herein, we present a systematic study on bio-efficacy of methanolic extract of latex from *E. caducifolia* Haines against three species of vector mosquito larvae; a simple approach to fractionation of methanol extract by HPLC, and characterise the chemical constituents therein, on the basis of the chemical test, GC-MS, FTIR and NMR studies.

2. Experimental

2.1 General

High-Performance Liquid Chromatography (HPLC) was carried out on a HPLC unit with binary pump and Holochrome UV-Vis detector (220 nm, 0.5X100 mV AUFS) from Gilson Medical Electronics, France. Infrared spectra were recorded on Shimadzu FTIR 8101 using KBr pellets. ^1H and ^{13}C -NMR were recorded on BRUKER DPX - 300 using CDCl_3 as solvent and TMS as an internal standard. 1D NMR spectra

were measured at 500 MHz for ^1H and 125 MHz for ^{13}C . The GC-MS analysis was carried out on Thermo Trace GC DSQ-MS instrument with an Alltech Econo Cap-EC-5. Analytical grade chemicals and HPLC grade solvents from E. Merck were used throughout the experiment. Melting points were determined in soft glass capillaries in an Electrothermal melting point apparatus and are uncorrected.

2.2 Plant Materials

The latex (500 gm) was collected from mature branches of *E. caducifolia* (from Kailana area, Jodhpur, Rajasthan, India) after giving an incision with the help of a sharp blade, the oozing latex was collected in sterile vials and was stored under refrigeration as crude latex. Eye protection and hand gloves were used while collecting the latex [16]. A voucher specimen no. HS/EC/CHEM/01 for *E. caducifolia* has been deposited at Botanical Survey of India, Arid Zone Circle, Jodhpur-342008, under Certificate no. SI/ AZC/ I.12012/ Tech./ 2012-13 (Pl.Id.)-260.

2.3 Extraction

The solvent assisted extraction of latex was done with solvents of different polarities such as acetonitrile, methanol, ethanol, acetone, methylene chloride and hexane to remove the proteineous part of the latex. The latex is mixed with solvents in a ratio 1:3 (v/v), stirred in an ultrasonic bath (Elma, Germany) for five minutes, centrifuged (Model Mikro Rapid-K, Hettich, Germany) at 5000 rpm for 10 min to remove and discard the residual proteineous matter. Methanol (dielectric constant, $\epsilon = 32.6$) was found to be the most suitable solvent for effective and efficient separation of insoluble part of latex as residue and supernatant liquid. The supernatant enriched solvent extract was filtered through sterile millex-GV0.22 micro-filter unit (Millipore, S.A. Molsheim, France). The solvent was evaporated under a soft stream of nitrogen, the resultant powdered residue (50mg/mL methanolic extract or 150mg/1000mg of

crude latex) was reserved for bio-efficacy trials, TLC and HPLC studies.

2.4 Bioassay

Methanolic extract and HPLC fractions were screened for larvicidal efficacy against laboratory reared⁵ vector mosquito larvae species viz. *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* following the standard WHO techniques [17]. Laboratory reared 40 late third instar larvae were exposed to different concentrations of latex extract ranging from 0.01 to 1 % (w/v) along with control under laboratory conditions of 28±1°C temperature and 70–80% relative humidity. The larvae were introduced into 250 ml of water, inoculated and equilibrated with required concentration of latex/ methanol extract/HPLC fraction, in a 500 ml beaker. For each concentration three replicate and one control assays were made and the mortality count was made after 24hrs of exposure period. The experiments showing more than 20% control mortality was discarded and repeated. The corrected mortality was calculated by applying Abbott's formula [18] and results were subjected to Statistical analysis of the experimental data using the computer software "NCSS 2007".

2.5 Chromatographic Separation and Isolation

The chromatographic screening of methanol extract was carried out by high performance liquid chromatographic (HPLC) on a reverse phase μ -Bondapak C18 column (300 mm X 3.9 mm id) with 10 μ m particle size and 125 Å pore size (Waters, WAT 027324) employing isocratic elution with methanol: water (70:30 v/v) as mobile phase at a flow rate of 1ml/min with UV detector at 220 nm. Preparative HPLC–UV was carried out on a reverse phase μ -Bondapak C18 column (300 mm X 19 mm id) with 15–20 μ m particle size and 125 Å pore size employing isocratic elution with methanol: water (70:30 v/v) at a flow rate of 20 ml/min. The fractions corresponding to major chromatographic peak

under preparative HPLC mode were collected and subjected to preparative TLC at silica gel G coated 20X20 cm glass plates, employing petroleum ether: ethyl acetate (80:20, v/v) solvent system and visualized under saturated chamber of iodine vapours. The bands obtained under preparative TLC mode were extracted in methanol, purified by re-crystallization with methanol and were reserved further for chemical tests, HPLC, IR, GC–MS and NMR studies.

2.6 Spectral Assay

2.6.1 Compound 1

Molecular formula, $C_{31}H_{52}O$ m.p. 112 °C; IR (KBr ν_{max}): 3560 (O–H stretching), 3045 (C–H stretch of cycloalkane), 2940, 2840 (C–H stretching, alkane), 1675 (tetrasubstituted double bond), 1460 (C–H bending), 1376, 1360 (gem dimethyl group), 1140 (C–O stretching of β -OH), 1320, 1015, 935, 660 cm^{-1} ; GC: Peak at R_t = 14.04 min; MS: m/z = 440; 216 (100%), 203, 407, 201, 425, 422, 217, 353, 379, 300; ^1H and ^{13}C NMR are shown in (Table S1).

2.6.2 Compound 2

Molecular formula, $C_{30}H_{50}O$: m.p. 135 °C. IR (KBr ν_{max}): 3595 (O–H stretching), 2960, 2853 (C–H stretching), 1660, 820, 720 (trisubstituted double bond), 1385, 1365 (gem dimethyl group), 1455 (C–H bending), 1330 (O–H bending) and 1130 (C–O stretching of β -OH), 820, 780, 720, 660 cm^{-1} . GC: Peak at R_t = 24.35 min; MS: m/z = 426 (100%), 274, 259, 275, 134, 163, 123, 109, 149, 205; ^1H and ^{13}C NMR are shown in (Table 1).

3. Results and Discussion

Biological activity of methanol extract of latex of *E. caducifolia* recorded as mosquito larvicidal efficacy, revealed that 0.6% and 0.9% (w/v) methanol extract of latex could bring 100% larval mortality in 24 hrs for *Aedes aegypti* (vector of dengue/ DHF) and *Anopheles stephensi* (vector of malaria) respectively

Table 1. ¹H and ¹³C NMR spectral data of compound **1** and **2**.

Table S1.

Position	1		2	
	δ C(ppm)	δ H(ppm)	δ C(ppm)	δ H(ppm)
1	32.9 (CH ₂)	1.44, 1.74(2H,m)	18.6 (CH ₂)	1.42, 1.75(2H,m)
2	30.2(CH ₂)	1.60(2H,m)	28.3(CH ₂)	1.62 (2H,m)
3	78.7(CH)	3.21(dd,1H,11.2,4.9) 2.01 (1 H, 7.2)	76.2(CH)	3.22(dd,1H,11.2,0) 1.80 (1 H, 6.8)
4	40.5(C)	---	40.9(C)	---
5	47.2(CH)	0.87(1H,m)	142.9(C)	---
6	24.5(CH ₂)	1.29, 1.62(2H, m)	121.0(CH)	5.34 (1H, m)
7	26.7(CH ₂)	1.88(2H,m)	24.1 (CH ₂)	2.05, 2.22 (2H,m)
8	47.3(CH)	2.12 (1H,m)	44.5(CH)	2.12 (1H,m)
9	20.0(C)	---	35.3(C)	---
10	26.3(C)	---	50.2 (CH)	1.87 (1H, m)
11	26.1(CH ₂)	1.50, 1.61 (2H,m)	33.9(CH ₂)	1.44, 1.60 (2H,m)
12	32.3(CH ₂)	1.36, 1.45 (2H, m)	30.3(CH ₂)	1.27, 1.58 (2H, m)
13	44.7(C)	---	46.1(C)	---
14	49.3(C)	---	49.4(C)	---
15	35.2(CH ₂)	1.32(2H,m)	34.2(CH ₂)	1.32(2H,m)
16	27.0(CH ₂)	0.96, 1.87 (2H,m)	28.2(CH ₂)	0.96, 1.86 (2H,m)
17	52.9(CH)	1.39 (1H,m)	49.9(CH)	1.40 (1H,m)
18	18.4(CH ₃)	0.65 (3H,s)	15.4(CH ₃)	0.64 (3H,s)
19	29.7(CH ₂)	0.50 (d,4.1), -0.04(d,4.1) (2H)	19.1(CH ₃)	1.04 (3H, s)
20	36.4(CH)	1.47 (1H,m)	35.6(CH)	1.44 (1H,m)
21	18.4(CH ₃)	0.87 (d,3H,6.3)	18.4(CH ₃)	0.88 (d,3H,6.0)
22	34.6(CH ₂)	1.64, 1.22(2H,m)	36.1(CH ₂)	1.64, 1.22(2H,m)
23	31.4(CH ₂)	2.12, 2.18 (2H,m)	25.2(CH ₂)	2.12, 2.18 (2H,m)
24	128.9(C)	---	125.3(CH)	5.17 (1H, d)
25	125.0(C)	---	131.2(C)	---
26	20.2(CH ₃)	1.77 (3H,s)	17.7(CH ₃)	1.61(3H,s)
27	20.2(CH ₃)	1.77 (3H,s)	25.7(CH ₃)	1.55 (3H,s)
28	18.5(CH ₃)	1.75 (3H,s)	26.0(CH ₃)	1.18 (3H,s)
29	15.9(CH ₃)	0.97 (3H,s)	28.6(CH ₃)	1.18 (3H,s)
30	24.3(CH ₃)	0.97 (3H,s)	19.0(CH ₃)	0.71 (3H,s)
31	19.1(CH ₃)	0.80 (3H,s)	---	---

however for *Culex quinquefasciatus* (vector of filariasis) only 50% larval mortality could be achieved even at higher dose of extract. LC_{50} and LC_{90} values for methanol extract of latex against larvae of *Ae. aegypti* were 0.282 % and 0.743% ($\chi^2=13.50$, df (p-value) = 12(0.17)) and that for *An. stephensi* values were 0.580 % and 0.920% ($\chi^2=15.50$, df (p-value) = 10(0.14)) respectively.

The bio-efficacy results prompted us to perform phytochemical analysis of bioactive fraction of latex of *E. caducifolia* and. The preliminary chemical analysis [19, 20] of insoluble part of latex revealed its proteinous nature whereas, methanol extract revealed the presence of terpenoids and cardiac glycosides. The TLC studies of methanol extract of latex revealed a single spot ($\sim R_f$ 0.61) using MeOH: $CHCl_3$ (9:1) as the solvent system. The HPLC resolution revealed well-resolved chromatogram (**Figure 1**), showing one major peak ' α ' ($\sim R_t$ 2.5 min) and a closely associated peak ' β ' ($\sim R_t$ 3 min) followed by eight minor peaks within 20 minutes of HPLC run.

The isolated fractions F1-F2 and F3-F10 under preparative HPLC mode, were screening independently for mosquito larvicidal efficacy; wherein, fractions F1-F2 (combined) revealed significant larvicidal action, comparable to that of the methanol extract. Therefore, further studies on isolation and characterisation of bioactive principles were focused on fraction F1-F2, corresponding to HPLC peak α and β . The isolated fraction F1-F2 revealed two spots ($R_f=0.57$ and $R_f=0.39$) on a TLC plate. The extracted and purified compounds from two bands (under preparative TLC mode), revealed a single peak each (**Figure 2**) under HPLC screening, confirms the presence of a single compound in each band corresponding to peak α and β . The powdered residue (38mg/mL) obtained from isolated fraction F1-F2 revealed two spots ($R_f=0.57$ and $R_f=0.39$) on a TLC plate.

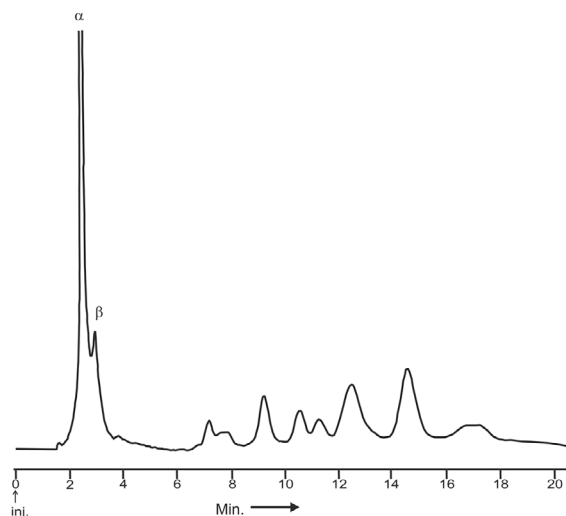


Figure 1. High performance liquid chromatogram of methanol extract of latex from *E. caducifolia* Haines. λ_{max} 220 nm; C-18 RP Column; mobile phase: methanol-water (70:30, v/v); flow rate of 1ml/min.; inj. 10 μ l.

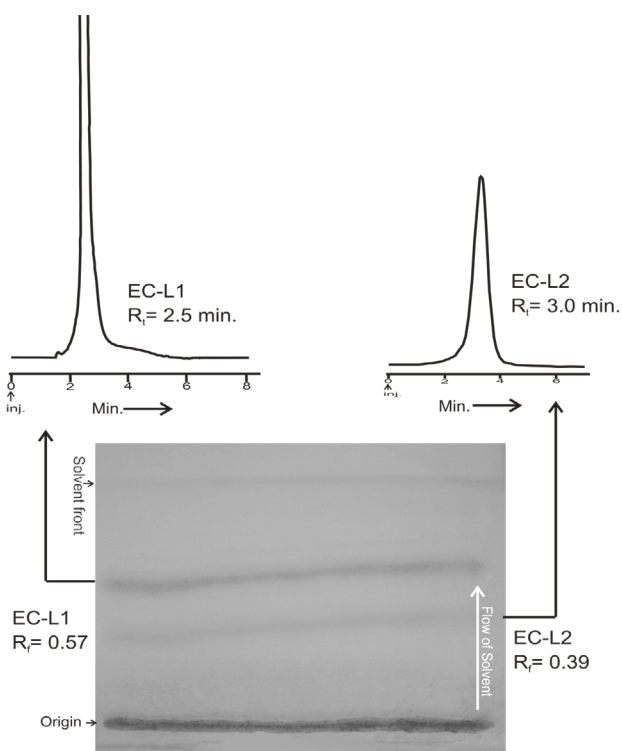


Figure 2. Isolated fraction F1-F2 under preparative TLC plate showing two bands and corresponding HPLC record of extracted compound 1 (ECL-1) and 2 (ECL-2).

24-Methyl-9,19-cyclolanost-24-en-3-ol (a lanostereone- type terpene) (**Figure 3**). The study of ^1H NMR spectrum of compound-1 shows that presence of eight methyl group, out of them one secondary methyl group appeared at δ 0.87 δ ppm as a doublet with 6.3Hz coupling constant, while three tertiary methyl group appeared at down field as compared to secondary as a singlet of 3H and 6H at 1.75 and 1.77 δ ppm respectively. The methyl group which is present as substituents of steroid nucleus shown in the range of 0.65 to 0.97 δ ppm as a complex of 12 protons along with 24 protons of steroid nucleus and alkyl chain. The methine proton of steroid nucleus which attached with hydroxy group appeared as a double doublet at 3.21 δ with 11.2 and 4.9Hz coupling constant respectively

The characteristic cyclopropyl methylene

protons appeared as two doublets at -0.04 (4.1) and 0.50 (4.1). The ^{13}C NMR spectra exhibited 31 carbon signals which also supported the given structure. The peaks at δ 128.9 and 125.0 were characteristic of a double bond between C-24 and C-25 atoms. The signal at δ 78.7 for C-3 showed linkage of hydroxyl group moiety at this carbon atom and the signals at δ 18.4, 18.4, 20.2, 20.2, 18.5, 15.9, 24.3 and 19.1 showed the presence of eight methyl groups in the proposed structure.

Compound **2**, m.p. 135°C also responded positively to Salkowski test and Libermann Buchard test, characteristics of terpenoids. The IR spectrum (KBr) of **2** displayed broad absorption bands at 3595 cm^{-1} confirming the presence of hydroxyl group. Characteristic absorptions at 2960 cm^{-1} and 2853 cm^{-1} for C-H

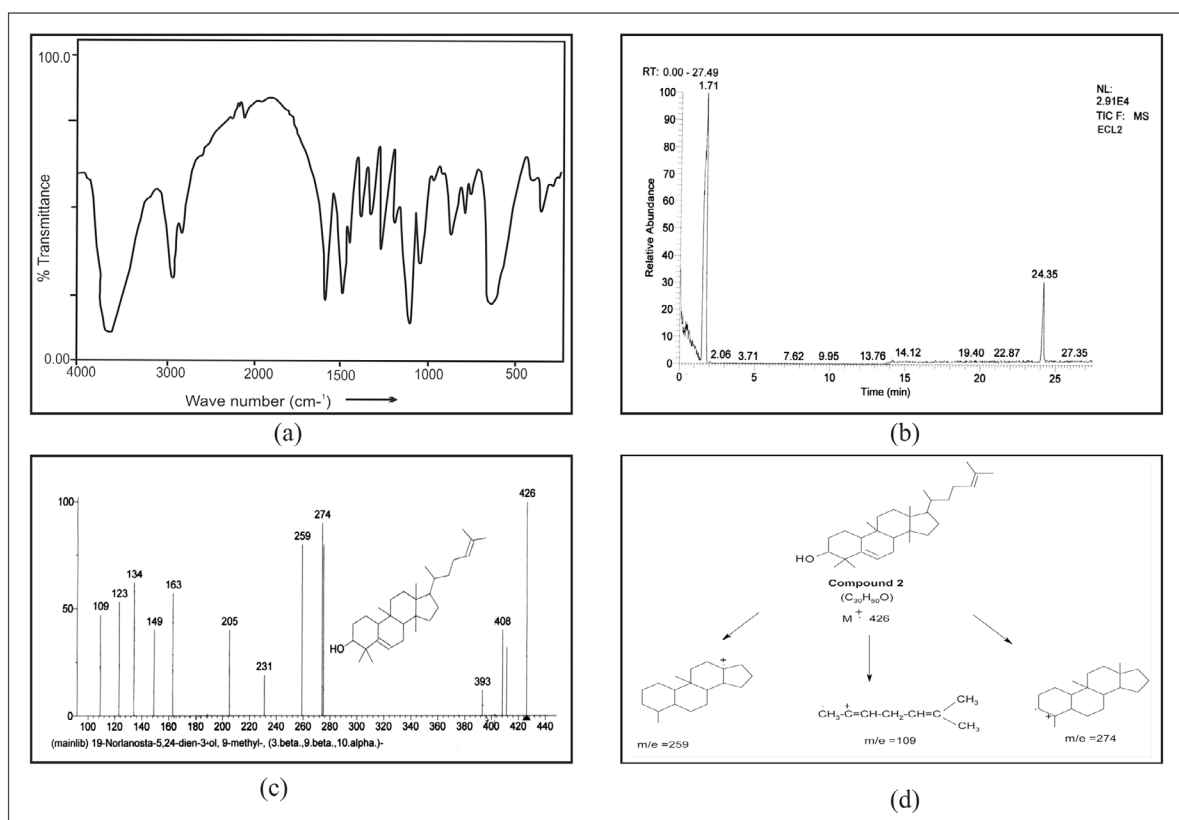


Figure 4. Spectral analysis for the identification of compound **2** (a) FTIR spectrum; (b) GC spectrum; (c) Mass spectrum; (d) Mass fragmentation pattern.

stretching vibration of alkyl group, 1660 cm^{-1} , 820 cm^{-1} , 720 cm^{-1} for trisubstituted double bond, 1455 cm^{-1} for C-H bending, a doublet at 1385 cm^{-1} , 1365 cm^{-1} for gem-dimethyl group, 1330 cm^{-1} for O-H bending and 1130 cm^{-1} for C-O stretching of β -OH were recorded.

In mass spectrum of compound molecular ion, the peak was observed at m/z at 426 corresponding to molecular formula $\text{C}_{30}\text{H}_{50}\text{O}$ together with peaks at m/z 274, 259, 275, 134, 163, 123, 109, 149 and 205 according to their relative abundance. On the basis of above evidences and on comparing the mass spectra of compound with the MAINLIB Computer Library software, compound **2** was identified as **19-Norlanosta-5, 24-dien-3-ol, 9-methyl-, (3 β , 9 β , 10 α)-**, (tetracyclic terpene) (**Figure 4**). The ^1H NMR spectra of compound- **2** showed signal at δ 3.22 (dd, 1H, $J=11.2, 4.9$ Hz) of methine proton which is attached with hydroxy group. The proton present in a steroid nucleus with double bond appeared as a triplet at 5.34 δ ppm and the proton of alkyl chain with double bond also appeared as a triplet at 5.17 δ ppm. The three protons singlets at δ 0.64, 1.04, 0.88, 1.61, 1.55, 1.18, 1.18 and 0.71 were due to the presence of eight methyl groups supported the proposed structure. The absence of doublets at -0.04 (4.1) and 0.50 (4.1) reveals that Cyclopropane ring absent in compound **2**.

The ^{13}C NMR spectra showed 30 carbon signals. The signals at δ 76.2 showed the presence of hydroxyl group at C-3 atom. The signals at δ 142.9, 121.0, 125.3 and 131.2 showed the presence of double bonds between C-5, C-6 and C-24, C-25 atoms respectively. The presence of eight methyl groups was further supported by ^{13}C NMR signals at δ 15.4, 19.1, 18.4, 17.7, 25.7, 26.0, 28.6 and 19.0.

4. Conclusions

Methanol extract *E. caducifolia* Haines revealed significant larvicidal activity against *Aedes aegypti* (vector of Dengue/DHF) and *Anopheles stephensi* (vector of Malaria) mosquitoes. Two terpenes were isolated and identified as

9, 19-Cyclolanost-24-en-3 β -ol, 24-methyl - lanostereone (**1**) and 19-Norlanosta-5, 24-dien-3-ol, 9-methyl-, (3 β , 9 β , 10 α) tetracyclic triterpenoids (**2**) from bioactive fraction of methanol extract of latex. To best of our knowledge, these terpenes have not been reported earlier from the latex of *E. caducifolia*.

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References

1. Singhi M., Joshi V., Sharma R.C., Adha S., & Dixit K., Larvicidal efficacy of *Calotropis procera* against the vector of dengue, malaria and lymphatic filariasis in the arid zone of Rajasthan. *Ann. Arid. Zone*, (2005) 44, 185–190.
2. Thomas T.G., Raghavendra K., Lal S., Saxena, V. K., Mosquito larvicidal properties from unripe fruits of *Carica papaya* Linn. (Caricaceae), *J. Comm. Dis.*, (2004) 36(4), 290–292.
3. Srivastava V. K., Singh S.K., Rai M. & Singh A., Toxicity of *Nerium indicum* and *Euphorbia royleana* lattices against *Cx. quinquefasciatus* mosquito larvae. *Nig. J. Nat. Product Med.*, (2003) 7, 1–4.
4. Yadav R., Srivastava V.K., Chandra R., & Singh A., Larvicidal activity of latex and stem bark of *Euphorbia tirucalli* plant on the mosquito *Culex quinquefasciatus*, *J. Comm. Dis.*, (2002) 34(4), 264–269.
5. Parkash V., Somani H., Thomas T.G., & Kumbhat S., Mosquito larvicidal properties of the latex of *Pedilanthus tithymaloides* (L) (family : Euphorbiaceae). *J. Comm. Dis.*, (2009) 41(2), 129–131.
6. Bhandari M.M. (1978) *Flora of the Indian Desert*, Scientific Publishers, Jodhpur, India.
7. Sen D.N., Chawan D. D., Leafless euphorbia on Rajasthan rock, *Sov J Ecol.* (1974) 4, 373–379.
8. Maqbool M. A., Hashmi S., & Ghaffar A., Effect of latex extracts from *Euphorbia caducifolia* and *Calotropis procera* on root-node nematode *Meloidogyne incognita* infesting tomato and egg, *Pakistan J. Nematol.*, (1987) 5(1), 43–47.
9. Goyal M., Nagori B.P., Sasmal D., Wound healing activity of latex of *Euphorbia caducifolia*, *J. Ethnopharmacol.* (2012) 144(3), 786–790.
10. Goyal M., Sasmal D., Nagori & B.P., GCMS analysis and antimicrobial action of the latex of *Euphorbia caducifolia*, *Intercult Ethnopharmacol.* (2012) 1(2), 119–123.
11. Afza N., Yasmeen B., Khan A.Q., Malik A., & Khan

- Q. A., Phytochemical investigation on the fresh latex of *Euphorbia caducifolia*. *Fitoterapia*, (1988) 59 (3), 253–254.
12. Afza N., Khan A.Q., Malik A., & Yasmeen B., Cyclocaducinal, a cycloartane type triterpene from *Euphorbia caducifolia*, *Phytochem.*, (1989) 28(7), 1982–1984.
13. Govardhan C., Reddy R. Prasad & Sundararamaiah T., 3-epi-cyclolaudenol and known triterpenes from *Euphorbia caducifolia*, *Phytochem.*, (1984) 23(2), 411–413.
14. Ahmed S., Seligmann O., Wagner H., & Hussain G., Caudicifolin, new diterpenes from *Euphorbia caducifolia*, *Phytochem.*, (1977) 16, 1844.
15. Bhattacharyya S.K., Kar S., Chakraborty S., Dasgupta S., Mukhopadhyay R. & Bandyopadhyay A., Technical analysis of *euphorbia caducifolia* Haines latex of South-East Asian origin - Part I, *Polymers from Renewable Resources*, (2013) 4(3), 133-151.
16. Taviad K., Vekariya S., Bhati H. & Patgiri B.J., Exposure to Snuhi Ksheera (*Euphorbia caducifolia* Haines. latex) resulting in ocular injury: A case report. *J. Ayu. Herb. Med.*, (2017) 3(1), 1- 4.
17. Hill F. (1998). *Hill's Economic Botany*. Harvard University, U.S.A.: Tata McGraw Hills Publ. Co. Ltd.; WHO (2005). Guidelines for laboratory and field testing of mosquito larvicides. Geneva, World Health Organization, (WHO/CDS/WHOPES/GCDPP/2005.13).
18. Abbott W.B., A method for computing the effectiveness of insecticides. *J. Am. Mosq. Control. Assoc.*, (1987), 3(2), 302–303.
19. Falodun, A., Ali, S., Quadir, I.M., & Choudhary, Iqbal M. I., Phytochemical and biological investigation of chloroform and ethyl acetate fractions of *Euphorbia heterophylla* leaf (Euphorbiaceae), *J. Medicinal Plants Res.*, (2008) 2(12), 365–369.
20. Harbone, J. B., *Phytochemical Methods: A guide to modern techniques of plant analysis*, (1973). London: Chapman and Hall Ltd.