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In vitro neutralization of *Eachis carinatus* and *Naja naja* venom by *Canthium parviflorum* and its GC–MS analysis

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Abstract

Canthium parviflorum is used in the traditional therapy to treat snakebite victims. In the present study, partially purified methanol root extract of *C. parviflorum* was used for the neutralization of *Eachis carinatus* and *Naja naja* venom. The extract inhibited *in vitro* phosphomonoesterase, phosphodiesterase, acetylcholinesterase, hyaluronidase, protease, phospholipase A_2 and 5'nucleotidase activities of both venoms. One of the promising band exhibited neutralization of all the *in vitro* enzyme activities and was further subjected to GC-MS analysis which revealed the presence of eight active phytocompounds. These phytochemicals might be responsible for *in vitro* enzyme neutralization. Methanol extract possesses potent active phytochemicals against the site specific toxins and hydrolytic enzymes analyzed.

Introduction

World Health Organization highlights snakebite as a "Neglected disease". It is a medical emergency particularly in the South East Asian Region including India (WHO, 2007; Banerjee, 1978). There were 52 poisonous snakes reported in India of which four species (*Naja naja*, *Bungarus caeruleus*, *Daboia russelii* and E*chis carinatus)* are responsible for mortality and maximum deaths (Bawaskar 2004). Neurotoxic nature of *N. naja* (Indian cobra) possesses a dangerous public health problem. *E. carinatus* (saw scaled viper) bite kills more people than those of any other genus (Warrell et al., 1977).

The most effective and accepted therapy for snakebite patient is the immediate administration of polyvalent anti-venom after the envenomation which is associated with several drawbacks. Antivenom binds and neutralizes the venom, further preventing the adverse effects due to venom. However, it cannot reverse the damages already caused by the venom (Gomes et al., 2010).

Several medicinal plants have been used in folk medicinal system but there is no scientific validation. *Canthium parviflorum* belonging to the family Rubiaceae is used in the traditional medicine system to treat the snakebite. However, there is no scientific validation for its application. Ethnomedicinal reports and personal survey on antivenom plant from Karnataka reveal that the plant can be used for snakebite treatment (Hiremath and Taranath 2010; Mahishi et al., 2005).

The present study focuses on the antivenom potential of thin layer chromatography (TLC) separated methanol extract of plant against *E. carinatus* and *N. naja* venom. Partially purified band was subjected to GC-MS analysis.

Materials and Methods

The lyophilized venoms of *E. carinatus* and *N. naja* were procured from Irula Snake Catcher's Co-operative Society, Kancheepuram, Chennai, Tamil Nadu, India.

The venom was dissolved in the physiological saline and was used for further studies. Di-sodium-p-nitrophenol phosphate (DNPP), L-leucine, diansidine hydrochloride, horseradish peroxidase, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, hyaluronic acid, cetyltrimethylammonium bromide and lecithin were purchased from Himedia Laboratories and casein from Sigma-Aldrich laboratories, USA. All the other reagents were analytical grade.

Collection of plant material

The roots of *C. parviflorum* were collected in the month of October from Kalasa, from Kudremukh, Karnataka. The plant was identified and authenticated at National Ayurveda Dietetics Research Institute, Bangalore, Karnataka, India (RRCBI-MUS/03).

Preparation of extracts

C. parviflorum roots were shade dried powdered and extracted by using soxhlet extractor using ethyl acetate and methanol extracts equentially. The extracts were concentrated by rotary vacuum evaporator and the residue was dried and used for further studies.

Thin layer chromatography

The methanol extract was partially purified using TLC on analytical plates over silica gel (TLC grade-Merck India). The solvent system used for separation was ethyl acetate and methanol (8:2 v/v). Each band was scrapped, separated and dissolved in methanol. Methanol was evaporated and individual band (100 µg/mL) was studied for enzyme inhibitory activities.

In vitro enzyme inhibition studies using TLC separated bands

Neutralization of phosphomonoesterase activity

Phosphomonoesterase activity was determined using DNPP with slight modifications (Bessey et al., 1946). Reaction mixture consists of 1.0 mL Tris-HCl buffer (pH 8.0), 1.0 mL DNPP and 0.5 mL 0.25% crude venom (w/v) and incubated at 37°C for 3 hours. The absorbance was measured at 425 nm. Inhibition studies were carried out by pre-incubating *E. carinatus* and *N. naja* venom with TLC separated bands for 30 min at 37°C. All tests were conducted in triplicates.

Neutralization of phosphodiesterase activity

Phosphodiesterase activity was determined using disodium-p-nitrophenol phosphate as substrate (Lo et al., 1966). Reaction mixture containing 0.5 mL of 0.25% venom solution/fraction was added to an assay mixture containing 0.5 mL 0.0025 M sodium-p-nitrophenyl phosphate, 0.3 mL 0.01 M $MgSO_4$, 0.5 mL 0.17 M Tris-HCl buffer (pH 8.0). The absorbance was measured at 400 nm. Inhibition study was carried out by pre-incubating *E. carinatus* or *N. naja* venom with TLC separated bands for 30 min at 37°C. All tests were conducted triplicates.

Neutralization of acetylcholinesterase activity

Acetylcholinesterase was determined using acetylthiocholine iodide as the substrate (Ellman et al., 1961). 50 µL of 0.1% crude venom was incubated with 3 mL phosphate buffer (pH 8.0) at room temperature for 5 min. 10 µL of DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) and 20 µL substrate acetylthiocholine iodide were added. The increase in absorbance was measured at 412 nm. Inhibition study was carried out by pre-incubating *E. carinatus* or *N. naja* venom with TLC separated extract for 30 min at 37°C. All tests were conducted triplicates.

Neutralization of hyaluronidase activity

Hyaluronidase assay of crude venom was determined turbidometrically using hyaluronic acid as a substrate (Pukrittayakamee et al., 1988). The assay mixture contained buffer of Tris-HCl (pH 8.0), 50 mg hyaluronic acid (0.5 mg/mL in buffer) and enzyme volume was made up to 1.0 mL. The mixture was, then, incubated for 15 min at 37°C and the reaction was stopped by the addition of 2 mL 2.5% (w/v) cetyltrimethylammonium bromide (cTAB) in 2% (w/v) NaOH. The absorbance was read at 400 nm (within 10 min) against a blank containing 1 mL of the Tris buffer and 2 mL of 2.5% (w/v) cTABin 2% (w/v) NaOH. Inhibition study was carried out by pre-incubating *E. carinatus* or *N. naja* venom with TLC separated bands for 30 min at 37°C. All tests were conducted triplicates.

Neutralization of protease activity

Protease assay of crude venom was determined using casein as a substrate (Greenberg, 1955). The reaction mixture consists of 0.5% casein, 1.0 mL of Tris-HCl buffer (pH 8.0), 0.5 mL 0.25% of crude venom was added and the reaction mixture incubated for 4 hours at 37°C. At the end of 4 hours the reaction was terminated by adding trichloroacetic acid (TCA) and then filtered. 1.0 mL of filtrate was used for protein estimation was carried out using L-tyrosine as a standard (Lowry et al., 1951). Inhibition study was carried out by pre-incubating venom with plant extract for 45 min. Inhibition study was carried out by pre-incubating *E. carinatus* or *N. naja* venom with TLC separated extract for 30 min at 37°C. All tests were conducted triplicates.

Neutralization of phospholipase A_2 assay

Phospholipase A_2 assay was determined according to the acidimetric method with little modification (Tan and Tan, 1988). Briefly, egg yolk suspension was prepared by mixing 1% lecithin, 18 mM calcium chloride and 8.1 mM sodium deoxycholate. The pH of the suspension was adjusted to 8.0 with 0.1 M of sodium hydroxide and stirred for 10 min to ensure

homogenous mixing. 0.1 mL of 0.1% of venom solution was added to 15 mL of egg yolk suspension to initiate the hydrolysis. The initial decrease in pH was measured by a pH meter. Inhibition study was carried out by pre-incubating *E. carinatus* or *N. naja* venom with TLC separated bands for 30 min at 37°C. All tests were conducted triplicates.

Neutralization of 5′-nucleotidase activity

5′-Nucleotidase inhibition was carried out using 5′ AMP as a substrate (Rowe et al., 1980). The substrate solution contained 1 mL of Tris-HCl buffer (pH 8.0), 0.1 mL of 0.1 M $Mgcl_2$ and 0.8 mL of 0.15% 5′ AMP, 0 .25 mL of 0.1% crude venom was added to the substrate solution and incubated at 37°C for 15 min. At the end of 15th min the reaction was stopped by adding trichloro-acetic acid and filtered. The filtrate was assayed for inorganic phosphate at 625 nm using potassium dihydrogen phosphate as standard (Fiskie and Subbarow, 1925). Inhibition study was carried out by pre-incubating *E. carinatus* or *N. naja* venom with TLC separated extract for 30 min at 37°C. All tests were conducted triplicates.

GC-MS analysis of TLC separated band

TLC separated bands from methanol extracts of the plant were subjected to GC-MS analysis on the instruments Thermo GC-trace ultra ver: 5.0, Thermo MS DSQ II equipped with column DB - 5MS capillary standard non-polar (length 30 m x inner diameter 0.25 mm film thickness 0.25 µm) was used for analysis. Initially oven temperature was maintained at 70°C and raised to 260° C at 60 min. 1.0 µL of sample was injected for analysis. Helium gas used as a carrier gas and flow rate was 1.0 mL/min. The m/z ratio was plotted on graph which is the finger print of the molecule.

Identification of the compound

Interpretation on mass spectrum GC-MS analyses was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was stored in the NSIT library. Unknown compared with relative retention time and mass spectra of the known components stored in the NIST library.

Results

Methanol extract was subjected to TLC using ethyl acetate and methanol (8:2 v/v) as solvent system (Figure 1). 100 µg/mL of TLC separated methanol extract was used for enzyme inhibition studies. Phosphomonoesterase activity, phosphodiesterase activity, acetylcholinesterase, hyaluronidase, protease, phospholipase A_2 enzymes from *E. carinatus* or *N. naja* venom neutralized by TLC separated methanol extracts

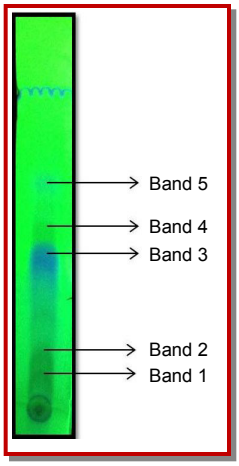

Figure 1: TLC separation of methanol extract of *C. parviflorum*. Ethyl acetate and methanol extract used as solvent system (8:2 v/v)

and exhibited various percentage of inhibition on the enzyme activities (Figure 2-3). Of all the bands analyzed, band number 5 showed maximum inhibitory response.

GC-MS analysis of TLC separated band 5 of methanol extract of *C. parviflorum* revealed the presence probable active phytochemicals (Figure 4, Table I). The spectrum of the TLC separated band 5 of methanol root extract was compared with the spectrum of known compound present in NSIT library. GC-MS chromatogram showed 9 prominent peaks with retention time (RT) 6.67-35.42 min (Figure 4). The peak at retention time 6.7 min showed the presence of 1,1,(Methylthio)canthin-6-one,1 -methyl-2,2-bis(4-methoxyphenyl)cyclopropane-carboxylate, 9.1 min was cyclohexasiloxanedodeca-methyl, 11.9 min showed tetradecamethylcyclohepta-siloxane. The peak at retention time 15.3 min revealed the presence of cyclooctasiloxane, hectadecamethyl, at 18.5 min the compound was cyclooctasiloxaneoctadeca-methyl, 21.5 min the compound was 1-(benzyloxy)-2-fluoro-2-phenyl-3-(p-toleuenesulsulfonyloxy)propanea, 25.4 min probable compound octadecanoic acid, methyl ester. Peak at retention time 30.5 min was 13-docosenoic acid, methyl ester, (Z)-(CAS), at 35.4 min compound identified as 2R,3S)-3-allyloxy-2-[3-{iv-allyl-oxypropoxymethyl)tetrahydropyran, N-formyl-calycinine, N-[2-bromo-6-(4-chlorophenyl)benzamide.

Discussion

C. parviflorum was well-studied for various pharmacological properties. Ethanolic leaf extract was neutralized *in vitro* and *in vivo* anti-cancer activity on DLA and HeLa cell lines (Prabhu et al., 2011). *C. parviflorum* possesses anti-oxidant activity in alloxan-induced diabetic rats (Sathishkumar et al., 2008). *Canthium coromadelicum* leaves posses antimicrobial and anti-HIV activity (Chinnaiyan et al., 2013).

In the present study, partial purification was carried out

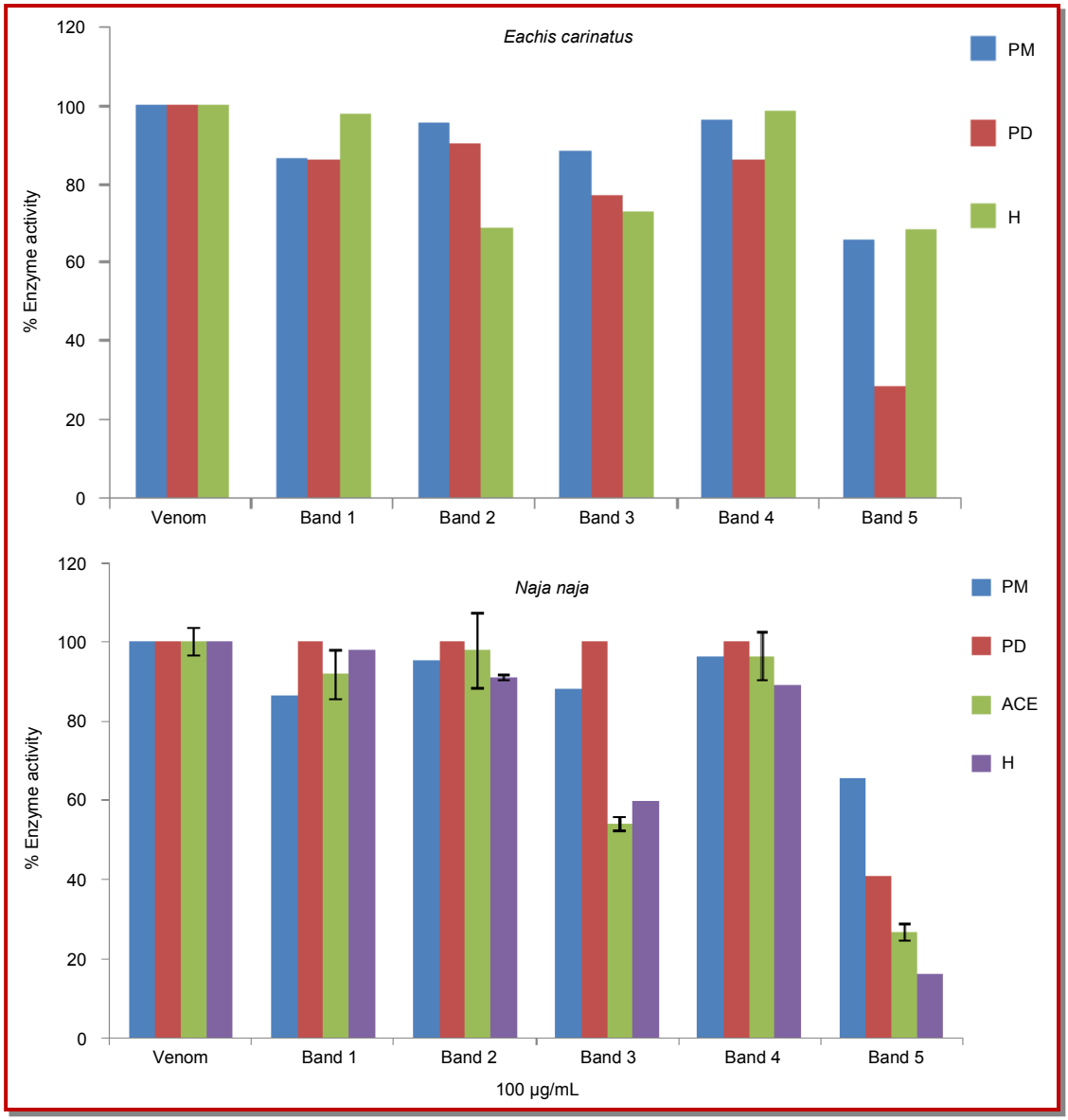

Figure 2: Neutralization of phosphomonoesterase (PM), phosphodiesterase (PD), acetylcholinesterase (ACE), hyaluronidase (H) activities of *E. carinatus* or *Naja naja* venom by TLC separated methanol extracts. Results were expressed mean ± SEM

using TLC to identify the bioactive compound against the *E. carinatus* or *N. naja* venom. TLC separated band 5 showed maximum %inhibition compared to other bands and inhibited all the enzyme activity.

Snake venom is a complex mixture of hydrolytic enzymes and some site specific enzymes. Phospholipase A_2, metalloprotenase, 5′ nucleotidase are major hydrolytic and hyaluronidase is a site specific enzymes responsible for earlier reaction of envenomation (Ushanandini et al., 2006). Hyaluronidase known as spreading factor results in membrane degradation of hyaluron lead to easy spreading of other toxic enzymes. The degradation of extracellular matrix is a continuous process and anti-venom fails to neutralize the local tissue damages (Homma and Tu, 1970; Girish et al., 2004). Lethal toxicity and pharmacological activity is combined action of all these toxic enzymes. Various plants have been studied for inhibition of these toxic enzymes effects by *in vitro* using various solvent extracts. *In vitro* inhibitory activity of *Carissa spinarum* leaf extracts against Krait and Viper russellitoxic snake venom enzymes was studied (Janardhan et al., 2014). *Tabernaemontana alternifolia* inhibits *in vitro N. naja* and *E. carinatus* venom enzyme activities (Vineetha et al., 2014).

Numerous plants species have been scientifically investigated for quest new phytoconstitute responsible for snake bite. Aristolochia species has been studied against *Trimeresurus flavoviridis* and *Viper russellii* venom effects (Vishwanath et al., 1987). Aristolochic

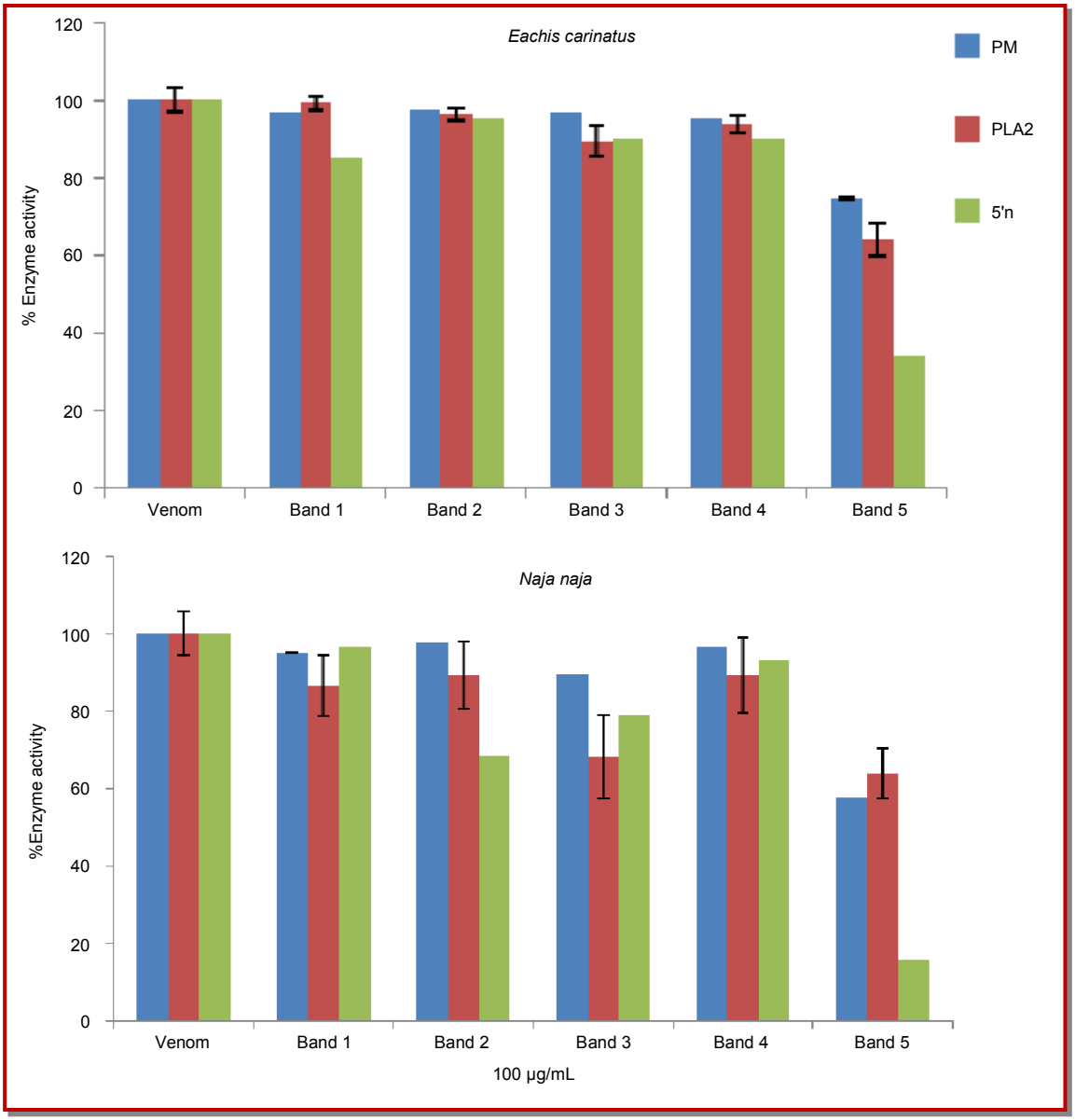

Figure 3: Neutralization of Protease (P), Phospholipase A_2 (PLA2), 5′ nucleotidase (5′n) activities of *E. carinatus* or *Naja naja* venom by TLC separated methanol extracts. Results were expressed mean ± SEM

acid, a bioactive phytochemical from Aristolochia, inhibited phospholipase which is responsible for edema formation and other pharmacological activities (Vishwanath et al., 1987). 2-OH-4-methoxy benzoic acid and lupeol acetate was isolated from *Hemidesmus indicus* which possesses potent inhibition activity against *N. naja* venom (Alam and Gomes, 1998; Chatterjee et al., 2006). A multiform of glycoprotein isolated from *Mucuna pruriens* seeds neutralized *E. carinatus* venom effects (Guerranti et al., 2004).

The sequential ethyl acetate extract of *C. parviflorum* exhibited enzyme activities (unpublished data). In the present study TLC separated bands of methanol root extract of *C. parviflorum* neutralized all the enzyme activities from both the venoms. The chromatogram of TLC separated *C. parviflorum* methanol root extract shows 9 prominent peak in retention time range between 6.7-35.4 min. In the previous studies, *C. parviflorum* ethanol leaf extract was reported 22 constituents present in the leaves and D-mannitol and squalene responsible for anti-cancer properties (Prabhu et al., 2013). GC-MS reveals presence of some active phytoconstituents which has various biological activities. Octadecanoic acid methyl ester, 13-docosenoic acid, methyl ester, (Z)-(CAS) have various biological activities present in the TLC separated methanol root extract. Presence of hexadecadecanoic acid ethyl ester compound in leaves were reported in ethanolic extract

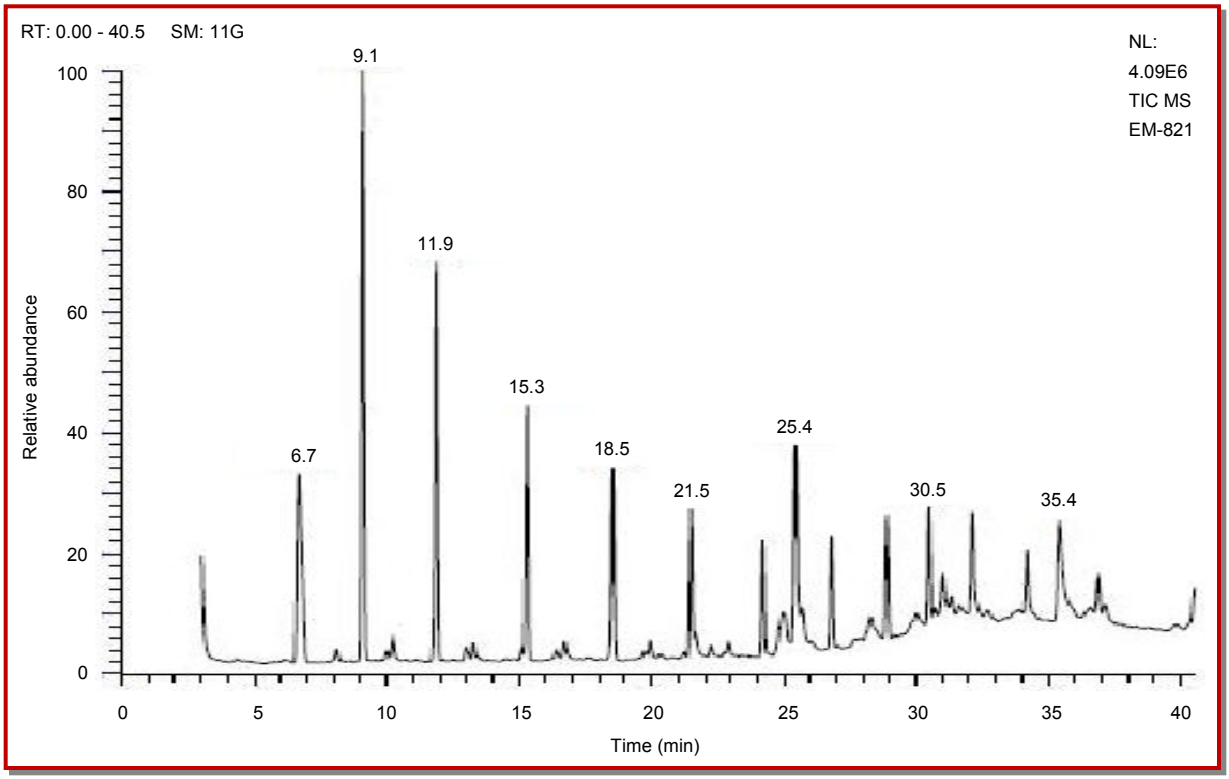

Figure 4: GC-MS chromatogram of TLC separated band 5 of methanol extract of *C. parviflorum*

Table I

Chemical profile identified by GC-MS analysis of methanol root extract of TLC separated band 5

RT (min)	%Area	Molecular formula	Compound	MW
6.7	9.7	$C_{15}H_{10}N_2OS$	11(Methylthio)canthin -6-one	266
	9.7	$C_{20}H_{22}O_4$	1-Methyl-2,2-bis(4-methoxyphenyl)cyclopropane-carboxylate	326
9.0	16.5	$C_{12}H_{36}O_6Si_6$	Cyclohexasiloxane, dodecamethyl	444
11.9	11.9	$C_{14}H_{42}O_7Si_7$	Tetradecamethylcycloheptasiloxane	518
15.3	6.9	$C_{16}H_{48}O_8Si_8$	Cyclooctasiloxane, hectadecamethyl	592
18.5	5.1	$C_{18}H_{54}O_9Si_9$	Cyclooctasiloxaneoctadecamethyl	666
21.5	0.3	C23H23FO4S	1-(Benzyloxy)-2-fluoro-2-phenyl-3-(p-toleuenesulsulfonyloxy) propane	414
25.4	7.2	C19H3802	Octadecanoicacid, methyl ester	298
30.5	1.5	C23H44O2	13-Docosenoic acid, methyl ester, (Z)-(CAS)	352
35.4	4.4	C22H38O6	(2R,3S)-3-Allyloxy-2-[3-{iv-allyloxypropoxymethyl)tetrahydropyran	398
	4.4	C19H17NO5	N-Formyl-calycinine	339
	4.4	C20H15BrCINO	N-[2-Bromo-6-(4-chlorophenyl)benzamide	399

of *C. parviflorum* leaves (Prabhu et al., 2013). An oleic ester derivative 9-octadecanoic acid (z)-phenylmethyl ester was reported in *Sauropus bacciformis* stem (Jenecius et al., 2012). 11(Methylthio)canthin-6-one is an alkaloid, the probable compound in 6.7 RT is not reported in Rubiaceae family to best of our knowledge. Two canthin-6-one alkaloids isolated from *Eurycoma longifolia* from Simaroubaceae family have anti-cancer effects in HT-1080 human sarcoma cell-lines (Miyake et al., 2010). Quinolinealkaloid is reported in Rubiaceae family which also the probable compound at RT 4.3 min but area % is 0.1. In the presence of siloxane compound might be due to interference of silica which was used during TLC separation.

Conclusion

Partially purified *C. parviflorum* methanol root extract inhibited phosphomonoesterase, phosphodiesterase, acetylcholinesterase, hyaluronidase, protease, phospholipase A_2, 5′ nucleotidase activities of *E. carinatus* and *N. naja* venom. TLC separated band 5 was promising when compare to other bands. The GC-MS analysis revealed presence of bioactive phytocompounds which might be responsible for neutralization of toxic enzymes. As per our knowledge and scientific literature survey, *C. parviflorumn* was not studied for antivenom properties. Therefore, the present study adds on for an additional scientific validation to the society.

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Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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