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Isolation and Characterizations of new alkaloid 3-deoxy- 3, 11-epoxy cephalotaxine from *Clitoria ternatea*

R. Manivannan

Department of Chemistry, Government Arts College (Autonomous), Kumbakonam, Tamilnadu-612001, India

ABSTRACT

The present study describes the isolation and structural elucidation of new alkaloid 3-deoxy-3, 11-epoxy cephalotaxine (1) from the flowers of *C. ternatea*. It was isolated by careful column chromatographic separation of the crude extract on silica gel 60. The structure was established based on UV, IR, ^{1}HNMR, ^{13}CNMR and GC-MS spectroscopy methods. The antibacterial activity of various bacterial and fungal strains and anti-inflammatory activities of the isolated compound and its crude methanol extract was studied. The highest zone of inhibition (13.0 and 12.0 mm) was shown by 1 at a dose of 200 µg/kg against *E. coli* and *S. aureus* strains and (16.0, 12.0 mm) against anti-fungal strains of *C. albicans* and *A. flavus*. The results indicated that at both dose levels (100 mg and 200 mg / kg) of isolated compound **1** had significant anti-inflammatory activity from 2nd hour onwards. The bioactive compound isolated from this plant can be employed for antimicrobial activity, also for the treatment of various bacterial and fungal infections and to show pronounced anti-inflammatory effects after three hours of injection.

Keywords: *Clitoria ternatea,* Alkaloid, 3-deoxy-3, 11-epoxy cephalotaxine, Anti-microbial activity, Anti-inflammatory activity

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***Address for Correspondence:**

Dr. R. Manivannan, Assistant Professor of Chemistry, Government Arts College (Autonomous), Kumbakonam, Tamilnadu-612001, India

INTRODUCTION

The cephalotaxine was first described and reported in the genus cephalotaxus[1]. Homoerythrina alkaloids also present in *Cephalotaxus* extracts and that cephalotaxinone usually occurs as a minor constituent of *C. harringtonia* [2]. Four *Cephalotaxus* alkaloids in lesser amounts, all closely related to esters of cephalotaxine, possess marked activity against experimental leukemia in mice [3, 4]. Cephalotaxine consisting of five fused polycyclic rings have a novel arrangement which is unique in nature, i.e. a benzodiazepine onto which a spiro-pyrrolidine-pentanediol system is fused. The only reactive function is a secondary alcohol located at a third position. The methyl enol ether is located at a second position and is potentially sensitive to proton attack [5, 6]. Considering all that, we did not find any previously published original articles about epoxy Cephalotaxine of *C. ternatea* and in order to provide a traditional medicinal value of this species, the present study was designed. The chemical composition and the biological activities such as antibacterial and anti-inflammatory activities of the 3-deoxy-3, 11-epoxy Cephalotaxine (**1)** from the flowers of *C. ternatea* were evaluated and reported for the first time.

C. ternatea (Fabaceae) is propagated through seeds. It is a perennial twining herb, commonly known as Butterfly pea with blue and white flowers [7]. The whole plant is used in the treatment of chronic bronchitis, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat and tumors. It is also traditionally used for sexual ailments, like infertility and gonorrhea, to regulate the menstrual discharge and as an aphrodisiac [8]. A wide range of secondary metabolites like alkaloids, triterpenoids, flavonol glycosides, anthocyanins, and steroids were isolated from *C. ternatea* [9]. The objective of the present study describes the isolation, structural elucidation of the alkaloid obtained for the first time from the chloroform fraction of *C. ternatea,* further antimicrobial activity of the isolated compound against Gram-positive and Gram-negative bacteria by disc diffusion method and to establish its therapeutic potential in the treatment of anti-inflammatory activity in carrageenan induced paw edema bioassay in male albino rats.

MATERIALS AND METHODS

General

Melting points were determined using SMP20 melting point apparatus (Fisher Scientific Ltd, UK) without correction.

Optical rotations were measured with a JASCO P-2000 polarimeter (Tokyo, Japan). UV spectra were recorded on an ultraviolet spectrometer (UV-3010, Perkin Elmer, USA), IR spectra were measured on FT-IR spectrophotometer (PE 1600, Perkin Elmer, USA) with KBr tablets from 4000 to 400 cm^{-1} to resolution 2 cm^{-1}. Supporting evidence for the structure of the compound is provided by the ^{1}H ($CDCl_3$, 500 MHz) and $^{13}CNMR$ (500 MHz, $CDCl_3$) spectra, which was recorded on Bruker AMX 500 instrument (Bruker Company, Faelladen, Switzerland). GC-MS analyses were performed on a Perkin Elmer Clarus 500 GC-MS system. The fused-silica HP-5 MS capillary column (30 m - 0.25 mm ID, the film thickness of 0.25 mm) was directly coupled to the MS. Column chromatography (CC) was performed on 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 110ºC for an hour prior to be used. TLC was performed on 0.25 mm Brinkman percolated silica gel 60 F254 plates (silica gel, 60 / 230-400 meshes, 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 was collected in the month of March from the family garden in and around Kumbakonam, Thanjavur District (India) and authenticated by Dr. N. Ramakrishnan, Head & Associate Professor, Department of Botany, Government Arts College (Autonomous), Kumbakonam, Tamilnadu, India and a voucher specimen (GACBOT-121) was deposited in the Herbarium of the Department of Botany for future reference. The flowers were air-dried under shade for extraction procedure and evaluation.

Extraction and isolation

The air-dried flowers (750 g) were extracted with 95% MeOH (6 x 500 mL) by cold percolation and soaked for 4 days at room temperature (30±2°C). The suspension was stirred from time to time and then filtered using Whatman No.1. The MeOH extract was filtered and concentrated to dryness using a rotary evaporator under reduced pressure at a temperature of 45ºC for complete solvent removal. After that, the dried MeOH extract (30.4 g) was fractionated using petroleum ether in a simple column chromatography packed with silica gel (column grade). The petroleum ether extract was washed with 50 mL of 5 % aqueous hydrochloric acid. The aqueous layer was made basic to pH 10, by adding Na_2CO_3, and extracted with chloroform (5 x 100 mL). The chloroform extract was evaporated to dryness *in vacuo* to afford a brown amorphous material (11.50 g) and it was subjected to silica gel column chromatography (230 - 400 mesh, 10 × 40 cm) with 500mL of ethyl acetate as eluents to afforded (**1)** (7.15 g, 62 %) which gave a single spot on TLC; R_f 0.85.

Acute toxicity studies

Acute toxicity studies were carried out male albino rats (200 – 250 g) of Wistar strain, according to OECD (Organization for economic cooperation and development guidelines no. 425, 2006). Test groups (each containing six animals) were received oral doses of 100, 200 and 300 mg/kg (p.o.) of 3 - deoxy - 3, 11- epoxy cephalotaxine and its MeOH extract, while the control group received the vehicle (saline). The groups were observed for 48 h and mortality (at the end of this period) was recorded in each group [10]. The LD50 (50% lethal dose) was determined by probit test using the log of the dose versus probit [11]. No adverse effect or mortality was observed during the period of the observation (48 h). Based on the study, respective doses were selected for further pharmacological evolution.

Anti-bacterial activity by Disc diffusion method

Anti-bacterial activity was carried out by the method suggested by Bauer et al [12]. Bacterial strains of *Escherichia coli, Staphylococcus aureus and* fungal strains of *Aspergillus flavus, Candida albicans* were obtained from Microbial Type Culture Collection Centre (MTCC), Chandigarh, India. The bacterial cultures were swabbed onto Muller Hinton agar media and autoclaved at 15 lbs pressure for 20 minutes and cooled to 45ºC. To the cooling medium 10 mL of L-tartaric acid (10 %) was added (anti-bacterial agent) and poured onto the sterile Petri plates and allowed for solidification. A total of 6 mm diameter wells was punched into the agar and filled with plant extracts along with 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 antibacterial 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 was evaluated in albino rats employing the method suggested by Diwan *et al* [13]. Male albino rats (200 – 250 g) of Wistar strain were procured from the animal house of our college. Animals were fasted overnight and divided into control; standard and different test groups each consisting of six animals. The different test concentration (100 and 200 mg / kg of isolated compound **1**, 300 mg/kg methanolic extract and 10 mg / kg diclofenac sodium (98 %, purchased from Sigma-Aldrich, India) was administered to the animals by the oral route. Control group animals were received 1% DMSO in sterile saline orally (10 mL/Kg). They are housed in cages and maintained under standard conditions (at 26 ± 2°C, relative humidity of 60 – 65 %, 12 hours light and 14 hours dark cycles a day) for one week. All animals were fed with the standard rodent pellet diet and UV purified and filtered water, ad libitum. 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).

Acute inflammation was induced by the subplantar 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-hour intervals [14]. The efficacy of the 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

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

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

Statistical analysis

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

***3-deoxy-3, 11-epoxy cephalotaxine* (1) -** Colorless needles ($CHCl_3$); mp 158-160°C; R_f 0.85 (TLC); $[\alpha]^{20}_D$: -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). Supporting evidence for the structure of the compound **1** was provided by the analysis of ^1H NMR and ^{13}C NMR data were presented in Table 1.

RESULTS AND DISCUSSION

Identification of Active Compound

During chemical analysis the chloroform extract of *C. ternatea* flowers was separated by column chromatography with ethyl acetate as eluents to yield compound **1.** The NMR spectrum of compound **1** showed similarities to those of the previously isolated cephalotaxine and cephalotaxinone skeleton [2, 3]. Compound **1** was isolated as colorless needles (mp 158-160°C) with a specific rotation of $[\alpha]_{20D}$= −59.0 (c = 0.30 in $CHCl_3$). The GC-MS spectrum showed a molecular ion peak at m/e 312.97, which in accordance with the molecular formula of $C_{18}H_{19}NO_4$ (MW 313). This molecular formula was deduced from the elemental analysis as follows, anal. C - 68.85 (calcd, 68.94); H - 6.42 (calcd, 6.17); N - 4.49 (calcd, 4.65) %. The UV-VIS spectrum in MeOH displayed absorption bands at 240 and 288 nm. The IR spectrum indicates the presence of C-H stretching (-OCH_3) within the region of 2800-3000 cm^{-1}, C-C stretching at 1448, 1060 and 1015 cm^{-1}, C-O-C stretching at 1230 cm^{-1} and 920 cm^{-1}, N-C-H at 3288 cm^{-1}, C-N stretching at 1107 cm^{-1}, and C=C stretching frequency at 1646 cm^{-1}. The ^{13}CNMR spectra of **1** suggested the five-membered spiro-fused ring annular to the benzazepine skeleton, and it has shown 18 resonance signals due to the cephalotaxine system (Fig. 1). The chemical shifts and coupling constants of the ^1H NMR data indicated that two aromatic signals were observed at (δ_H 6.63, H-14 and 6.66, H-17) and the doublet at δ_H 5.88 ppm (d, 2H, *J* = 1.5 Hz), integrating for two protons was assigned as the methylenedioxy group shows that the second aromatic ring was totally substituted [1, 15]. Compound **1** further showed one-proton singlet at δ 4.90 (1H, s, H-1), which is identified as an olefinic proton, thus confirming the presence of a nonaromatic -C=C- linkage in the alkaloid. In addition, the methoxy group signals was present at δ 3.69 ppm (3H, s) and at δ_C 57.30 indicates that attached to an unsaturated carbon; which showed the carbon resonance at δ_C 186.30 (C-2). Characteristic low-field signal appears at δ 4.70 (1H, d, *J* = 6.6 Hz, H-3) and this proton is coupled to one whose signal appears at δ 4.03 (1H, *J* = 6.6 Hz, H-4) and the H-10 protons were easily assigned to the center of the AB resonance system located at δ 3.06 and δ 2.57 with a large coupling constant (*J* =13.7 and 5.9 Hz). The ^{1}HNMR spectrum contains one more proton signal at δ 4.84 (H-11a), it implies that, the hydroxyl group in the C-3 in cephalotaxine is replaced by 11th beta hydrogen by ether linkage in compound **1.** The ^{1}HNMR data of compound **1** instantaneously notify that it refers to great similarities with compound cephalotaxine & cephalotaxinone, showing only differences in the H-3 and H-11 proton signals [1, 16]. The NMR spectra and chemical shift assignments for compounds **1** were summarized in Table 1. Therefore, the compound **1** was assigned the name 3-deoxy-3, 11-epoxy cephalotaxine based on the above experimental spectroscopic data.

Anti-bacterial activity

The antibacterial activity of compound **1** isolated from *C. ternatea* was studied in two different concentrations (100 and 200 µg/mL) and its methanol extract at 300 µg/mL 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 based on its application purpose for further formulation study. This plant was particularly used as a traditional remedy to treat vaginal infections and infertility. Vaginal infections are caused by a fungus *C. albicans*. The anti-bacterial and anti-fungal potential of the test samples were assessed in terms of zone inhibition of bacterial growth, and the results were compared with standards (Ciprofloxacin 30 µg/mL and Amphotericin – B 20 µg/mL). Test sample at 100 and 200 µg/mL doses revealed moderate anti-bacterial activity in a zone of inhibition ranging from 10.0 to 16.0 mm to all pathogens (Table 2). The highest zone of inhibition (13.0 and 12.0 mm) was shown by **1** at a dose of 200 µg/mL against *E. coli* and *S. aureus* strains and (16.0, 12.0 mm) against anti-fungal strains of *C. albicans* and *A. flavus*. The results revealed that in the methanol extract, the bacterial activity against *E. coli* and *S. aureus* shows inhibition zone measured at (9.0 and 8.0 mm) and against *C. albicans* and *A. flavus* at 8.0 and 7.0 mm. These observations may be due to the presence of biological active constituent and which is responsible for anti-bacterial and anti-fungal activities.

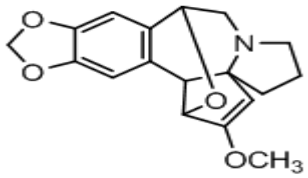
Anti-inflammatory activity

Inhibition of carrageenan induced inflammation in rats is one of the most suitable test procedures to screen anti-inflammatory agents. The paw edema induced by carrageenan involves several chemical mediators such as histamine, serotonin, bradykinin, and prostaglandins [17]. The intraperitoneal injection of the carrageenan produced an inflammatory edema which decreased gradually, reaching its maxima at the 4th hour after injection. In this study, the isolated compound **1** tested at doses of 100 and 200 mg / kg exhibited significant anti-inflammatory activity in carrageenan induced rat paw edema model (Table 3). The results indicated that at both dose levels (100 mg and 200 mg / kg) of isolated compound **1** had significant anti-inflammatory activity from 2nd hour onwards. Paw volume found to be reduced in both doses as compared to control group to rats. 200 mg / kg was more active than 100 mg / kg and caused significant inhibition in the development of paw edema on the 2nd, 3rd, 4th hours (3.43 ± 0.02, 3.06 ± 0.02 and 2.92 ± 0.05) and at a dose of 100 mg / kg caused maximum inhibition (3.44 ± 0.02) of the paw volume at the 4th hour. This shows a performance well comparable with the standard drug diclofenac sodium (10 mg / kg) treated group caused significant inhibition of oedema on the second, third and fourth hour (3.40 ± 0.03, 3.19 ± 0.01 and 2.86 ± 0.06) after carrageenan injection. Also, after the administration of carrageenan with the methanol extract of *C. ternatea* at the dose of 300 mg / kg exerted a considerable inhibitory effect on paw edema in rats starting from the 2nd hour (3.58 ± 0.04). The maximum inhibition (2.99 ± 0.04) exhibited by the methanol extract was recorded in the 4th hour. In the carrageenan induced rat paw edema model, 3-deoxy-3, 11- epoxy cephalotaxine (**1**) showed a significant inhibitory effect on edema formation. This effect starts from the second 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.

CONCLUSION

Based on the results, we can conclude that chloroform extracts of *C. ternatea* is the good sources of 3-deoxy-3, 11-

epoxy Cephalotaxine (**1**). The bioactive compound **1** isolated from this plant can be employed for antimicrobial activity, also for the treatment of various bacterial and fungal infections and to show pronounced anti-inflammatory effects after three hours of injection. In addition, these results provided an initial scientific validation of the widespread use of this plant as a medicine against microbial and inflammatory processes.



1

Figure 1: Chemical structure of compound 1

Table 1: ^{1}H and ^{13}CNMR data of 3-deoxy-3, 11-epoxy cephalotaxine (1) isolated from *Clitoria ternatea*

^{13}C NMR (δ)		^{1}H NMR (δ in ppm, J in Hz)	
Carbon	**δ_C**	**H**	**δ_H**
1	126.40	1	4.90 (1H, s)
2	163.80	2	-
3	74.81	3	4.70 (1H, d, J = 6.8 Hz)
4	42.01	4	4.03 (1H, d, J = 6.8 Hz)
5	64.10	5	-
6	19.92	6	1.86 (1H, m, Ha)
			1.77 (1H, m, Hb)
7	29.98	7	1.53 (1H, m, Ha)
			1.70 (1H, m, Hb)
8	51.60	8	3.06 (1H, dd, J = 13.7, 5.9 Hz, Ha)
			2.57 (1H, m, Hb)
10	54.28	10	3.06 (1H, dd, J = 13.7, 5.9 Hz, Ha)
			2.57 (1H, m, Hb)
11	77.29	11	4.84 (1H d, J = 5.9, 1.6 Hz, Ha)
12	131.10	12	-
13	130.20	13	-
14	113.20	14	6.63 (1H, s, aromatic)
15	146.40	15	-
16	145.80	16	-
17	109.40	17	6.66 (1H, s, aromatic)
18	100.84	18	5.88 (s, 2H, J = 1.5 Hz, OCH_2O)
OCH_3	57.30	OCH_3	3.69 (3H, s)

Table 2: Anti-bacterial activity of 3-deoxy-3, 11-epoxy cephalotaxine (1) isolated from *Clitoria ternatea*

S. No.	Micro organisms	Zone of inhibition mm in diameter (M ± SD)			
		3-deoxy-3, 11-epoxy cephalotaxine		Methanolic Extract	Standard
		100 µg / mL	200 µg / mL	300 µg / mL	*- 30 µg / mL **- 20 µg / mL
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 µg / mL)

Fungal Standard** - Amphotericin - B (20 µg / mL)

Values are expressed in Mean ± Standard Deviation (M ± SD) (n=3)

Table 3: Anti-inflammatory activity of 3-deoxy-3, 11-epoxy cephalotaxine (1) isolated 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% carrageenan	3.81 ± 0.05	3.60 ± 0.16	3.52 ± 0.12	3.51 ± 0.04
3	Diclofenac sodium (10 mg)	3.85 ± 0.08	3.40 ± 0.03	3.19 ± 0.01*	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 ± 0.02
5	3-deoxy-3, 11-epoxy cephalotaxine (200 mg)	3.74 ± 0.04	3.43 ± 0.02	3.06 ± 0.02*	2.92 ± 0.05*
6	Methanolic Extract (300 mg)	3.79 ± 0.03	3.58 ± 0.04	3.46 ± 0.04	2.99 ± 0.04*

Data presented above are mean ± standard deviation (M ± SD) values of three replicates. *p < 0.05 compared to control

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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