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Pharmacognostic evaluation of *Eranthemum indicum* extracts for its *in-vitro* antioxidant activity, acute toxicology, and investigation of potent bioactive phytocompounds using HPTLC and GCMS

Flavius Phrangsngi Nonglang[1], Abhijeet Khale[2], Wankupar Wankhar[3] and Surya Bhan[1*]

Abstract

Background: Northeast India has a rich resource of herbal plants, and it is essential to validate their therapeutic activity with proper scientific evidence. This study aims to identify active phytocompounds found in the extracts of *Eranthemum indicum* (*E. indicum*) and to determine its antioxidative activities and toxicity.

Results: *In vitro* free radical scavenging activity of the aqueous extract (AE) and methanol extract (ME) of *E. indicum* (leaves) was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic-acid (ABTS), ferric reducing antioxidant power (FRAP), and total antioxidant activity (TAC). ME depicted better inhibitory concentration when compared to AE. This indicates the effective extraction capacity of methanol, which is consistent with the fact that ME had a higher polyphenol and flavonoid, resulting in their antioxidative activity. HPTLC analysis using the solvent system of ethyl acetate/methanol/ammonia 28–30% (40:10:10) showed better fingerprinting separation, especially in the ME. Furthermore, DPPH radical solution, when used as a derivatizing agent in HPTLC analysis, confirmed that ME has better *in vitro* antioxidant activities than AE. GCMS analysis of AE identified 3-beta-hydroxy-5-cholen-24-oic-acid as active compound, while in ME Beta.-l-arabinopyranoside and 2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane were identified as the major bioactive compound. Acute toxicological investigations have shown that both *E. indicum* extracts have a high L.D. 50 value of 1533 mg/kg b.w for AE and 1567 mg/kg b.w for ME making them safe and non-toxic.

Conclusions: Extraction and identification of these phytocompounds in the extracts of *E. indicum* can help us scientifically document its medicinal importance, and its benefit in pharmaceutical industries. Since it showed promising free radical scavenging activity, it can also be a potent antioxidant source.

Keywords: Phytocompounds, Antioxidants profile, *E. indicum*, HPTLC, GCMS, Natural product, Toxicology

1 Background

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a dual role in both toxic and beneficial effects [1]. The presence of a high concentration of these free radicals plays a significant role in the genesis of chronic and degenerative illnesses such as cancer, arthritis, aging, autoimmune disorders,

*Correspondence: bhansurya900@gmail.com

[1] Department of Biochemistry, North Eastern Hill University, Shillong, Meghalaya 793022, India
Full list of author information is available at the end of the article

cardiovascular disease, and neurological diseases [3, 4]. On the other hand, antioxidants prevent the oxidation of easily oxidizable biomolecules such as lipids, proteins, and DNA [5]. Antioxidants delay or inhibit cellular damage through their free radical scavenging property by converting them to non-radical species, breaking the auto-oxidative chain reaction initiated by ROS/RNS [6, 7]. Natural antioxidants are more pharmacologically efficient and have fewer adverse effects, making them ideal compounds for medicine, food, and cosmetics [8].

Identification of natural bioactive chemicals is crucial in proving the traditional uses of medicinal plants. High-performance thin-layer chromatography (HPTLC) is a robust, simple, quick, reliable, and effective analytical approach for the quantitative analysis of compounds based on the principle of thin-layer chromatography (TLC). It is one of the analytical tools that allow detection, separation, and analysis of a broad number of phytocompounds present in the herbal extract with better analytical precision and accuracy in a short time at a moderate cost [9–11]. Gas chromatography-mass spectroscopy (GCMS), on the other hand, is another advanced and sophisticated technology used to identify and quantify unknown phytocompound in a complex mixture. Their separation is based on their volatility and can be determined by matching it with the reference library spectra [12, 13].

Meghalaya, a northeastern state in India, is known for its rich diversity of flora and fauna, accounting for roughly 18 percent of the country's overall flora, with 3128 blooming and 1237 endemic plant species [14]. It is also home to a rich variety of medicinal plants, many of which have been extensively used by local people for generations to treat various diseases. One such important medicinal plant is *Eranthemum indicum,* a suffrutescent perennial herb belonging to the Acanthaceae family. It is found predominantly in the Northeast region of India, Myanmar, Bhutan, Sikkim, and Nepal [15]. Leaves are simple, petiolate, lineolate, and usually entire, while the flowers are blue, purple, violet, or purplish-white in terminal or axillary, simple, and branched dense spikes [16]. Locally known as Hur mynsaw (Khasi name), local practitioners traditionally use its leaves for their wound healing potential and ability to help bone fracture recovery. As limited studies have been documented on the plant, particularly Meghalaya, phytochemical profiling and identifying its bioactive compounds for potential therapeutic and pharmacological purposes is very important.

2 Methods

2.1 Collection and identification

The leaves of *E. indicum* were collected from the tropical forest of Shallang, located in West Khasi Hills District of Meghalaya, India, and authenticated by Dr. Chaya Deori, Scientist—in Charge, Botanical Survey of India (BSI), Eastern Regional Centre (Accession no BSI/ERC/Tech/2019–20/655). The collected plant was separated from undesirable materials, thoroughly washed, and dried. The dried leaves were then grounded into a coarse powder with the help of a grinder. The powder was then stored in an airtight container and kept in a cool, dark, dry place until the analysis commenced.

2.2 Plant extraction

2.2.1 Preparation of methanol extract

30 g powder of dried leaves of *E. indicum* was dissolved in 300 mL of a solvent comprising a 4:1 mixture of methanol and water. A polytetrafluoroethylene stir bar with a magnetic stir plate was then used to agitate and stir the mixture for 48 h. After that, a Whatman No 1 filter paper was used to filter the mixture. The filtrate was then collected, and the solvent was evaporated using a rotary evaporator before being dried thoroughly using a Scanvac cool safe freeze drier (internal condenser temperature of − 80 to − 120 °C) to achieve a dry concentrated extract of the plant sample. After that, the dried crude sample was weighed and kept at − 20 °C until biochemical analysis.

2.2.2 Preparation of aqueous extract

30 g powder of dried leaves of *E. indicum* was dissolved in 300 mL of distilled water. A polytetrafluoroethylene stir bar with a magnetic stir plate was then used to agitate and stir the mixture for 48 h. After that, the mixture was filtered using Whatman No 1 filter paper. The filtrate was collected, and the solvent was removed using lyophilization, yielding a dry concentrated extract of the plant material. After that, the dried crude sample was weighed and kept at − 20 °C until biochemical analysis. The extraction efficiency was quantified by determining the weight of each of the extracts, and the percentage yield was calculated.

2.3 *In vitro* free radical scavenging assay

2.3.1 DPPH radical scavenging assay

Principle The DPPH free radical scavenging technique is commonly utilized to assess plant extract's antioxidant capabilities. When added in a concentration-dependent manner, the extract converts DPPH (violet color solution), a free radical that is stable at room temperature, to diphenylpicryl hydrazine, a yellow-colored product [17].

1 mL of varying concentration of plant extract/standard ascorbic acid was reacted with 2 mL of DPPH solution. The mixture was allowed to stand for 30 min at room temperature. The control was prepared by adding 2 mL of DPPH to 1 mL of methanol. Ascorbic

acid was used as standard. The absorbance was measured at 517 nm against blank [17].

$$\% \text{ Inhibition } = \text{Abs (control)} - \text{Abs(Sample)} / \text{Abs(control)} \times 100$$

2.3.2 ABTS free radical scavenging activity

Principle In the presence of hydrogen-donating antioxidants, the blue-green ABTS radical is decreased in ABTS radical scavenging activity (ABTS radical formed by interacting with a strong oxidizing agent, e.g., potassium persulfate with the ABTS salt) [18].

1 mL of varying concentration of plant extract/standard ascorbic acid was reacted with 2 mL of ABTS radical cation working solution and incubated at room temperature for 4 min. The control was prepared by adding 2 mL of ABTS radical cation working solution to 1 mL of distilled water or 0.025% ethanol (Abs control). The absorbance was taken at 734 nm against blank [18]. The % Inhibition is also calculated as follows:

$$\% \text{ Inhibition } = \text{Abs(control)} - \text{Abs(Sample)} / \text{Abs(control)} \times 100$$

2.3.3 Ferric reducing antioxidant properties

Principle The FRAP test assesses sample antioxidant capacity by converting ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) with antioxidants present in the samples, resulting in a blue color that can be read calorimetrically at 593 nm.[19].

The working FRAP reagent was prepared by mixing (300 mmol/L, pH-3.6) acetate buffer, (10 mmol/L) TPTZ solution in HCl, and (20 mmol/L) $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 in volume. An amount of 100 µL of varying concentrations of plant extract was mixed with 1.5 mL FRAP reagent in test tubes and was vortexed and mixed properly. The same steps were followed for standard ascorbic acid. The samples were then incubated in the water bath at "37˚C for 30 min," and the absorbance of the samples was determined against blank at 593 nm [19]. The ferric reducing antioxidant activity was then expressed in "mg AAE/g dry weight" of the extract.

2.3.4 Total antioxidant assay—phosphomolybdenum assay

Principle The phosphomolybdenum technique for assessing total antioxidant capacity (TAC) is based on the ability of the antioxidants present in the sample to reduce Mo (VI) to Mo (V), generating a "green phosphate/Mo (V) complex" at acidic pH that can be colorimetrically measured at 695 nm [20].

The ability of *E. indicum* extract to diminish molybdate ion was determined according to the technique given by Prieto et al. [20]. The sample of 0.3 mL at different concentrations was mixed with 3 mL of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were covered and incubated in a water bath at 95 °C for 90 min. Once cooled at room temperature (28 °C), the absorbance of the solution was measured at 695 nm against a blank. Ascorbic acid at different concentrations was used as the standard, and the total antioxidant capacity was stated as "mg AAE/g dry weight" of the extract.

2.4 Quantitative analysis of some of the component contents.

2.4.1 Flavonoid determination

1 mL of varying concentrations of standard rutin/plant extracts was reacted with 1 mL of 2% aluminum chloride and incubated at room temperature for 10 min. A blank solution was prepared by adding 1 mL of 2% aluminum chloride to 1 mL distilled water. The absorbance was taken at 430 nm. The flavonoid contents were quantified against the rutin standard calibration curve. The results were expressed as "mg Rutin equivalents/g dry weight" of the extracts [21].

2.4.2 Total polyphenol determination

1 mL of the standard gallic acid/plant extracts was reacted with 5 mL of the diluted Folin–Ciocalteu reagent. "After ~ 5 min," 4 mL of sodium carbonate (7.5%) was added. The test tubes were then covered and kept for two hours in the dark at room temperature. The blue complex formed was quantified spectrophotometrically at an absorbance of 740 nm against a gallic acid standard curve [22]. Results were expressed as "mg GAE/g dry weight" of the extract (GAE = gallic acid equivalents).

2.5 High-performance thin-layer chromatography (HPTLC) profiling analysis:

2.5.1 Sample preparation and application

10 mg/mL plant (aqueous and methanol) extracts and 1 mg/mL of standard reference chemicals, namely ascorbic acid (vitamin C—class of polyphenol), caffeine (class of alkaloid), pyrogallol, gallic acid (class of phenolic compound), quercetin and rutin (class of flavonoid compound), were made in chromatographic grade methanol. Prepared samples were then applied on a "TLC aluminum sheets silica gel" 60 F 254 (Merck) using ATS 4 CAMAG sample applicator set at a speed of 150 (nL/s). The plant extracts (aqueous and methanol) and standard

reference were applied in a 100.0 × 100.0 mm plate, each having a band length of 8.0 mm.

2.5.2 Developing solvent system

Several solvent systems were tried to obtain a better resolution and a maximum number of bands; however, the following solvent system, *ethyl acetate/methyl ethyl ketone/formic acid/water* in the ratio of *5:3:1:1* (Solvent A) and *ethyl acetate/methanol/ammonia 28–30%* in the ratio of *40:10:10* (Solvent B) provided the most satisfactory result, whereby adequate resolution and separation of compounds were observed.

2.5.3 Development of chromatogram

The chromatograms were developed for 20 min at room temperature in a CAMAG twin trough glass chamber 10 × 10 saturated with the solvent system combination mentioned above for a distance of 80 mm. The resolved bands' retardation factor (Rf) values and color were recorded.

2.5.4 Scanning and detection of spots

Spots were visible at 254 nm (Deuterium lamp, absorption mode filter K320) and 366 nm (Mercury lamp, Fluorescence filter K400) wavelengths. On a CAMAG TLC Scanner 4, a deuterium lamp with a wavelength of 254 nm and a spectrum speed of 100 nm/s was used to scan the produced plate's spectrum. Baseline correction of the lowest slope with the noise of 0.05, peak detection-Gauss (legacy) with a sensitivity of 0.1, a threshold of 0.1, and separation of 1 was set. The Rf values of the chromatogram peaks were recorded. To confirm the *in vitro* antioxidant activity of the separated compounds, the plate was derivatized by spraying with DPPH (250 mg DPPH in 250 mL of ethanol).

2.6 Gas chromatography-mass spectroscopy (GCMS) profiling analysis

Chemical profiling of the active phytocompounds in the aqueous and methanol extracts of *E. indicum* was carried out using GCMS analysis. The analysis was carried out on a PerkinElmer Turbo Mass Spectrophotometer (USA) with the model Clarus 680 Gas chromatography/Clarus 600 Mass spectrometer (G.C. having Liquid Autosampler). Chromatography was performed on a PerkinElmer Elite-35MS capillary column of length "60 m and an internal diameter of 0.25 mm." The electron impact technique (5.0 mV) was used. Pure helium gas (99.99%) was used as the carrier gas with a flow rate of 1 mL/min. The injector and detector temperatures were 280 °C and 0 °C, respectively. The oven temperature was programmed initially at 60 °C with an initial hold of 1.0 min, equilibration time of 2.0 min, then finally increasing to 350 °C (maximum) with Ramp 1 rate of 7.0 °C/min to 200 °C and holding time of 3.0 min and Ramp rate 2 of 10.0 °C/min to 300 °C and holding time of 5 min. 1.5 µL of the prepared 1% extract diluted with methanol was injected (normal speed), and a split injection technique (10.1:1 split ratio) was used. The total run time was 39 min, and the sampling rate was 1.5625 pts/s. Data were evaluated using a total ion chromatogram (TIC) for compound identification and quantification. The identification of the compounds was based on a comparison of their retention indexes (R.I.) and retention time (R.T.). They were also confirmed by comparison of their mass spectra with the National Institute of Standard and Technology (NIST) library spectra database and published literature data.

2.7 Acute toxicology studies

Healthy female mice were used as they are more sensitive to toxic compounds (LD50) [23], and all experiments were carried out in accordance with the Institutional ethics guidelines (Animal model). The median lethal dosage or lethal dose 50 (LD50), the dose of a chemical that causes death in 50% of a population of test animals, was calculated according to the method of Wilbrandt [24]. Prior to the administration of extracts, mice were starved for 4 h, and then, a limit dose of 2000 mg/kg b.w was administered intraperitoneally. If the mice died at this limit, the extracts were delivered in escalating concentrations (from 400–2000 mg/kg b.w.) to check for L.D. 50 value. Furthermore, animals in all groups were monitored for up to 2 weeks for any signs of pain, convulsion, coma, or death.

$$\mathrm{LD50} = \text{Maximum dose (mg/kg)} - \text{Product}\ \frac{a \times b}{\text{No of animals in a group}}$$

2.8 Statistical analyses

Data were expressed as means ± standard deviation (S.D.) of triplicate. Statistical calculations were carried out using SAS.

3 Result

3.1 % Extraction yield

Results summarized in Table 1 show that the methanolic extract (ME) of *E. indicum* has a higher percentage extraction yield of 11.77% as compared to the aqueous extract (AE) with 10%.

Table 1 Extractive yield (%) obtained from the methanol and aqueous extracts of the leaves of *E. indicum*

Plant name	Part used	Dry powder weight (w′) g/mL	Dry weight extract (w) g/mL	% Yield (w/w′) (%)
E. indicum (Aqu)	Leaves	30 g in 300 mL Methanol/Water (4:1) (0.1 g/mL)	3 g from 300 mL Methanol/Water (4:1) (0.01 g/mL)	10
E. indicum (Met)	Leaves	30 g in 300 mL water (0.1 g/mL)	3.53 g from 300 mL water 0.0117 (g/mL)	11.77

3.2 In vitro antioxidant activities

DPPH (Fig. 1a), ABTS (Fig. 1b), FRAP (Fig. 1c), and TAC (Fig. 1d) were used to access the antioxidant properties of the AE and ME of *E. indicum. In vitro* free radical scavenging activity of *E. indicum* using the DPPH model depicted IC50 value of 2.885 mg/mL (50% radical scavenging activity at 2.885 mg/mL) and 1.792 mg/mL (50% radical scavenging activity at 1.792 mg/mL) for AE and ME, respectively, while for ascorbic acid. IC 50 is 0.013 mg/mL. On the other hand, ABTS radical scavenging activity depicted an IC50 value of 0.689 mg/mL and 0.419 mg/mL for AE and ME, respectively, while for ascorbic acid, IC 50 value is 0.011 mg/mL. FRAP assay, using concentrations ranging from 250 to 1000 µg/ml, showed increased absorbance with increasing concentrations and showed the antioxidant activity of 4.55 ± 0.18 and 8.65 ± 0.64 mg AAE/g dry weight of extract for AE and ME, respectively. Similarly, the total antioxidant activity of 12.9 ± 0.85 and 19.2 ± 1.7 mg AAE/g dry weight of extract was observed for A.E. and M.E., respectively.

3.3 Total flavonoid and polyphenol content

The result summarized in Table 2 showed that the total polyphenol content of *E. indicum* is 326.35 ± 0.4 and 412.6 ± 11.9 mg gallic acid equivalent/g dry weight ($Y = 1.0101x - 0.1256$; $R^2 = 0.9994$) and total flavonoid content is 16 ± 0.28 and 30.9 ± 1.06 mg rutin equivalents/g dry weight for AE and ME, respectively ($Y = 15.666x + 0.0105$; $R^2 = 0.9994$).

3.4 High-performance thin-layer chromatography (HPTLC) profiling analysis:

HPTLC chemical profiling of *E. indicum* AE and ME showed various bands separated differently in the chromatogram plate (different Rf value) when viewed under ultraviolet light (254 nm and 366 nm) and white light. These separated bands depict various phytocompounds present in the plant. Various solvent system combinations have been used to achieve the best separation. Two best solvent system combinations were determined, and the separation process was carried out. Solvent system A is a combination of *ethyl acetate/methyl ethyl ketone/formic acid/water in the ratio of 5:3:1:1,* where good separation was observed in both extracts. ME of *E. indicum* showed more bands and had better separation when compared to AE (Fig. 2a–d). After applying 10µL of AE, plates were developed on the solvent system to separate the compounds, whereby 6 chromatogram peaks were observed on scanning. Peak 1 showed Rf value at 0.335 (3.89% area composition), peak 2 Rf value at 0.476 (having maximum area composition of 49.39%), peak 3 Rf value at 0.602 (32.14%), peak 4 Rf value at 0.674 (5.32%), peak 5 Rf value at 0.760 (2.09%), and peak 6 Rf value at 0.910 (7.17%) (Fig. 3). Similarly, separation was again performed for ME, and 7 chromatogram peaks were observed, with peak 1 having an Rf value of 0.190 (3.28%), peak 2 Rf value of 0.325 (15.92%), peak 3 Rf value of 0.466 (having the maximum area composition of 38.86%), peak 4 Rf value at 0.608 (18.61%), peak 5 Rf value at 0.678 (5.34%), peak 6 Rf value at 0.765 (13.22%), and peak 7 Rf value at 0.906 (4.76%) (Fig. 4). In solvent system A, the standard reference compound also showed good separation with ascorbic acid Rf value at 0.427, caffeine Rf value at 0.598, pyrogallol Rf value at 0.897, gallic acid Rf value at 0.865, quercetin Rf value at 0.925, and rutin Rf value at 0.341. These standard references were run in the same solvent system to ensure a uniform running procedure. DPPH derivatized plates (Fig. 2d) did not show antioxidant activities for the compounds separated in the plant extract, however (as evident from no change in color of the separated bands). On the other hand, solvent system B was a combination of *ethyl acetate/methanol/ammonia 28–30% in the ratio of 40:10:10.* It was observed that ME of *E. indicum* showed more bands and had better separation as compared to AE (Fig. 5a–d). After applying 10µL of AE, plates were developed on the solvent system to separate compounds, whereby 4 chromatogram peaks were observed on scanning. Peak 1 showed Rf value at 0.341 (having the maximum area composition of 53.01%), peak 2 Rf value at 0.380 (22.59%), peak 3 Rf value at 0.698 (7.25%), and peak 4 Rf value at 0.778 (17.14%) (Fig. 6). On the other hand, on applying 10µL of ME to the plate, 9 chromatogram peaks were observed, with peak 1 showing Rf value at

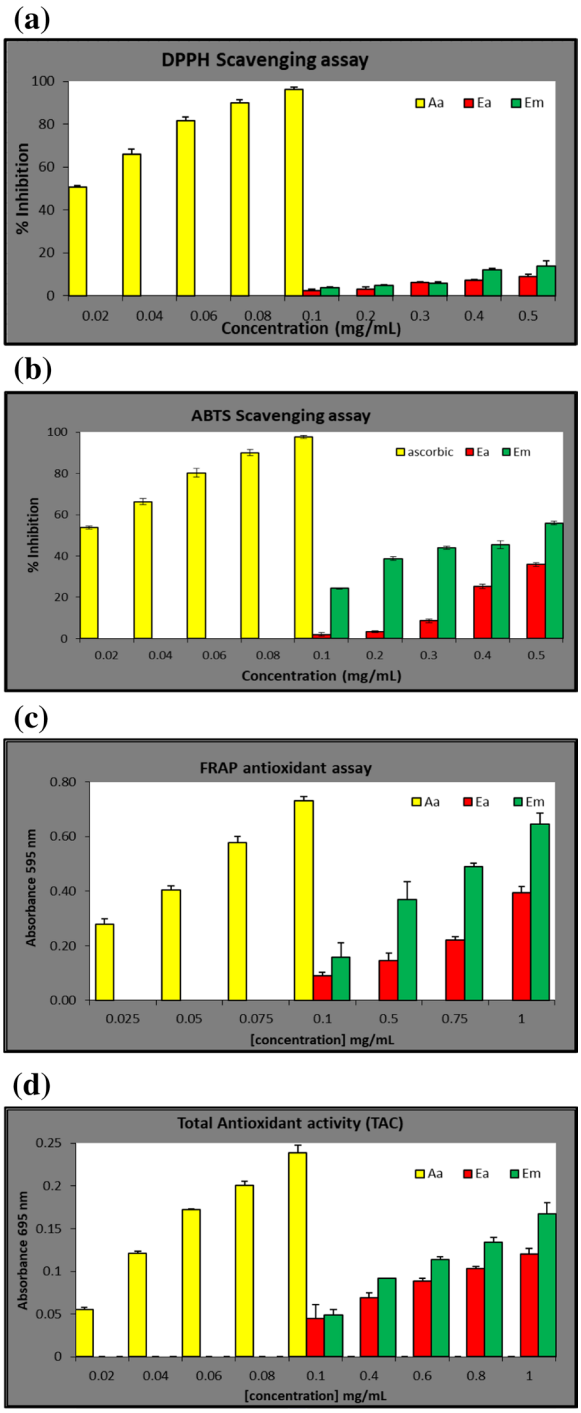


Fig. 1 **a** DPPH scavenging assay, **b** ABTS scavenging assay, **c** FRAP assay, **d** TAC assay

0.054 (3.25%), peak 2 Rf value at 0.179 (8.99%), peak 3 Rf value at 0.329 (having the maximum area composition 31.42%), peak 4 Rf value at 0.364 (14.49%), peak 5 Rf value at 0.412 (7.20%), peak 6 Rf value at 0.451 (12.87%), peak 7 Rf value at 0.596 (0.624%), peak 8 Rf value at 0.698 (10.71%) and peak 9 Rf value at 0.766 (9.92%) (Fig. 7). In this solvent system, the standard reference did not show good separation compared to solvent system A, with only caffeine showing good separation with an Rf value of 0.588. DPPH derivatized plate (Fig. 5d), on the other hand, shows that the plant compounds in the extract separated in this solvent system showed potent antioxidant activities (as evident from the change in color of the separated bands).

Table 2 Quantitative analysis of total flavonoid and polyphenol content

Components	Plants	
	E. indicum **(Aq) (mg/g)**	***E. indicum*** **(Met) (mg/g)**
Polyphenol content (mg GAE/g dry weight extract)	326.35 ± 0.4	412.6 ± 11.9
Flavonoid content (mg Rutin equivalent/g dry weight extract)	16 ± 0.28	30.9 ± 1.06

3.5 Gas chromatography-mass spectroscopy (GCMS) analysis

A peak chromatogram of AE (Fig. 8) and ME (Fig. 9) of different phytocompounds present in the extracts was obtained using GCMS analysis. The compounds' identification was based on comparing their retention indexes, retention time, and mass spectra with the NIST—library spectra. It may be noted that identification was performed for the predominant compound with good separation. The major active compound identified in AE was 3-beta-hydroxy-5-cholen-24-oic acid (8.092% area composition), while the other compounds such as 3-cyclopentylpropionic acid, 2-methylpropyl ester (0.276% area composition), 4-t-butyl-1-(1-Methylallyl) cyclohexanol (0.693% area composition), 1-decanol, 9-[(trimethylsilyl) oxy]-trifluoroacetate (0.555% area composition), oleic acid (0.922% area composition), 2-trimethylsiloxy-6-hexadecenoic acid, methyl ester (0.642% area composition), decanoic acid, 10-fluoro-, trimethylsilyl ester (0.592% area composition), 1,25-dihydroxyvitamin D3, TMS derivative (0.303% area composition), 11-bromoundecanoic acid (0.599% area composition) were also identified (Table 3). The phytocompounds identified in AE, along with their structure and biological activities, are shown in Table 4. The major active compound identified in ME was beta.-l-arabinopyranoside, methyl (18.729% area composition) and 2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane (19.992% area composition), while other compounds such as N-decanoic acid

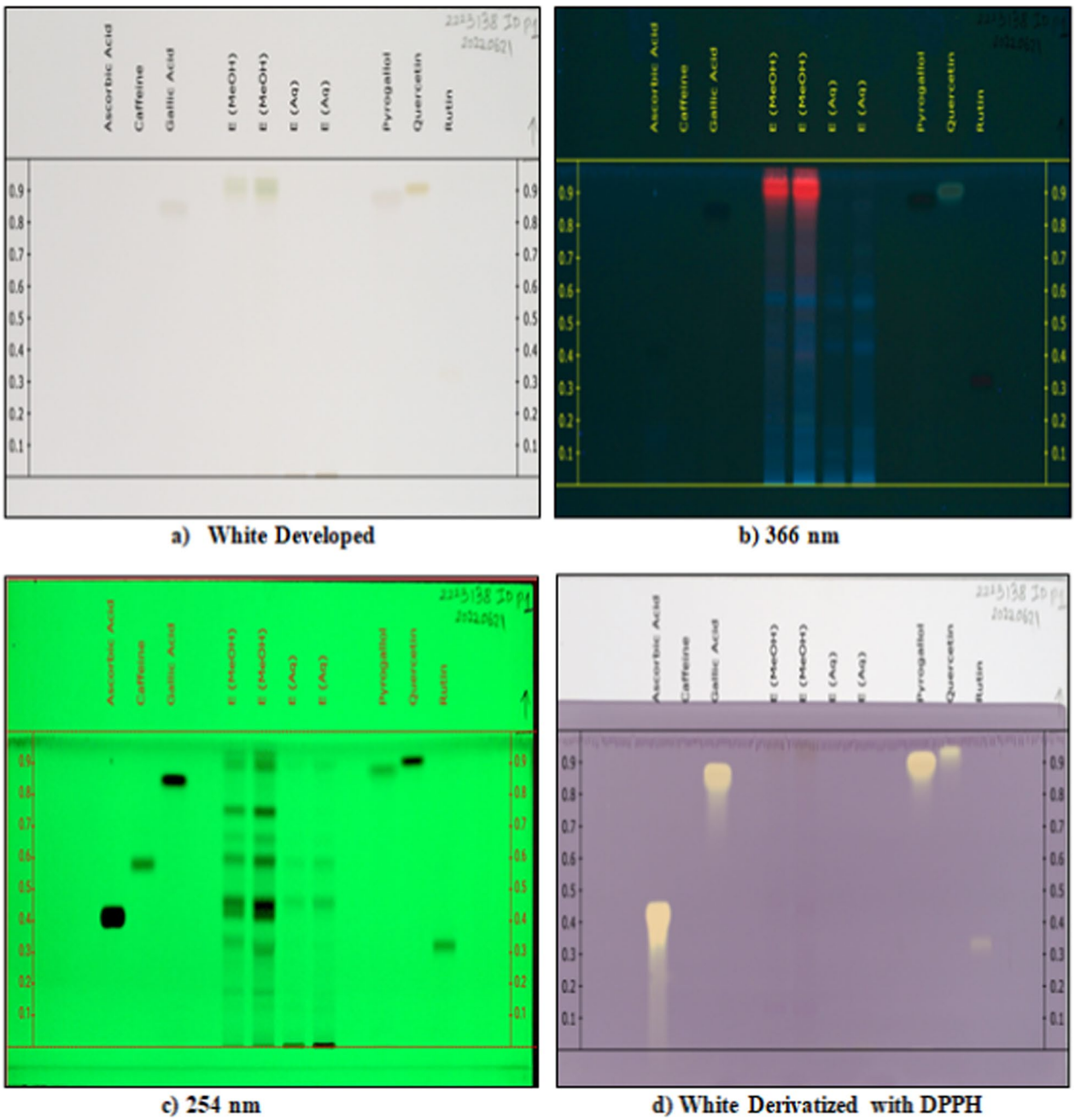
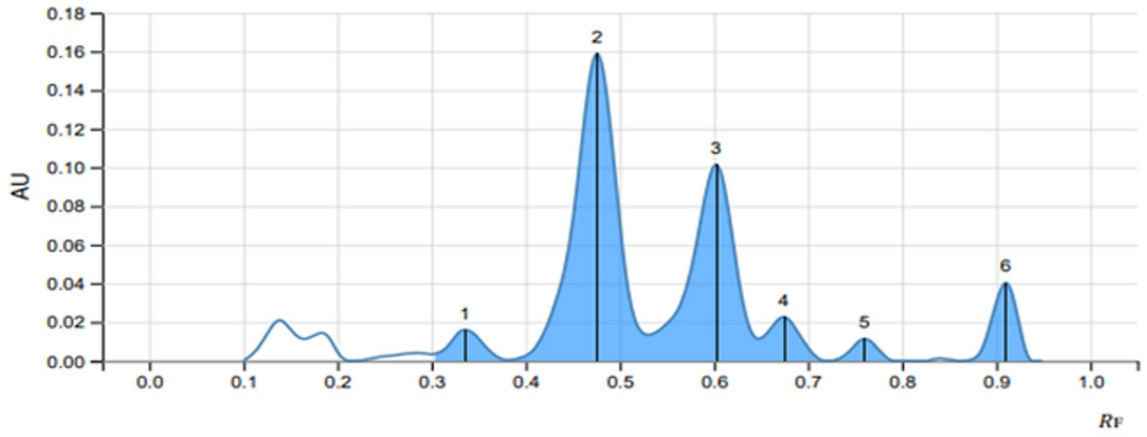


Fig. 2 HPTLC band chromatogram using solvent system A (ethyl acetate/methyl ethyl ketone/formic acid/water in the ratio of 5:3:1:1)

(0.494% area composition), octadecanoic acid (0.534% area composition), 2-propenoic acid, butyl ester (0.534% area composition), 3-methyl-2-(2-oxopropyl) furan (2.198% area composition), oleic acid (1.110% area composition), butyl 9-hexadecenoate (5.061% area composition), 2,5-diisopropyl-1,3,2-dithiaborinane (0.989% area composition), decane, 2-cyclohexyl (1.321% area composition), and 2-trimethylsiloxy-6-hexadecenoic acid, methyl ester (0.266% area composition) were also identified (Table 5). Phytocompounds identified in the ME, along with their structure and biological activities, are shown in Table 6.

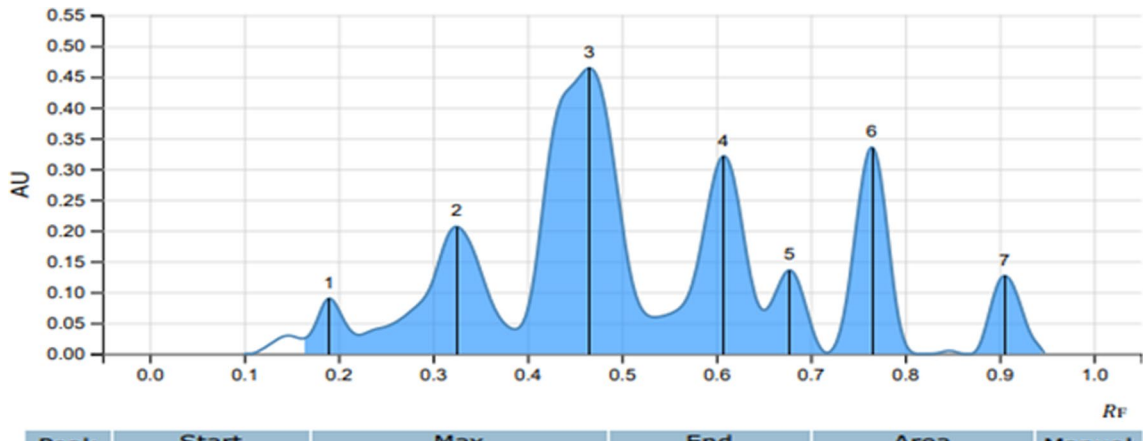
3.6 Acute toxicology studies

Acute toxicology studies in Swiss albino mice Balb/c showed that the LD50 of the AE was 1533 mg/kg b.w (Table 7), while the LD50 of the ME was 1567 mg/kg b.w (Table 8).



Peak #	Start		Max			End		Area		Manual peak
	R_F	H	R_F	H	%	R_F	H	A	%	
1	0.300	0.0035	0.335	0.0160	4.57	0.381	0.0005	0.00066	3.89	No
2	0.381	0.0005	0.476	0.1588	45.33	0.530	0.0133	0.00843	49.39	No
3	0.530	0.0133	0.602	0.1014	28.95	0.648	0.0113	0.00548	32.14	No
4	0.648	0.0113	0.674	0.0226	6.46	0.719	0.0000	0.00091	5.32	No
5	0.723	0.0000	0.760	0.0112	3.21	0.793	0.0000	0.00036	2.09	No
6	0.865	0.0000	0.910	0.0403	11.49	0.939	0.0000	0.00122	7.17	No

Fig. 3 Peak chromatogram band of *E. indicum* aqueous extract using the solvent system A (ethyl acetate/methyl ethyl ketone/formic acid/water in the ratio of 5:3:1:1)



Peak #	Start		Max			End		Area		Manual peak
	R_F	H	R_F	H	%	R_F	H	A	%	
1	0.162	0.0246	0.190	0.0898	5.35	0.222	0.0307	0.00332	3.28	No
2	0.222	0.0307	0.325	0.2065	12.30	0.386	0.0399	0.01611	15.92	No
3	0.387	0.0399	0.466	0.4640	27.63	0.535	0.0595	0.03933	38.86	No
4	0.535	0.0595	0.608	0.3210	19.12	0.651	0.0709	0.01883	18.61	No
5	0.651	0.0709	0.678	0.1357	8.08	0.717	0.0017	0.00540	5.34	No
6	0.717	0.0017	0.765	0.3353	19.97	0.813	0.0000	0.01338	13.22	No
7	0.870	0.0000	0.906	0.1267	7.55	0.948	0.0007	0.00481	4.76	No

Fig. 4 Peak chromatogram band of *E. indicum* methanol extract using the solvent system A (ethyl acetate/methyl ethyl ketone/formic acid/water in the ratio of 5:3:1:1)

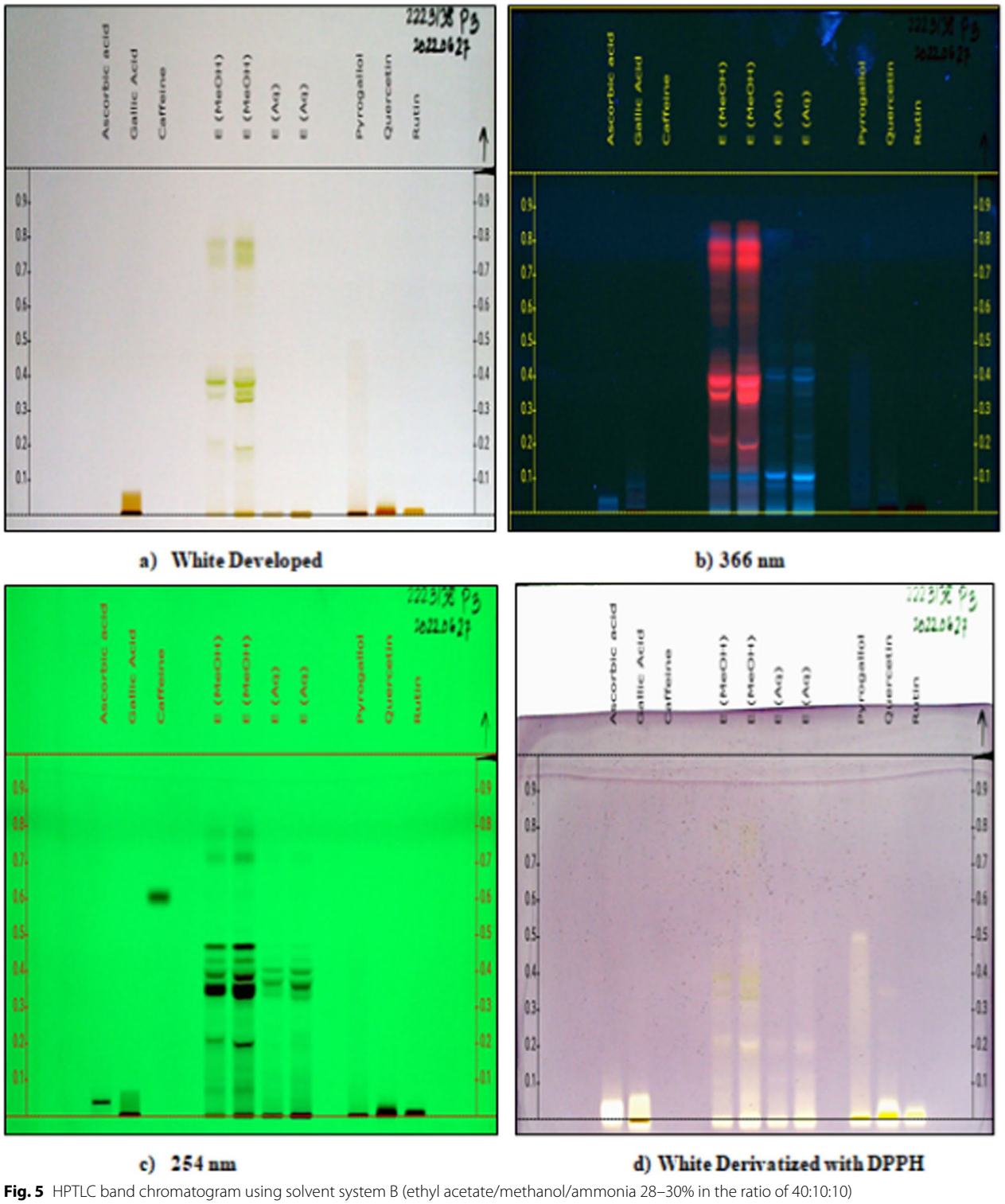
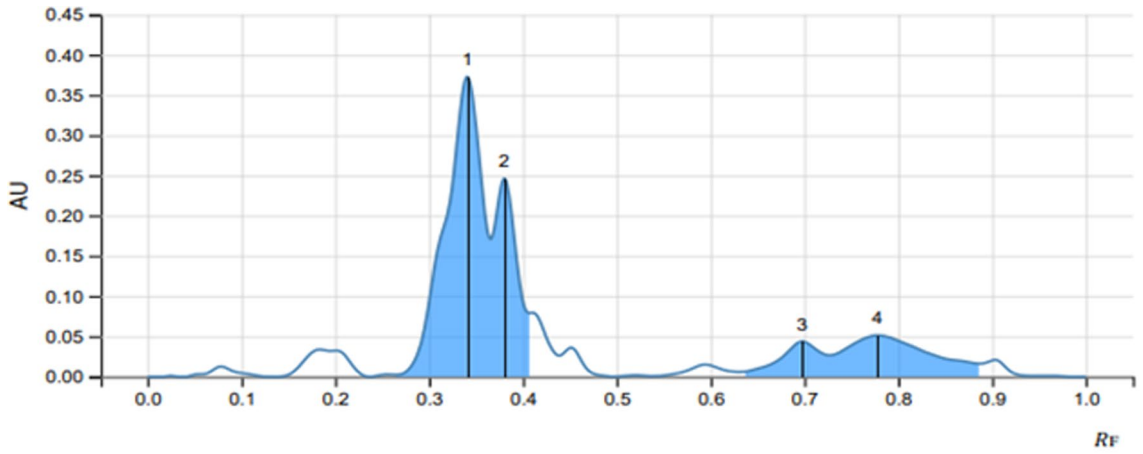
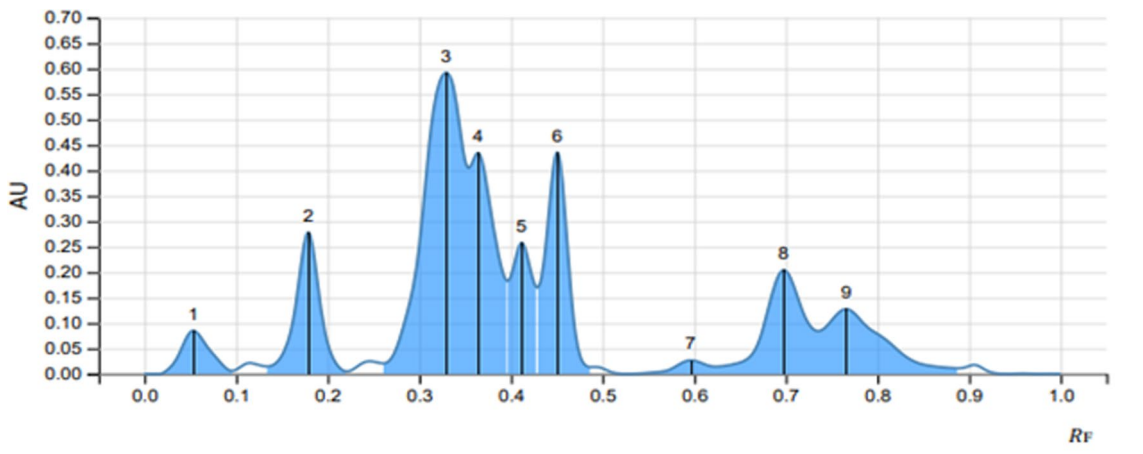


Fig. 5 HPTLC band chromatogram using solvent system B (ethyl acetate/methanol/ammonia 28–30% in the ratio of 40:10:10)



Peak #	Start		Max			End		Area		Manual peak
	R_F	H	R_F	H	%	R_F	H	A	%	
1	0.265	0.0025	0.341	0.3726	52.19	0.365	0.1710	0.01691	53.01	No
2	0.365	0.1710	0.380	0.2461	34.46	0.407	0.0788	0.00721	22.59	No
3	0.631	0.0058	0.698	0.0439	6.14	0.727	0.0266	0.00231	7.25	No
4	0.727	0.0266	0.778	0.0514	7.20	0.886	0.0162	0.00547	17.14	No

Fig. 6 Peak chromatogram band of *E. indicum* aqueous extract using the solvent system B (ethyl acetate/methanol/ammonia 28–30% in the ratio of 40:10:10)



Peak #	Start		Max			End		Area		Manual peak
	R_F	H	R_F	H	%	R_F	H	A	%	
1	0.017	0.0000	0.054	0.0849	3.48	0.096	0.0060	0.00312	3.25	No
2	0.134	0.0146	0.179	0.2778	11.39	0.221	0.0048	0.00864	8.99	No
3	0.260	0.0207	0.329	0.5919	24.27	0.354	0.4048	0.03021	31.42	No
4	0.354	0.4048	0.364	0.4340	17.80	0.396	0.1828	0.01393	14.49	No
5	0.396	0.1827	0.412	0.2574	10.55	0.428	0.1700	0.00692	7.20	No
6	0.429	0.1697	0.451	0.4350	17.83	0.487	0.0133	0.01238	12.87	No
7	0.529	0.0000	0.596	0.0267	1.09	0.624	0.0143	0.00111	1.15	No
8	0.624	0.0143	0.698	0.2038	8.36	0.735	0.0842	0.01030	10.71	No
9	0.735	0.0842	0.766	0.1274	5.22	0.887	0.0109	0.00953	9.92	No

Fig. 7 Peak chromatogram band of *E. indicum* methanol extract using the solvent system B (ethyl acetate/methanol/ammonia 28–30% in the ratio of 40:10:10)

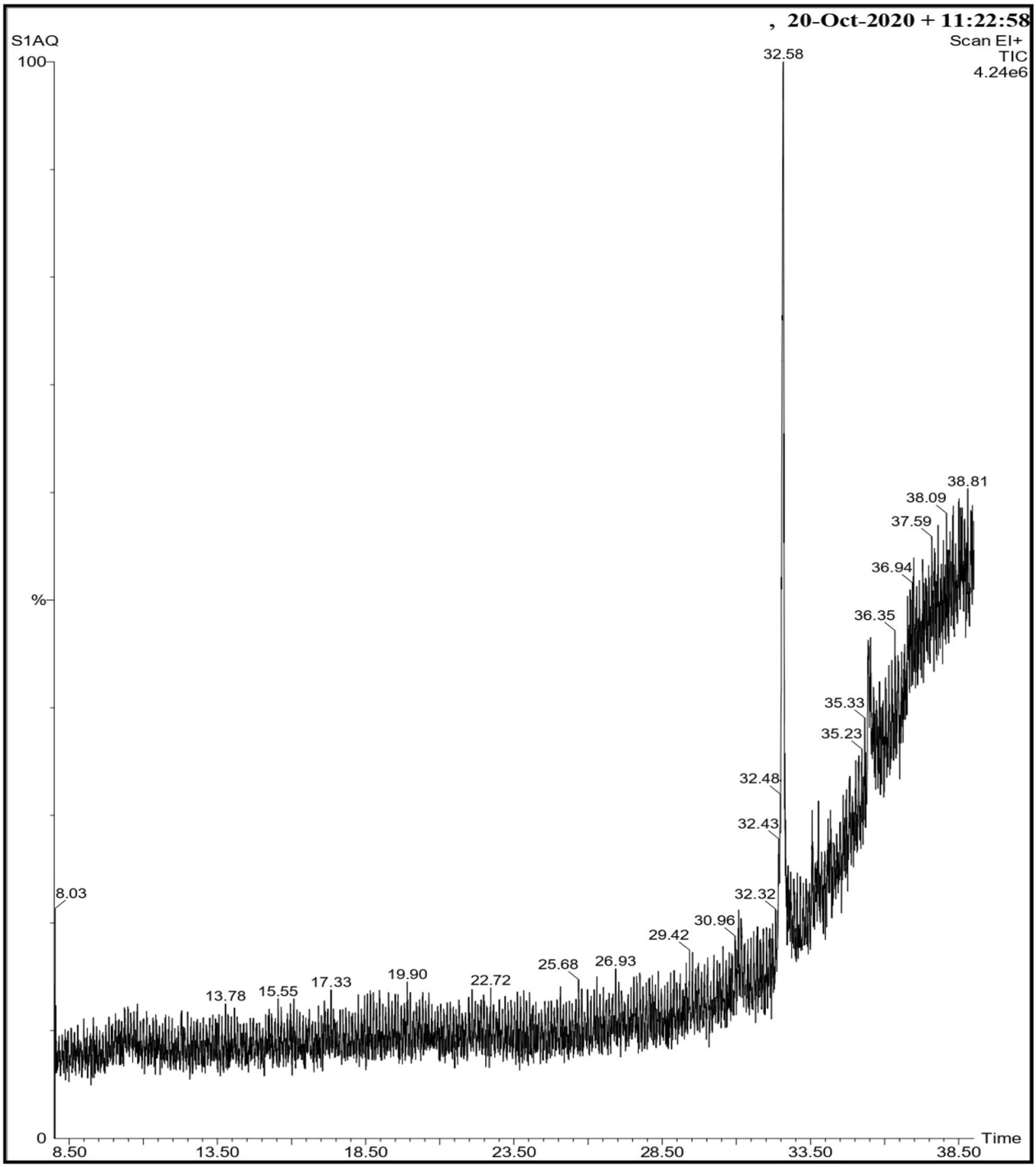


Fig. 8 GCMS chromatogram of *E. indicum* (aqueous extract)

4 Discussion

The plant *E. Indicum* (Fig. 10) is one of the most widely used herbal medicines utilized by traditional practitioners for decades in Meghalaya. Water and methanol were used as solvent systems in this study as water is the most extensively used and acceptable solvent for ingestion. In contrast, methanol extract was used to see if the activity and chemical composition of the plants changed when extracted with a different solvent.

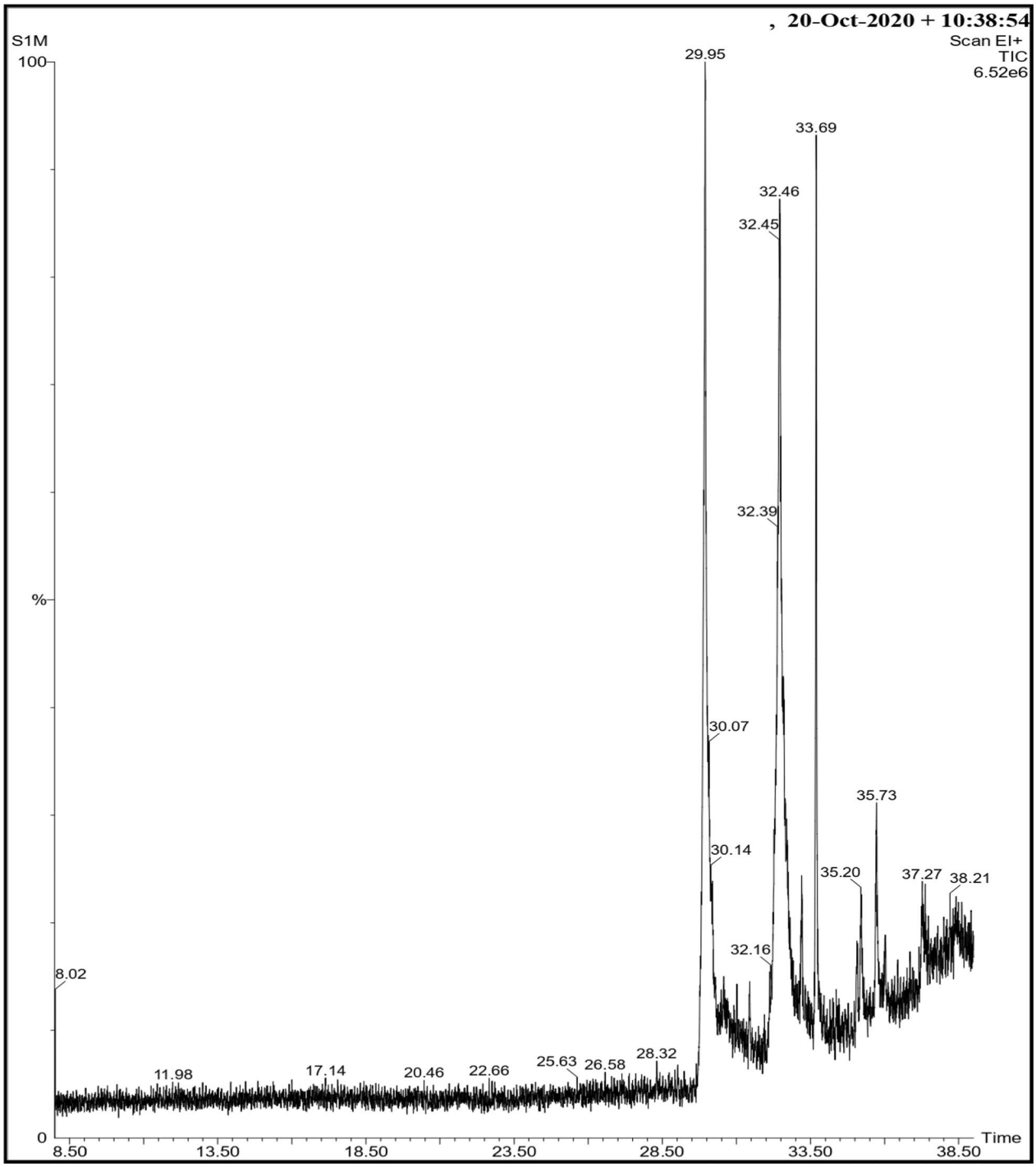


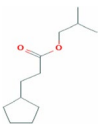
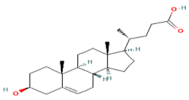
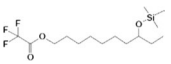
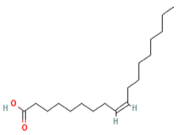
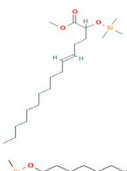
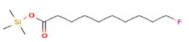
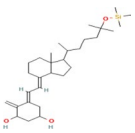
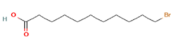
Fig. 9 GCMS chromatogram of *E. indicum* (methanol extract)

Preliminary profiling, like antioxidative activity, is critical for understanding the essential pharmacological action of the plant. Various antioxidant assays give diverse results, and it is necessary to characterize the antioxidant activities by different methods [25]. This study demonstrates that the ME depicts a better free radical scavenging activity when compared to AE. However, the AE and ME of *E. indicum* exhibit potent antioxidant activity compared to standard compound ascorbic acid (vitamin C). The free radical scavenging capacity of the

Table 3 GCMS library search for *E. indicum* (aqueous extract)

S. no.	Retention time (RT)	Height	Area	Area %	NIST library	Compound name	Mol. Wt g/mol
1	32.323	295,961	10,180.0	0.276	22,445	3-Cyclopentylpropionic acid, 2-Methylpropyl ester	198.1
2	32.433	528,404	25,572.5	0.693	144,304	4-t-Butyl-1-(1-Methylallyl) Cyclohexanol	210.35
3	32.583	3,526,663	328,345.9	8.092	247,775	3-Beta.-Hydroxy-5-Cholen-24-oic acid	374.6
4	34.174	443,324	20,472.2	0.555	52,309	1-Decanol, 9-[(Trimethylsilyl)Oxy]-, Trifluoroacetate	342.47
5	35.449	624,114	34,017.1	0.922	247,193	Oleic acid	282.5
6	35.529	593,831	23,693.3	0.642	41,294	2-Trimethylsiloxy-6-Hexadecenoic acid, Methyl ester	356.6
7	36.455	483,299	21,827.7	0.592	42,155	Decanoic acid, 10-Fluoro-, Trimethylsilyl ester	262.44
8	37.590	418,981	11,174.4	0.303	19,952:	1,25-dihydroxyvitamin d3, tms derivative	488
9	38.621	482,878	22,110.4	0.599	30,967	11-Bromoundecanoic acid	265.15

Table 4 Phytocompounds identified in the aqueous extract using GCMS with their structure and biological activity

S. no.	Compound	Mol. wt	Biological activity/reference	Structure
1	3-Cyclopentylpropionic acid, 2-Methylpropyl ester	198.1 ($C_{12}H_{22}O_2$)	Not known	
2	4-T-Butyl-1-(1-Methylallyl) Cyclohexanol	210.35	Not known	Not found
3	3-Beta.-Hydroxy-5-Cholen-24-oic acid	374.6 ($C_{24}H_{38}O_3$)	Belongs to the class of bile acids bearing a hydroxyl group. Bile acids facilitate fat absorption	
4	1-Decanol, 9-[(Trimethylsilyl)Oxy]-, Trifluoroacetate	342.47 ($C_{15}H_{29}F_3O_3Si$)	Not known	
5	Oleic acid (Omega-9 fatty acid)	282.5 ($C_{18}H_{34}O_2$)	Antioxidant, Antimicrobial, Antifungal, anticonvulsive activity, Anti-atherosclerosis, Anesthetic, Anti-helminthic, Antianxiety, Antibacterial, Anti-beriberi, Antibiotic, Anticancer, Anti-convulsion, Anti-diabetic, Anti-diarrheic, Anti-fertility, Anti-gastric, Anti-inflammatory, Anti-obesity, Anti-ulcer, Anti-tuberculosis, Anti-cold, Anti-hepatotoxic and Anti-viral activity [29, 30]	
6	2-Trimethylsiloxy-6-Hexadecenoic acid, Methyl ester	356.6 ($C_{20}H_{40}O_3Si$)	Not known	
7	Decanoic acid, 10-Fluoro-, Trimethylsilyl ester	262.44 ($C_{13}H_{27}FO_2Si$)	Not known	
8	1,25-Dihydroxyvitamin D3, TMS derivative	488 ($C_{30}H_{52}O_3Si$)	Vitamin D derivative	
9	11-Bromoundecanoic acid	265.15 ($C_{11}H_{21}BrO_2$)	Not known	

plant can be linked to its flavonoid and phenolic content, which are compounds responsible for antioxidant activity [26, 27]. This is in agreement with our finding as ME had greater total flavonoid and polyphenolic content as compared to AE.

HPTLC is a sophisticated and powerful analytical tool for acquiring chromatographic information about complex combinations of medicines, natural products, and other substances [9]. Different Rf values of the phytocompound obtained after performing HPTLC analysis

Table 5 GCMS library search for *E. indicum* (Methanol extract)

S. no.	Retention time (RT)	Height	Area	Area %	NIST library	Compound Name	Mol. Wt g/mol
1	29.947	6,131,049	966,003.1	18.729	30,974	Beta.-l-Arabinopyranoside, Methyl	164.16
2	30.287	390,568	25,493.4	0.494	252,106	N-Decanoic acid	172.68
3	31.453	503,103	27,521.8	0.534	245,247	Octadecanoic acid	284.48
4	32.163	536,641	27,194.3	0.527	19,885	2-Propenoic acid, Butyl ester	128.17
5	32.463	5,067,954	1,031,125.8	19.992	35,425	2-Methyl-3-(3-Methyl-But-2-enyl)-2-(4-Methyl-pent-3-enyl)-Oxetane	222.37
6	33.698	1,223,586	113,374.3	2.198	24,759	3-Methyl-2-(2-oxopropyl)furan	138.16
7	33.203	917,267	57,240.7	1.110	247,193	Oleic acid	282.5
8	33.694	5,522,194	261,010.2	5.061	19,859	Butyl 9-Hexadecenoate	310.5
9	35.069	661,134	50,984.7	0.989	52,287	2,5-Diisopropyl-1,3,2-Dithiaborinane	202
10	35.204	953,389	68,122.9	1.321	254,396	Decane, 2-Cyclohexyl	224.42
11	38.211	444,250	13,709.2	0.266	41,294:	2-Trimethylsiloxy-6-Hexadecenoic acid, Methyl ester	356

will offer a brief idea of and characteristics of the plant components, such as their polarity and separation [28]. In this study, two solvent systems that showed good separation and detections of the phytocompounds (seen as bands) were selected. Solvent system A is a combination of ethyl acetate/methyl ethyl ketone/formic acid/water in the ratio of 5:3:1:1, and solvent system B is a combination of ethyl acetate/methanol/ammonia 28–30% in the ratio of 40:10:10. Also when derivatized the plates with DPPH to confirm their antioxidant activities, the phytocompounds separated in the solvent system B showed better antioxidant activities when compared to the phytocompounds separated in solvent system A. This means that solvent system B allows for the detection and better separation of phytocompounds (in both the extracts) that have antioxidant activities. From HPTLC analysis, it can be confirmed that the ME has a better antioxidant capacity than the AE (Fig. 5d). Thus, it shows that the methanolic solvent is more effective and efficient in extracting phytocompounds than the aqueous solvent.

GCMS analysis shows the identification of various active phytocompounds present in the AE and ME of *E. indicum,* with 3-beta.-hydroxy-5-Cholen-24-oic acid (8.092% area composition) identified as the major active compound in AE and beta.-l-arabinopyranoside, methyl (18.729% area composition), 2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane (19.992% area composition) identified as the major active compound in the ME. The compounds identified in ME differ from those in the AE, implying that the phytocompounds are extracted differently depending on the solvent, and the identified compounds have distinct percentage compositions. Some of the phytocompounds identified in the AE have important medicinal properties, such as 3-beta.-hydroxy-5-cholen-24-oic acid, an essential class of bile acid that helps in felicitating fat absorption, oleic acid (omega-9 fatty acid) which have a wide variety of important medicinal properties including antioxidant activity, antimicrobial, anti-diabetic, anticancer, anti-viral, etc. [29, 30], 1,25-dihydroxyvitamin D3, TMS derivative (a vitamin D derivative). Some phytocompounds identified in ME have important medicinal properties, such as beta.-l-arabinopyranoside, methyl having antioxidant activity, anticancer activity (liver, lung, breast, and prostate), and methyl donor methyl guanidine inhibitor [30, 31], N-decanoic acid has antibacterial and antifungal activity [32], octadecanoic acid has antioxidant activity and acts as an emulsifying agent, solubilizing agent, tablet, and capsule lubricant[33], 3-methyl-2-(2-oxopropyl) furan has antioxidant, antimicrobial, bacteriocide, antipyretic, anti-inflammatory activity [30, 34], and oleic acid is also found in the ME.

According to the OECD Guidelines for Chemical Testing, the limit dose for rodents is 2000 mg/kg b.w. If no toxicity was observed at this level, the plant extracts were deemed non-toxic for mice [29]. According to observations and calculations, the LD50 value of both AE and ME of *E indicum* was found to be more than 1500 mg/kg dose, suggesting that the extract is safe and not toxic to mice even at a larger dose. As a result, *E. indicum* extracts can be regarded safe for *in vivo* pharmacological investigations.

5 Conclusions

The presence of numerous phytocompounds in the extracts of *E. indicum* explains its antioxidant activities. This is especially true for ME, owing to their potent flavonoids and polyphenol extraction capacity. Fingerprinting profile using solvent system B, a combination of Ethyl acetate/methanol/ammonia 28–30% in the ratio of 40:10:10

Table 6 Phytocompounds identified in the methanol extract using GCMS with their structure and biological activity

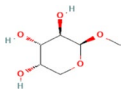
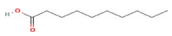
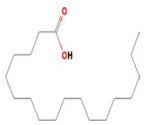
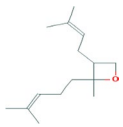
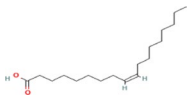
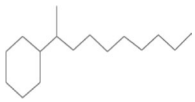
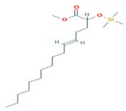
S. no.	Compound	Mol. wt	Biological activity	Reference
1	Beta.-l-Arabinopyranoside, Methyl	164.16 ($C_6H_{12}O_5$)	Methyl donor Methylguanidine inhibitor Catechol-O-methyltransferase inhibitor, 17-beta-hydroxysteroid dehydrogenase inhibitor, beta-adrenergic receptor blocker, anticancer (liver, lung, breast, and prostate), antioxidant [30, 31]	
2	N-Decanoic acid (Lauric acid)	172.68 ($C_{10}H_{20}O_2$)	Antibacterial and antifungal activity [32]	
3	Octadecanoic acid	284.48 ($C_{18}H_{36}O_2$)	Antioxidant, used as an emulsifying agent, solubilizing agent, tablet, and capsule lubricant [33]	
4	2-Propenoic acid, Butyl ester	128.17 ($C_7H_{12}O_2$)	Not known	
5	2-Methyl-3-(3-Methyl-But-2-enyl)-2-(4-Methyl-pent-3-enyl)-Oxetane	222.37 ($C_{15}H_{26}O$)	Not known	
6	3-Methyl-2-(2-oxopropyl)furan	138.16 ($C_8H_{10}O_2$)	Antioxidant, antimicrobial and bacteriocide, Antipyretic, anti-inflammatory activity [30, 34]	
7	Oleic acid (Omega-9 fatty acid)	282.5 ($C_{18}H_{34}O_2$)	Antioxidant, Antimicrobial, Antifungal, anticonvulsive activity, Anti-atherosclerosis, Anesthetic, Anti-helminthic, Antianxiety, Antibacterial, Anti-beriberi, Antibiotic, Anticancer, Anti-diabetic, Anti-diarrheic, Anti-fertility, Anti-gastric, Anti-inflammatory, Anti-obesity, Anti-tuberculosis,, Anti-hepatotoxic and Anti-viral activity [29, 30]	
8	Butyl 9-Hexadecenoate	310.5 ($C_{20}H_{38}O_2$)	Not known	
9	2,5-Diisopropyl-1,3,2-Dithiaborinane	202	Not known	Not found
10	Decane, 2-Cyclohexyl	224.42 ($C_{16}H_{32}$)	Not known	
11	2-Trimethylsiloxy-6-Hexadecenoic acid, Methyl ester	356 ($C_{20}H_{40}O_3Si$)	Not known	

Table 7 Determination of LD50 of *E. indicum* (aqueous extract)

Doses in mg/kg body weight	No. of animals	No. of dead animals	Mean Mortality (a)	Dose differences (b)	Product (a × b)
400	6	0	0	0	0
800	6	0	0	400	400
1200	6	0	0	400	400
1600	6	3	1.5	400	600
2000	6	4	3.5	400	1400

showed the best separation and detection of compounds in ME. GCMS analysis confirms that the bioactive molecule in the AE is 3-beta-hydroxy-5-cholen-24-oic-acid, whereas the bioactive ingredient in the ME is beta.-l-arabinopyranoside, methyl, and 2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane. All the other

Table 8 Determination of LD50 of *E. indicum* (methanol extract)

Doses in mg/kg body weight	No. of animals (n)	No. of dead animals	Mean mortality (a)	Dose differences (b)	Product (a × b)
400	6	0	0	0	0
800	6	0	0	400	400
1200	6	0	0	400	400
1600	6	3	1.5	400	600
2000	6	3	3	400	1200

Fig. 10 *E. indicum* (Nees) (leaf and flower)

compounds identified also have important medicinal properties. The high L.D. 50 value indicates that they are non-toxic and safe for further testing their in vivo pharmacological effects. Thus, with such promising preliminary results and findings, further studies can be done for future therapeutic and pharmaceutical applications.

Abbreviations

E. indicum: *Eranthemum indicum*; ME: Methanolic extract; AE: Aqueous extract; DPPH: α,α-Diphenyl-ρ-picryl hydrazyl; ABTS: 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; FRAP: Ferric reducing antioxidant potential; TAC: Total antioxidant capacity; IC 50: 50% Inhibitory concentration; TPC: Total polyphenol content; TFC: Total flavonoid content; GAE: Gallic acid equivalent; RE: Rutin equivalent; TLC: Thin-layer chromatography; HPTLC: High-performance thin-layer chromatography; Rf: Retardation factor; GCMS: Gas chromatography-mass spectroscopy; RI: Retention indexes; RT: Retention time; NIST: National Institute of Standard and Technology; LD50: Lethal dose 50.

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Author contributions

FPN contributed to conception and design of the work, collection and preparation of sample, analysis and interpretation of data, and manuscript preparation. WW was involved in manuscript preparation and review. AK was involved in HPTLC design and analysis of sample. SB contributed to concept of work and review of manuscript. We ensure and hereby declared that all authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests
We declare that we have no conflict of interest.

Author details
[1]Department of Biochemistry, North Eastern Hill University, Shillong, Meghalaya 793022, India. [2]Lab Division, Anchrom Enterprises (India) Pvt. Ltd. Mulund (E), Mumbai, Maharashtra 400081, India. [3]Faculty of Paramedical Science, Assam Down Town University, Panikhaiti, Guwahati, Assam 781068, India.

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