Heterocyclic Letters
Vol. 8| No.3|603-611|May-July|2018
ISSN : (print) 2231–3087 / (online) 2230-9632
CODEN: HLEEAI
http://heteroletters.org

A NEW ALKALOID ISOLATED FROM *CLITORIA TERNATEA* AND EVALUATION OF ANTI-BACTERIAL AND ANTI-INFLAMMATORY ACTIVITIES OF3-DEOXY- 3, 11-EPOXY CEPHALOTAXINE

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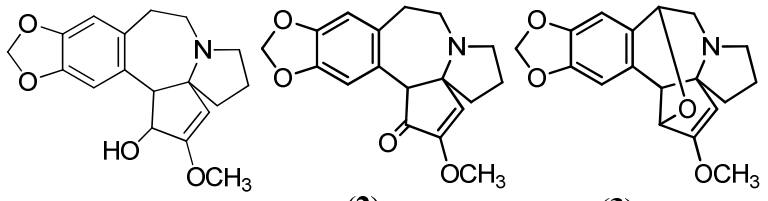
ABSTRACT
The isolation of 3-deoxy-3, 11-epoxy Cephalotaxine (**3**) alkaloid was obtained from *Clitoria ternatea* by careful column chromatography of the crude extract mixture on silica gel 60. The structure was established based on analysis of UV, IR, ^{1}H NMR, ^{13}C NMR, GC-MS spectroscopy methods. Moreover, the absence of toxicity of plant extracts and the isolation of active compounds are important to propose these plants as alternative approaches to resistance management and significant inhibitory effect on the oedema formation.

KEYWORDS:*Clitoria ternatea*,Fabaceae,Alkaloid,3-deoxy-3, 11-epoxyCephalotaxine.

INTRODUCTION
The cephalotaxine (**1**) first described by Paudler et al., has been reported only in the genus cephalotaxus[i]. Homoerythrina alkaloids are also present in *Cephalotaxus* extracts and that cephalotaxinone (**2**) usually occurs as a minor constituent of *C. harringtonia*[ii].Four lesser *Cephalotaxus* alkaloids, all closely related with esters of cephalotaxine, possess marked activity against experimental leukemia in mice[iii]. Cephalotaxine consisting of five fused poly cyclic rings which have a novel arrangement unique in nature, i.e. a benzodioxoazepine onto which is fused a spiropyrrolidino-pentenediiol system. The only reactive function is a secondary alcohol located in position three, the methyl enol ether located in position two being potentially sensitive to proton attack[iv, v]. We now wish to report the isolation of 3-deoxy-3, 11-epoxyCephalotaxine(**3**) in chloroform extracts of *Clitoria ternatea* is reported for the first time.



(**1**) (**2**) (**3**)

The botanical classification of these plant *C. ternatea* belongs to the family Fabaceaewhich is propagated through seeds. It is also commonly known as Butterfly pea belonging to the family. It is a perennial twinning herb with blue and white flowers[vi].It is used in the treatment of chronic bronchitis, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat and tumors. It is also used traditionally to cure sexual ailments, like infertility and gonorrhea, to control menstrual discharge and also as an aphrodisiac[vii].A wide range of secondary metabolites including alkaloid; triterpenoids, flavonol glycosides, anthocyanins and steroids have been isolated from *C. ternatea*[viii]. The present work has carried out to compare the efficacy of phytochemically extracted alkaloid in thechloroformfraction of *C. ternatea* and also to study its pharmacological effect has been chosen anti-bacterial activity by disc diffusion method and anti-inflammatory activity by carrageenan model.

MATERIALS AND METHODS

General

Melting points were determined on a Fisher Scientific melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-2000 polarimeter (Tokyo, Japan). UV analysis was carried out on UV-3010 Ultra violet spectrometer (Perkin Elmer Spectrophotometer); IR spectra were measured on FT-IR spectrograph (Perkin Elmer Spectrophotometer) with KBr tablets from 4000 to 400 cm^{-1} with resolution 2 cm^{-1}. Supporting evidence for the structure of the compound is provided by the ^{1}H ($CDCl_3$, 500 MHz) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectra were recorded on a Bruker AMX 500 NMR spectrometer (Bruker Company, Faelladen, Switzerland). GC-MS analyses were performed on Perkin Elmer Clarus 500 GC-MS system. The fused-silica HP-5 MS capillary column (30 m - 0.25 mm ID, film thickness of 0.25 mm) was directly coupled to the MS. The crude extracts were subjected to series of chromatographic columns using Silica gel 60 as stationary phase (particle size 0.04-0.036 mm, 230-400 mesh, ASTM E. Merck, Germany) and activated by heating at 110 ºC for one hour prior to use. Analyses of all compounds by TLC were accomplished on 0.25 mm Brinkman precoated Silica Gel F254 plates (silica gel 60 / 230-400 mesh, Merck). Analytical plates were developed with the solvent system $CHCl_3$-MeOH (9:1) and spots were visualized by spraying with bromothymol blue in EtOH.

Plant material

The beautiful blue flowers of *C. ternatea*, an ornamental plant, were collected during March from a family garden of a houses in and around Kumbakonam and the plant was taxonomically identified by Dr. N. Ramakrishnan, Associate Professor & Head, Department of Botany, Government Arts College (Autonomous), Kumbakonam, Tamilnadu, India. The flowers materials were dried up at the room temperature. The voucher specimen (GACBOT-121)was maintained in our research laboratory for future reference.

Extraction and isolation

The dried *C. ternatea* flowers were extracted about 750 g with 3 L of 95 % methanol and soaked for 4 days at room temperature (30 ± 2 °C). The suspension was stirred from time to time to allow the flowers powder to fully dissolve in the methanol and then filtered using Whatman no.1. Filtrate was vaporized in water bath at 45 ^{o}C. The filtrates (30.4 g) were evaporated to dryness *in vacuo*. Our main aim is to identify which crude extract was bioactive, before going to fractionation, so it draws our attention to do the sequential partition fraction of methanol crude extract in this regard. After that the crude extract were then made by using different solvent systems (Petroleum ether, Chloroform, Ethyl acetate) in a simple

column chromatography packed with silica gel (column grade) and first the alcoholic extract was then evaporated under reduced pressure (below 40 °C) and the concentrate (100 mL) was diluted with 0·75 L of 6 % of tartaric acid solution. The acidic solution was filtered and the filtrate was extracted with Petroleum ether (2 x 100 mL). The combined Petroleum ether extracts were washed with 50 mL of 5 % aqueous hydrochloric acid. The aqueous layer, combined with the hydrochloric acid extract was made basic with pH 10, in addition to Na_2CO_3 and extracted with chloroform (5 x 100 mL). The dried chloroform extracts were evaporated to dryness *invacuo* and yielded 11.50 g of brown amorphous material. Two further extractions of the plant residue, using the same procedure, yielded an additional 11.05 and 10.20 g respectively of crude material. Asolution of 11.50 g of crude extract in chloroform was chromatographed on 500 g of Silica gel 60, particle size 0.04-0.036 mm and 230-400 mesh. Elution with 100 mL of hexane and 100 mL of benzene yielded no material. Elution with 200 mL of petroleum ether yielded to **4** (0.54 g, 4 %). Elution of the column consecutively with 500 mL of ethyl acetate afforded to **3** (7.15 g, 62 %) gave a single spot on TLC; R_f0.85. After that elution of the column with 250 mL of 50 % ethyl acetate-methanol yielded compound **5** (0.89 g, 8 %).

Quantitative determination of alkaloid

5 g of compound **3** was added with 200 ml of 10% acetic acid in ethanol and allowed to stand for 4 hours and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated NH_4OH was added drop wise to the extract until the precipitation was complete and the precipitated was collected and washed with dilute NH_4OH and then filtered. The residue is the alkaloid, which was dried and weighed (4.25 g, 85 %). It was recrystallized on benzene, m.p. 158-160 °C. The melting point was not altered by further recrystallization. Further the compound **3** was visualized under short and long wave UV light, followed by spraying with Dragendorff's reagent. It was decolorized bromine in carbon tetrachloride and an aqueous solution of potassium permanganate, but it did not give a positive ferric chloride test, suggesting the absence of a phenolic hydroxyl group. It treated with hydrogen iodide consumes one mole of HI, producing one mole silver iodide (Zeisel methoxyl determination) and thus confirming the presence of one -OCH_3group[ix].

Anti-bacterial activity by disc diffusion method

The bacterial strains of *Escherichia coli* and *Staphylococcus aureus and* fungal strains of *Aspergillus flavus* and *Candida albicans* were obtained from Microbial Type Culture Collection Centre (MTCC), Chandigarh. The bacterial cultures were swabbed on to Muller Hinton agar media was prepared and autoclaved at 15 lbs pressure for 20 minutes and cooled to 45 ºC. The cooled media was added 10 mL / L tartaric acid (10 %) act as anti-bacterial agents and poured on to sterile petriplates and allowed for solidification. A total of 6 mm diameter wells were punched into agar and filled with isolated compound from plant extracts and also placed control and standard (Ciprofloxacin and Amphotericin B; < 95 % purchased from Sigma-Aldrich, India) discs. The plates were then incubated at 37 °C for 48 hours. The anti-bacterial activity was evaluated by measuring the zone of inhibition and expressed in mm.

Anti-inflammatory activity by Carrageenan induced rat paw edema

The anti-inflammatory activity of the test compounds were evaluated in albino rats employing the method[x]. Male albino rats (200 - 250 g) of Wistar strain were procured from the College animal house. Animals were fasted overnight and were divided into control, standard and different test groups each consisting of six animals.The different test concentration at the dose of 100 and 200 mg / kg of isolated compounds, 300 mg methanolic extracts and Diclofenac sodium (98 %, purchased from Sigma-Aldrich, India) at dose of 100 mg / kg were administrated to the animals by oral route. Control group animals were received

1 % DMSO at the dose of 10 mL / kg body weight. They housed in cages and maintained under standard conditions at 26 ± 2 °C and relative humidity 60 - 65 % and 12 hours light and 14 hours dark cycles each day for one week before and during the experiments. All animals were fed with the standard rodent pellet diet and water adlibitum. Before starting the experiment on animals, the experimental protocol was subjected to the scrutiny of the Institutional Animal Ethics Committee (IAEC), Bharathidasan University, Trichirappalli, Tamilnadu, India (Approval No. BDU/IAEC/2011/31/29.03.2011).

The acute inflammation was induced by the sub-plantar administration of 0.1 mL of 1 % carrageenan in the right paw. Paw volume was measured by using digital plethysmometer (Ugo Basile-Italy) before administration of carrageenan and after 1, 2, 3 and 4 hours intervals[xi]. The efficacy of different drug was tested on its ability to inhibit paw edema as compared to control group.

Volume of edema = Final Paw Volume - Initial Paw Volume

The Percentage inhibition of paw edema was calculated by the formula as below.

% Inhibition of Paw edema = [(VC – VT) / VC] x 100

Where, VC = Paw edema of control group and VT = Paw edema of treated group.

Statistical analysis

The experimental results were expressed as multiple comparisons of Mean ± SEM were carried out by one way analysis of variance (ANOVA) followed by Dunnet Multiple Comparisons Test and statistical significance was defined as $P < 0.05$.

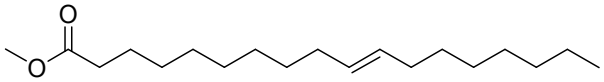
Acute toxicity studies

Acute toxicity studies were carried out according to the literature (Ghosh, 2005). Animals were fasted for eighteen hours and used. A dose of 200 mg / kg of isolated compound and 300 mg / kg methanolic extracts of*C. ternatea* were administrated orally to 12 rats, additionally three rats were kept as control. The control group received distilled water. Then they were observed for 72 hours. Since no mortality was observed and the behavioral pattern was unaffected. No depth was observed at the end of the study.

3-deoxy-3, 11-epoxy Cephalotaxine **(3)**:Colorless needles ($CHCl_3$); m.p.158-160°C;R_f 0.85 (TLC); $[\alpha]_D^{20}$: -59.0 (c = 0.30 in $CHCl_3$); UV λ_{max}^{MeOH} (log ε) 240 - 288 nm; IR (KBr):ν_{max} 3288, 2982, 2835, 1646, 1448, 1230, 1107, 1060, 1015 and 920 cm^{-1}; M+ m/e : 312.97(calcd for $C_{18}H_{19}NO_4$ 313.00); ^1H and ^{13}C-NMR ($CDCl_3$, 500 MHz) spectroscopic data: see Table 1.

10-octadecaneic acid, methyl ester **(4)**:Oily liquid (Petroleum ether); RT 18.63; M+ m/e: 294.85 (calcd for $C_{19}H_{36}O_2$ 296.00).

1, 2-15, 16 - Diepoxyhexadecane **(5)**:Oily liquid (Ethyl acetate-methanol); RT 22; M+ m/e: 254.40 (calcd for $C_{16}H_{30}O_2$ 254.22).



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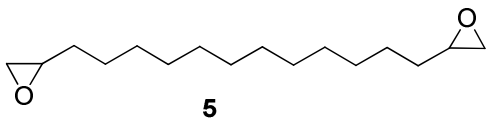

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RESULTS AND DISCUSSION

In this paper, the presence of 3-deoxy-3, 11-epoxy Cephalotaxine (**3)**in extracts of *Clitoria ternatea* is reported for the first time. The compound **3** was isolated as a yellow needles

(mp158-160 °C) with a specific rotation of $[\alpha]_D^{20} = -59.0$ (c = 0.30 in $CHCl_3$). The structure was established based on analysis of UV, IR, ^{1}H NMR, ^{13}C NMR(Table 1) and GC-MS spectroscopic data's and comparing those data with the published values[ii, xii]. The UV-VIS spectrum showed the peaks at 240 and 288 nm. The IRspectral data for this biosynthesized alkaloid is attributable to C-H stretch which exists at methoxy group in the region of 2800-3000 cm^{-1}. The three Peaks were showed at 1448, 1060 and 1015 cm^{-1} can be assigned to C–C stretching (in the ring) and also higher absorption band at 1230 cm^{-1} and another at 920cm^{-1} due to characteristic C-O-C stretching. In addition broad absorption peak showed at 3288cm^{-1} attributed to N-C-H and 1107 cm^{-1} contributes to the C-N stretching. The IR spectrum also confirms the presence of a C=C stretching frequency observed at 1646 cm^{-1}.

Table 1. ^{1}H and ^{13}C NMR data of compounds **1**, **2** and **3** ($CDCl_3$, 500 MHz, δ_H and δ_C in ppm, *J* = in Hz).

	1		**2**		**3**	
Position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	97.84	4.92 (s, 1H)	125.74	4.89 (s, 1H)	126.40	4.90
2	161.57	-	157.40	-	158.10	-
3	73.48	4.78 (d, 1H, 3-H) 1.70 (m, 1H, 3-OH)	200.34	-	186.30	4.03
4	59.01	3.68 (d, 1H)	59.49	3.55 (s, 1H)	60.24	4.03
5	70.52	-	64.62	-	64.10	-
6	21.16	1.96 (m, 1H, H-6_a) 1.83 (m, 1H, -6_b)	19.65	2.11 (q, 1H) 1.93 (m, 1H)	19.92	1.70
7	32.29	1.70 (m, 2H)	30.48	1.83 (m, 2H)	29.98	1.53-1.70
8	53.84	3.05 (m, 1H, H-8a) 2.55 (m, 2H, H-8a+10a)	51.98	3.08 (q, 1H) 2.70 (q, 1H)	51.60	3.06 2.57
10	44.00	2.98 (m, 1H, H-10a) 2.55 (m, 2H, H-8a+10a)	54.88	2.94 (q, 1H) 2.50 (m, 1H)	54.28	3.06 2.57
11	48.31	3.40 (m, 1H, -11b) 2.40 (m, 1H, -11a)	46.66	2.48 (m, 2H)	46.42	4.84
12	135.25	-	130.92	-	131.10	-
13	129.81	-	129.82	-	130.20	-
14	112.90	6.65 (s, 1H)	112.18	6.66 (s, 1H)	113.20	6.63
15	146.90	-	146.02	-	146.40	-
16	146.16	-	145.23	-	145.80	-
17	110.46	6.69 (s, 1H)	109.65	6.71 (s, 1H)	109.40	6.66
18	100.67	5.92 (s, 2H, OCH_2O)	100.62	6.41 (s, 1H, OCH_2O)	100.84	5.88
OCH_3	56.54	3.72 (s, 3H)	57.00	3.81 (s, 3H)	57.30	3.69

The ^{1}H-NMR spectrum revealed some readily assignable signals due to the Cephalotaxine (1) and cephalotaxinone (**2**) skeleton, including signals assigned to two aromatic protons appear at δ 6.63 (s, H-14) and at δ 6.66 (s,H-18), two methylenedioxy protons absorb at δ 5.88 (2H, s) were observedand it may be a second aromatic ring totally substituted (only two aromatic protons are present in the molecule)[i, xiii]. Also methoxy protons signal was present at δ 3.69 ppm (3H, s) which showed with the carbon resonance at δ 57.30 (C-2). This is also evidenced with strong absorption band at 1646 cm^{-1} in the infrared spectrum. In addition one proton singlet at δ 4.90 (m, H-3), which is identified as an olefinic hydrogen, thus confirming the presence of a non-aromatic -C=C- linkage in this alkaloid. Also it showed one proton singlet

at H-3 (δ 4.03) but cephalotaxine (**1**), which showed two proton doublets at H-3 (δ 4.78, 1.70) and cephalotaxinone (**2**) there is no proton signal present at H-3[i, xiv].In addition one proton signal shows at H-11 (δ 4.84) in compound **3** but two doublets at H-11 (δ 2.40 - 3.40) in compound **1** and **2**, because in the C-3-OH in compound **1** changed carbonyl group in compound **2**, but the hydroxyl group is replaced by one hydrogen (C-11) by ether linkage in compound **3**. These assignments also proved with infrared spectrum, which is devoid of the hydroxyl absorption (C-3) band at 3500 cm^{-1} present in compound **1**but carbonyl group present at 1720cm^{-1} in **2** and epoxy group at 1060-1107 cm^{-1} in **3**. The C-13 NMR spectra of **3** suggested the five-membered spiro-fused ring annular to the benzazepine skeleton makes as cephalotaxine system and it showed 18 resonance signals including a methoxy signals at δ 57.30. The GC-MS spectrum showed a molecular ion peak at m/e 312.97 in accordance with a molecular formula of $C_{18}H_{19}NO_4$ (MW 313). This molecular formula was deduced from elemental analysis, which exhibited an anal. C, 68.85; H, 6.42; N, 4.49 %, calcd for $C_{18}H_{19}NO_4$, C, 68.94; H, 6.17; N,4.65 %. These spectroscopic data's were consistent with structure **3** it's C-3 and C-11 ether links; the compound was assigned the name 3-deoxy-3, 11-epoxy Cephalotaxine (3).In petroleum ether fraction containing 10-octadecaneic acid, methyl ester (**4)** and 50% ethyl acetate-methanol fraction yielded 1, 2-15, 16 - Diepoxyhexadecane (**5)** but no NMR spectrum could be obtained due to too low amounts isolated.

Biological evaluation

Anti-bacterial activity

The anti-bacterial activity of compound(**3**) isolated from *C. ternatea*was studied in two different concentrations (50 and 100 mg / mL) and methanolic extract at 150 mg / kg against two pathogenic bacterial strains (*Staphylococcus aureus and Escherichia coli*) and two fungal strains (*Aspergillus flavus*and *Candida albicans*). All the bacterial and fungal strains were selected for the basis of its application purpose of further formulation study. Particularly this plant was a traditional remedy used to treat vaginal infections and infertility. Vaginal infections are caused by a fungus, mainly by *C. albicans*. Anti-bacterial and anti-fungal potential of test samples were assessed in terms of zone of inhibition of bacterial growth and the results were compared with standard (Ciprofloxacin and Amphotericin - B).The test samples doses at 50 and 100 mg / kgrevealed moderate anti-bacterial activity with zone of inhibition ranging from 10.0 to 16.0 mm and had shown to all pathogens (Table 2). The highest zone of inhibition (13.0 and 12.0 mm) was shown by the compound **3** at dose of 100 mg / kgagainst *E. coli* and *S. aureus* strains and (16.0, 12.0) against anti-fungal strains *C. albicans* and *A. flavus*(Fig. 1). The results revealed that in the methanol extracts for bacterial activity against *E. coli* and *S. aureus* shows inhibition zone measured at (09.0 and 08.0 mm) and (8.0 and 7.0 mm) against*C. albicans* and *A. flavus*. These observations may be due to the nature of biological active constituent that are responsible for anti-bacterial and anti-fungal activities.

Anti-inflammatory activity

Inhibition of carrageenan induced inflammation in rats is one of the most suitable test procedures to screen anti-inflammatory agents. The paw oedema induced by carrageenan involves several chemical mediators such as histamine, serotonin, bradykinin and prostaglandins[xv]. The intraperitoneal injection of the carrageenan produced an inflammatory oedema which increased gradually, reaching its maximum at the 4th hour after injection. In this study, the isolated compound **3**tested at doses of 100 and 200 mg / kg exhibited significant anti-inflammatory activity in carrageenan induced rat paw oedema model (Table 3). Diclofenac Sodium is a reference drug, a potent non-steroidal anti-inflammatory which

acts by inhibiting cyclooxygenage. It showed significant inhibitory effects were observed started at first hour after carrageenan administration. The compound **3** at the dose of 200 mg / kg had the highest percentage inhibition of the paw volume (2.92 ± 0.02) and at the dose of 100 mg / kg caused (3.44 ± 0.05) inhibition of the paw volume at 4 hours a performance well comparable with the standard drug Diclofenac Sodium (2.86 ± 0.06). Also after the administration of carrageenan with the methanol extract of *C. ternatea*at the dose of 300 mg / kg exerted considerable inhibitory effect on paw oedema in rats starting from the first hour. The maximum inhibition (2.99 ± 0.04; $p < 0.001$) elicited by the methanol extract was recorded at 4 hours. In the carrageenan-induced rat paw oedema model,3-deoxy-3, 11-epoxy Cephalotaxineshowed significant inhibitory effect on the oedema formation. This effect started from the first hour and was maintained in all the inflammatory phases, suggesting that the main mechanism of action of the tested compound may involve prostaglandin biosynthesis pathway and may influence other mediators of inflammation.

Table 2. Anti-bacterial activity of 3-deoxy-3, 11-epoxy cephalotaxine extracted from *Clitoria ternatea*

S. No.	Micro organisms	Zone of inhibition mm in diameter (M ± SD)			
		3-deoxy-3, 11-epoxy Cephalotaxine		Methanolic Extract (150 mg)	Standard
		50 mg	100 mg		
1	*Escherichia coli*	11 ± 0.40	13 ± 0.70	09±1.00	18±1.20*
2	*Staphylococcus aureus*	11 ± 0.70	12 ± 0.40	08± 0.40	17±0.90*
3	*Aspergillus flavus*	10 ± 0.10	12 ± 0.40	07±0.60	12±0.50**
4	*Candida albicans*	11 ± 0.20	16 ± 0.70	08±0.70	22±0.60**

Bacteria Standard* - Ciprofloxacin (30 mg); Fungal Standard** - Amphotericin - B (20 mg);
Values are expressed in Mean ± Standard Deviation (M ± SD) (n=3)

E. coli ***S. aureus*** ***A. flavus*** ***C. albicans***

Fig. 1. Anti-bacterial activity of 3-deoxy-3, 11-epoxy cephalotaxine

Table 3. Anti-inflammatory activity of 3-deoxy-3, 11-epoxy cephalotaxineextracted from *Clitoria ternatea*

S. No.	Treatment	Anti-inflammatory activity (cm) (M ± SD)			
		1 h	2 h	3 h	4 h
1	Normal Control	2.33±0.01	2.33±0.01	2.32±0.01	2.31±0.05
2	1% carragenan	3.81±0.05	3.60±0.06	3.52±0.02	3.51±0.04
3	Diclofenac sodium (100 mg)	3.85±0.08	3.40±0.13	3.19±0.14	2.86±0.06
4	3-deoxy-3, 11-epoxy Cephalotaxine(100 mg)	3.75±0.04	3.59±0.03	3.48±0.03	3.44±0.05
5	3-deoxy-3, 11-epoxy Cephalotaxine (200 mg)	3.74±0.04	3.43±0.02	3.06±0.04	2.92±0.02
6	Methanolic Extract(300 mg)	3.79±0.03	3.58±0.04	3.46±0.02	2.99±0.04

Data presented above are mean ± standard deviation (M ± SD) values of three replicates.

CONCLUSION

The absence of toxicity of plant extracts and the isolation of active compounds are important to propose medicinal plants *C. ternatea*as alternative approaches to resistance management and significant inhibitory effect on the oedema formation.By ways of solvent extraction, hydrolysis, precipitation and crystallization, high purity of alkaloid with cephalotaxine structure were acquired in our research. The method is suitable for isolation of alkaloid from the *C. ternatea* in industry.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Received on April 23, 2018.