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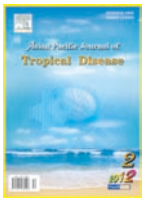

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Isolation and identification of antibacterial compound from Indo-Himalayan *Aconitum nagarum*

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ABSTRACT

Objective: To isolate and identify the antibacterial compound from *Aconitum nagarum* Stapf., a little known medicinal plant in Manipur (Indo-Himalaya). **Methods:** The enriched alkaloid extract from *Aconitum nagarum* root was fractionated through silica gel column chromatography and analysed for antibacterial activity against bacterial species including human pathogens, *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli* and *Bacillus subtilis*. The most potent compound (F_3C_2) isolated from this fraction was purified by chromatography and identified by spectral (IR and NMR) analysis. **Results:** Among different fractions (F1-F6), ether fraction (F3) was found to have maximum antibacterial activity. Based on activity guided fractionation, the compound was identified as a diterpenoid alkaloid aconitine [(1α,3α,6α,14α,16β)-8-(acetyloxy)-20-ethyl-3,13,15-trihydroxy-1,6,16-trimethoxy-4-(methoxymethyl)aconitan-14-yl benzoate]. **Conclusion:** The antibacterial compound showed activities against different human pathogens and has been identified as aconitine.

1. Introduction

Aconitum L. (Ranunculaceae) is a genus of perennial erect or rarely twinning herbs. Its extracts are traditionally used at diluted concentrations for curing fever, gastro-intestinal dysfunction, inflammation, cough, asthma and as external applications for treating neuralgia and other painful infections [1]. Some *Aconitum* species are also reported to possess antifungal, insecticidal and rodenticidal properties [2]. Botanical Survey of India reported two species namely, *Aconitum nagarum* Stapf. and *Aconitum elwesii* Stapf. from Manipur, India and until now their chemical composition and other biochemical properties have not yet been studied. The development of safe and effective antimicrobial drugs has revolutionized medicine in the last forty years and numerous studies proved that these antimicrobial agents from higher plants. [3] Bioassay guided research could thus reveal new, renewable and less expensive compounds in these plants. A wide variety of higher plants have been investigated for the presence of antibacterial compounds[4, 5, 6]. However, proper and scientific use of any herbal product needs detailed studies involving identification of their major bioactive components and mechanism of action [7]. From our previous study (Data unpublished) it was revealed, that alkaloid extract from *Aconitum nagarum* root is the most potent source amongst the sample studied of two species of *Aconitum* available in the region, with moderate to strong levels of antibacterial activities against *Staphylococcus aureus, Bordetella bronchiseptica, Bacillus subtilis, Pseudomonas putida* and *Xanthomonas campestris*. In the present study, we report isolation and identification of an antibacterial alkaloid from *Aconitum nagarum* collected from Manipur, India, and its efficacy was compared with standard antibiotics. To the best of our knowledge this is the first report of its kind on *Aconitum* species of this region.

2. Materials and methods

2.1. Plant material

The plant *Aconitum nagarum* Stapf. was collected from Shirui hill (2254 m above mean sea level), Ukhrul, Manipur, India. Specimens were authenticated by Central National Herbarium, Indian Botanic Garden Kolkata, India, *Aconitum nagarum* Stapf based on Sirohee: 19.9.1948, Mukherjee,

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3515. Voucher specimen (SYM-002201) was deposited at the herbarium of Department of Life Sciences, Manipur University, Imphal, India.

2.2. Alkaloid extraction and chromatography

Alkaloid was extracted from homogenate prepared using powdered dried roots in 1M HCl (1:10; w/v) by the method described earlier [8]. The supernatant was alkalized to pH 10 with ammonia solution (25%) and extracted thrice with equal volumes of chloroform. The extract was subjected to silica gel column chromatography (Keiselgel 60, 0.2-0.5 mm), eluted sequentially with hexane, hexane:ether (1:1), ether, chloroform, chloroform:methanol (1:1) and methanol and monitored by TLC using silica-gel 60 F254 plates (Merck, Germany). Plates were developed using diethyl ether:ethyl acetate (20:1) saturated with conc. ammonia [8] and visualized under UV lamp (Camag, Switzerland) at 254 nm. Similarly, preparative TLC (0.5 mm thickness) was performed.

2.3. Antibacterial activity and efficacy of antibacterial components

Antibacterial activity of the alkaloid fractions and purified compound dissolved in DMSO was tested using disc diffusion method by spread plating an aliquot (100 μl) of culture (~10^5 to 10^6 cfu / ml) and analysing the efficacy of the bioactive compound by comparing with standard antibiotics as reported earlier [9].

2.4. Spectral data analysis

Melting points (uncorrected), optical rotations, IR spectrum and NMR spectra were determined using a Fisher John apparatus (serial no. 21000275), JASCO-DIP 370 polarimeter (Jasco Corporation, Tokyo, Japan), JASCO FTIR 4100 spectrophotometer (Jasco Corporation, Tokyo, Japan) and Bruker AWANCE 300 MHz FT NMR spectrometer (Bruker, Fallanden, Switzerland), respectively. The chemical shifts in NMR spectra were reported relative to the chloroform ($CDCl_3$) with tetra methyl silane (TMS) as an internal standard.

3. Results

Alkaloid extract of *A. nagarum* root was found to have moderate to strong antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (NCIM 2063) while weak activity against *Salmonella typhimurium* (MTCC 98) and *Escherichia coli* (MG 1655) at the concentration of 100 μg/disc. The strength of antibacterial activity was grouped depending upon zones of inhibition (weak: 7-8 mm, moderate: 9-11 mm and strong: >12 mm) (data unpublished).

3.1. Assay guided isolation of major antibacterial compound

The root extract was subjected to silica gel column chromatography, which resulted in six different fractions (F1 - F6). The F1 (hexane), F2 (hexane-ether, 1:1), F4 (chloroform), F5 (chloroform-methanol, 1:1) and F6 (methanol) showed only weak antibacterial activity (7-8 mm) against sensitive bacterial species, whereas, F3 (ether) showed moderate level of antibacterial activity (9-11 mm) against *S. aureus*, *S. typhimurium*, *E. coli* and *B. subtilis* (Table 1). On qualitative thin layer chromatography (TLC), fraction F3 resolved in two major spots F3C1 and F3C2 at R_f values 0.55 and 0.41, respectively, in solvent systems of diethyl ether: ethyl acetate (20:1) saturated with conc. ammonia (Figure 1a). Out of these two major components, F3C2 (TLC purified) exhibited antibacterial activity up to moderate level against *S. aureus*, *S. typhimurium*, *E. coli* and *B. subtilis* at concentrations of 2 and 4 μg/disc similar to F3 (Table 1). The antibacterial activity of F3C1 was weak (upto 7 mm) against the tested bacterial species (Table 1). The antibiotics kanamycin (0.1 to 1.0 μg/disc), streptomycin (0.5 to 5.0 μg/disc), spectinomycin (5.0 to 20.0 μg/disc), ampicillin (0.005 to 5.0 μg/disc) and rifampicin (0.001 to 5.0 μg/disc) showed antibacterial activity similar to F3C2 (Table 1). Thus the findings indicated high potency of the F3C2 as an antibacterial agent. Its efficacy was found to be even higher than spectinomycin against these bacteria (Table 1). For *S. typhimurium*, its efficacy was higher than streptomycin, spectinomycin and rifampicin, whereas, for *E. coli* the value was higher than spectinomycin, ampicillin and rifampicin (Table 1).

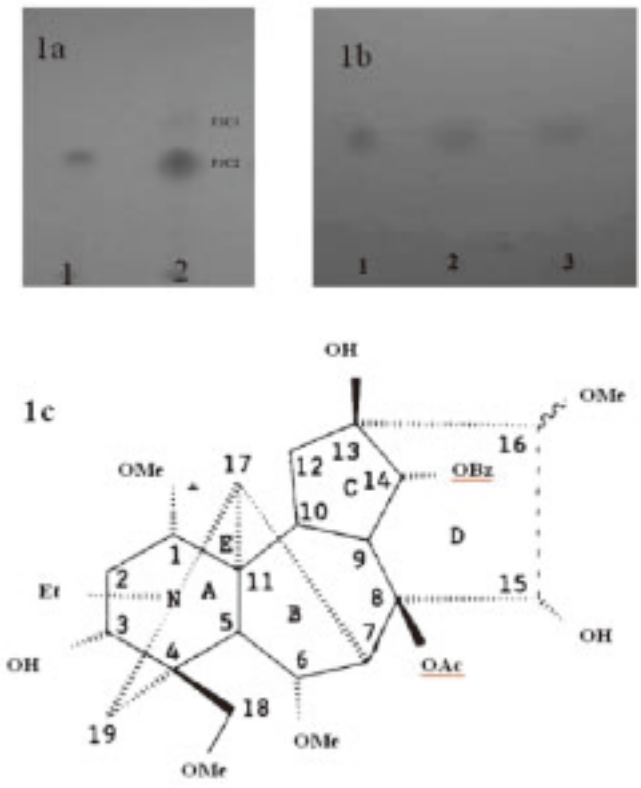


Figure 1. Identification of major antibacterial compound; (a) TLC of ether fraction (F3); Lane 1: Standard aconitine, Lane 2: Ether fraction (F3) showing two compounds F3C1 and F3C2; (b) Co-chromatography of aconitine with F3C2; Lane 1: Standard aconitine, Lane 2: F3C2 mixed with aconitine, Lane 3: F3C2; (c) Probable structure of F3C2 based on spectral data.

Table 1

Antibacterial activity of silica gel column eluted fractions (F1–F6) as well as purified compounds (F3C1 and F3C2) and antibiotic concentration to produce similar effect as F3C2.

Sample	Concentration(μg/disc)	S.a	S.t	E.c	B.s
		Zone of inhibition (mm)			
F1	2	$7^{x,r}$±1	$7^{x,r}$±1	NAc	NAc
	4	$8^{x,r}$±1	$7^{x,r}$±1	NAc	NAc
F2	2	$7^{x,r}$±1	NAc	NAc	$7^{x,r}$±1
	4	$7^{x,r}$±1	NAc	NAc	$7^{x,r}$±1
F3	2	$10^{y,r}$±1	$9^{x,r}$±1	$8^{x,r}$±1	$9^{x,r}$±1
	4	$11^{y,r}$±2	$9^{x,r}$±2	$9^{x,r}$±2	$10^{y,r}$±1
F4	2	NAc	$7^{x,r}$±1	$7^{x,r}$±1	$7^{x,r}$±1
	4	$7^{x,r}$±1	$7^{x,r}$±1	$7^{x,r}$±1	$8^{x,r}$±1
F5	2	$7^{x,r}$±1	NAc	NAc	$7^{x,r}$±1
	4	$7^{x,r}$±1	$7^{x,r}$±1	NAc	$8^{x,r}$±1
F6	2	$7^{x,r}$±1	NAc	NAc	$7^{x,r}$±1
	4	$7^{x,r}$±1	NAc	NAc	$7^{x,r}$±1
F3C1	2	$7^{x,r}$±1	$7^{x,r}$±1	$7^{x,r}$±1	$7^{x,r}$±1
	4	$7^{x,r}$±2	$7^{x,r}$±1	$7^{x,r}$±1	$7^{x,r}$±2
F3C2	2	$10^{y,r}$±1	$8^{x,r}$±1	$8^{x,r}$±1	$8^{x,r}$±2
	4	$11^{y,r}$±2	$9^{x,r}$±1	$9^{x,r}$±1	$10^{x,r}$±2
Standard aconitine	2	NAc	$7^{x,r}$±1	$8^{x,r}$±1	$8^{x,r}$±1
	4	NAc	$8^{x,r}$±1	$9^{x,r}$±2	$9^{x,r}$±2
Antibiotics		Concentration (μg/disc) for similar activity as F3C2			
Kanamycin		1.0	1.0	1.0	0.1
Streptomycin		1.0	5.0	1.0	0.5
Spectinomycin		20.0	10.0	5.0	5.0
Ampicillin		0.025	1.0	5.0	0.005
Rifampicin		0.001	5.0	5.0	0.01

Extract obtained using different solvent system: F1– hexane; F2– hexane–ether (1:1), F3– ether, F4– chloroform, F5– chloroform–methanol (1:1) and F6– methanol; S.a: *Staphylococcus aureus*; S.t: *Salmonella typhimurium*; E.c: *Escherichia coli*; B.s: *Bacillus subtilis*; NAc: No activity; Mean with different superscript column wise (x,y), and with different superscript row wise (r,s) differ significantly in one–way ANOVA (P<0.05) (n=4).

3.2. Identification of isolated compound

Further identification of the bioactive F3C2, a white amorphous powder, was carried out by determining melting point, IR spectrum, ^{1}H and ^{13}C NMR analysis. The observed values were as follows: m.p: 203–204℃, [α]24D: + 20° ($CHCl_3$, c = 0.65), IR υ^{KBr} (cm–1): 3491, 3019, 2928, 2855, 1719, 1452, 1280, 1215, 1100 (Figure 2a). ^{1}HNMR and ^{13}CNMR (CDCl3) exhibited the characteristic NMR spectral features of aconitine type diterpenoid bearing 4–methoxyl groups (dH 3.17 s, 3.26 s, 3.49 s, 3.73 s), an acetyl group (dH/C 1.72/21.4; dC 172.4 s), a benzoyl group (dH 8.01 d, 7.58 d, 7.45 dd) and (dC 70.5, 74.0, 80.2) (Table 2; Figure 2b & 2c). Among them the four–methoxyl groups could be located at C–1, C–6, C–16 and C–18, respectively. The acetyl signal at higher field (dH 1.72) and a doublet at dH 4.88 (H–14) suggested the acetyl and benzoyl groups were located at C–8 and C–14, respectively. Further co–chromatographic TLC with F3C2 and pure aconitine mixture confirmed the identity of this compound. The presence of single spot as well as same R_f as standard aconitine confirmed it as diterpenoid aconitine (Figure 1b). The probable structure of this compound [(1α,3α,6α,14α,16β)–8–(acetyloxy)–20–ethyl–3,13,15–trihydroxy–1,6,16–trimethoxy–4–(methoxymethyl)aconitan–14–yl benzoate] is being proposed in Figure 1c. The activity of F3C2 was found to be similar to standard aconitine with the exception that the isolate also showed high activity towards *S. aureus* (Table 1). This could be accounted by the possible presence of other minor constituents in F3C2.

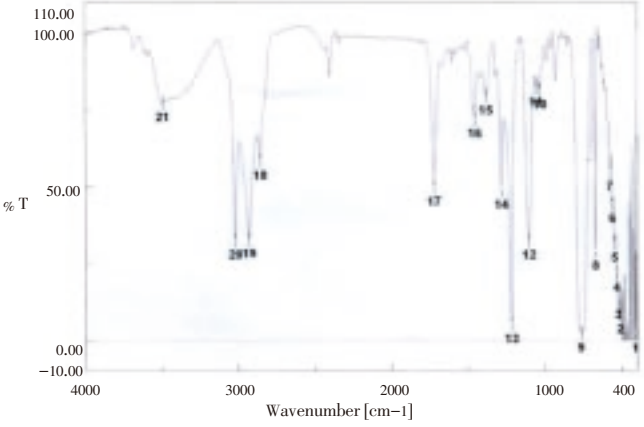
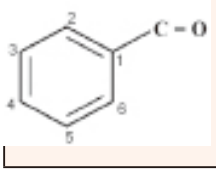


Figure 2a. IR spectrum of F3C2.

Table 2

^{13}CNMR and ^{1}HNMR data of the compound (F3C2).

Carbon Position	Chemical Shift (dC)	Peak Multiplicity	Proton Position	Chemical Shift (dH)	Peak Multiplicity
1	83.3	d	1	3.26	m
2	36.3	t	2	2.32, 2.37	m, m
3	70.5	d	3	3.75	m
4	43.1	s	4	–	–
5	46.6	d	5	2.11	d (J = 6.7)
6	82.3	d	6	3.95	d (J = 6)
7	44.6	d	7	2.90	s
8	91.95	s	8	–	–
9	44.3	d	9	2.84	dd (J = 7.5)
10	40.8	d	10	2.73	m
11	49.9	s	11	–	–
12	35.7	t	12	2.69, 2.16	m, m
13	74.0	s	13	–	–
14	78.8	d	14	4.88	d (J = 4.5)
15	80.2	d	15	4.47	d (J = 5)
16	89.9	d	16	3.24	d (J = 7)
17	60.9	d	17	3.18	Br s
18	76.5	t	18	3.29	m
19	47.1	t	19	3.30, 2.35	m, m
N–CH2–CH3	48.9	s	N–CH2	2.71, 3.40	m, m
N–CH2–CH3	13.3	q	N–CH2–CH3	1.07	t
1′	55.9	q	OMe	3.26	s
6′	57.9	q	OMe	3.17	s
16′	61.1	q	OMe	3.73	s
18′	59.1	q	OMe	3.49	s
			OH–3	3.77	d
			OH–13	3.97	s
			OH–15	4.39	d
CH3C=O	172.4	s		–	–
CH3C=O	21.4	q	CH3C=O	1.72	s
C6H5 C=O	166.0	s		–	–
	1–129.6	s			–
	2–128.6	d	2′, 6′	8.01	d (J = 7.3)
	3–129.3	d	3′, 5′	7.45	dd (J = 7.3)
	4–133.2	d	4′	7.58	d (J = 7.3)
	5–129.8	d			–
	6–128.6	d			–

d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, J = coupling constant (in Hz), δ = chemical shift in ppm.

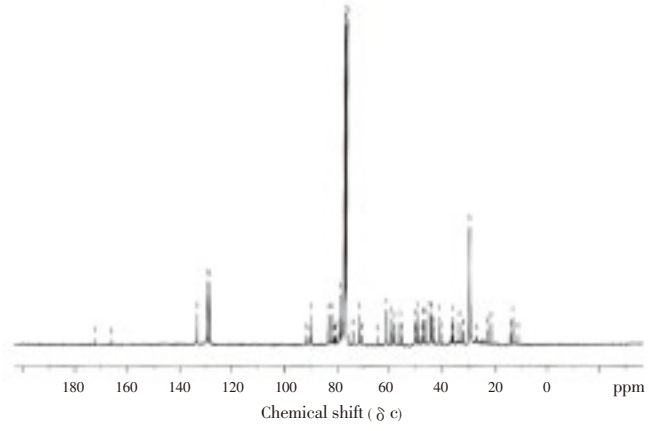


Figure 2b. ^{13}C NMR spectrum of F3C2.

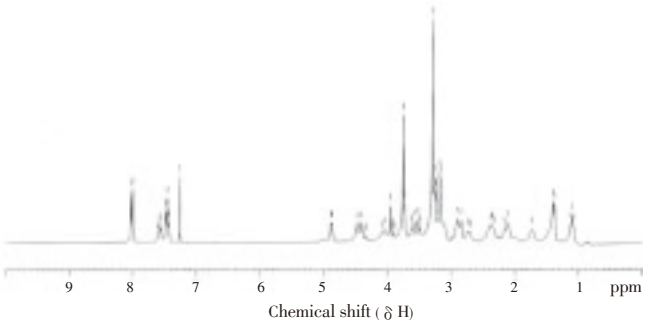


Figure 2c. ^{1}HNMR spectrum of F3C2.

4. Discussion

In recent years, a large number of studies have investigated the pharmacological characteristics of *Aconitum*, their main alkaloids and their derivates [10]. The current study was undertaken for the isolation and identification of the major antibacterial compound from *Aconitum nagarum* Stapf. The antibacterial activity was assessed against bacterial species including pathogens that involved in different human diseases sometimes resulting in acute health problem [11]. *S. aureus* infection mainly results in atopic dermatitis and toxic shock syndrome (TSS). *S. typhimurium* causes typhoid and gastroenteritis, whereas, *E. coli* infection results in diarrhea and dysentery-like syndrome in several cases.

Comparison of the ^{1}HNMR and ^{13}CNMR data of this compound with the literature [12, 13] suggested the compound (F3C2) as aconitine type C19-diterpenoid alkaloid. Several aconitine type diterpenoid alkaloids have been reported from *Aconitum* species [13, 14]. The diterpenoid alkaloid includes six types, aconitine, pseudoaconitine, bikhaconitine, neoline, isotalatizidine and lycoctonine type [15]. Recently, aconitine type alkaloids such as lycoctine and delphatine from *A. heterophyllum* have been reported to exhibit significant antibacterial activity [16].

Thus, the role of this compound in imparting antibacterial activity of this species is reported here. The study showed that aconitine type alkaloid has a good potential wide spectrum antibacterial activity against different human pathogens and thus its structural identification could provide clue to develop new potent antibacterial variants through synthetic route.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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