Open Access

SCHOLARENA

Journal of Medicinal Chemistry and Drug Design

RESEARCH ARTICLE

Isolation and Characterization of C_{19}-Diterpenoid Alkaloids from the Roots of *Aconitum Heterophyllum* Wall

Obaidullah[1], Muhammad Nisar Ahmad[2], Waqar Ahmad[3], Shafiq Ahmad Tariq[4], Najeeb ur Rahman[3], Shujaat Ahmad[3,5] and Manzoor Ahmad[3*]

[1]*Department of Pharmacy, University of Peshawar, Peshawar-25120, Pakistan*
[2]*Institute of Chemical Sciences, University of Peshawar, Peshawar-25120, Pakistan*
[3]*Departments of Chemistry and Pharmacy, University of Malakand, Chakdara Dir (L), Khyber Pakhtunkhwa, Pakistan*
[4]*Department of Pharmacolgy, Khyber Medical College, Peshawar, Pakistan*
[5]*Department of Pharmacy Shaheed Benazir Bhuttoo University, Sheringal, Dir (Upper), Pakistan*

[*]**Corresponding author:** Manzoor Ahmad, Department of Chemistry, University of Malakand, Chakdara, Dir (L), Khyber Pakhtunkhwa, Pakistan, Tel: +92-333-3586144, E-mail: manzoorhej@yahoo.com, manzoorahmad@uom.edu.pk

Citation: Obaidullah, Muhammad Nisar Ahmad, Waqar Ahmad, Shafiq Ahmad Tariq, Najeeb ur Rahman, et al. (2018) Isolation and Characterization of C19-Diterpenoid Alkaloids from the Roots of *Aconitum Heterophyllum* Wall. J Med Chem Drug Designing 1:101

Abstract

Two new C_{19}-diterpenoid alkaloids heterophylline-A (1) and heterophylline-B (2), along with a known C_{19}-diterpenoid alkaloids condelphine (3) have been isolated from the roots of the *Aconitum heterophyllum* Wall. The structures of the new compounds were deduced on the basis of spectroscopic techniques (EI-MS, HREI-MS, 1H NMR, ^{13}C NMR, HMQC and HMBC). The new compounds (1-2) showed significant antibacterial activity (MIC = 1.3 µg/mL, 2.1 µg/mL and 2.4 µg/mL) against *Escherichia coli. S. aureus and P. aeruginosa*, the compound 3 showed moderate antibacterial activity only against *P. aeruginosa* (MIC = 7.6 µg/mL). In case of antifungal activity, compound 1 exhibited significant antifungal activity (MIC = 3.4 µg/mL) against *T. longifusus*. The compound 2 showed significant antifungal activity (MIC = 2.7 µg/mL) against *M. canis*. Compound 3 presented moderate antifungal activity against *T. longifusus*.

Keywords: *Aconitum heterophyllum* Wall; C_{19}-diterpenoid Alkaloids; Heterophylline-A; Heterophylline-B; Antibacterial; Antifungal; Ranunculaceae

Introduction

Infectious diseases are still posing a major threat to the better quality of life for human beings. Fungal and bacterial infections have increased the complications of immunocompromised patients especially those undergoing cancer chemotherapy, organ transplant, and patients with AIDS [1]. Emergence of resistant bacterial and fungal strains requires timely strive for developing new potent and efficient molecules as antimicrobial agent both for bacterial and fungal infections [2]. Medicinal plants are still a reliable source for bioactive compounds. Thorough analysis of available scientific literature reveals the plants to be a source rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties.

Genus *Aconitum* is a rich source of diterpenoid alkaloids, many of which are already known to exhibit a broad spectrum of biological activities. Among others, lappaconitine hydrobromide has been used as an antiarrhythmic drug, while the methyllycaconitine perchlorate is used in curaremimetic preparation [3]. Some aconitine and mesaconitine derivatives possess potent analgesic and anti-inflammatory activities [4]. The methyllycaconitine and lycaconitine exhibited neuronal nicotinic acetylcholine receptor affinity [5]. Lycaconitine, obtained from several *Aconitum* species, was found to be effective against multi-drug resistance cancers. *Aconitum* plants are widely used in Chinese and Indian traditional systems of medicine [6,7]. Turkish *Aconitum* species are used externally in the treatment of rheumatic pain, sciatica and also against body lice [8].

In the current study our effort was to isolate potentially bioactive antibacterial and antifungal molecules. Here we describe the isolation, structure elucidation and antimicrobial potential of two new norditerpenoid alkaloids heterophylline-A (1), and heterophylline-B (2), along with a known alkaloid condelphine (3).

Results and Discussion

The alkaloidal constituents of the roots of *A. Heterophyllum* Wall., collected in flowering period (August 2005) from Swat district, Khyber Pukhtoonkhwa Province of Pakistan, were studied. The known alkaloid, condelphine, one new lycoctonine and one lycaconitine-type norditerpenoid alkaloid heterophylline-A (1) and heterophylline-B (2) have been isolated from this plant [9].

In an early study, heterophyllisine, heterophylline, heterophyllidine, heteratisine, atisine, atidine, F-dihydroatisine, heteratisine, atisenol and heterophyllinine-A, heterophyllinine-B, 6-dehydroacetylsepaconitine and 13-hydroxylappaconitine were reported from *A. Heterophyllum* Wall [10-13].

Heterophylline-A (1) was obtained as a white amorphous powder, and was assigned the molecular formula $C_{29}H_{41}N_2O_5$ on the basis of HREI-MS (m/z 496.6382, calcd. 496.6375). The IR spectrum of compound 1 showed absorption bands at 3492 (hydroxy), 1700 (carbonyl), 1600, 1280, 1250, and 750 cm^{-1} (1, 2-substituted aromatic ring), and 1083 (simple ether bonds). ^1H and ^{13}C NMR spectra of compound 1 exhibited a close resemblance to that of the known compound karakoline except the presence of aminobenzoyl group at C-14, instead of hydroxyl group in compound 1 [14,15].

The ^1H-NMR spectrum of compound 1 exhibited characteristic signals for *N*-ethyl, one methoxy group and several methine protons with geminal oxygen substituents. In the down field region of the spectrum a broad singlet of two protons at δ 5.64, was assigned to the amino group attached to benzoyl at C-2`. While a broad singlet of one proton at δ 4.56 was assigned to H-14. Triplet of three protons integration at δ 1.07 (J = 7.0 Hz), was due to the methyl of *N*-ethyl group. Similarly, in the down field region a triplet of one proton at δ 3.67 (J = 6.0 Hz), was assigned to the H-1, geminal to hydroxy group. A broad singlet of one proton at δ 2.55 was assigned to the H-17, while a broad singlet of one proton at δ 3.30 was assigned to H-7. The ^{13}C-NMR spectrum (BB, DEPT), showed twenty nine signals, including three methyls, seven methylene, thirteen methine, and six quaternary carbons (Table 1). The ^1H- ^{13}C correlation was determined by the HMQC spectrum, while the long range ^1H- ^{13}C connectivities were obtained through HMBC technique (Figure 1). The H-5 (δ 1.78) showed ^2J and ^3J correlation with C-4 (δ 38.0), C-11 (δ 50.1), C-6 (δ 21.2), C-7 (δ 48.1), and C-19 (δ 60.5), whereas H-7 (δ 3. 30), exhibited ^1J and ^2J correlation with C-6 (δ 21.2), C-7 (δ 48.1), C-8 (δ 72.9), and C- 17 (δ 62.5), while, H-14 (δ 4.56), showed correlation with C-13 (δ 45.8), C-16 (δ 81.3), C-9 (δ 49.2), C-6` ($\delta$ 130.5), and C-2` (δ 150.2). Thus on the basis of above spectral data, the structure of compound 1 was deduced as heterophylline-A.

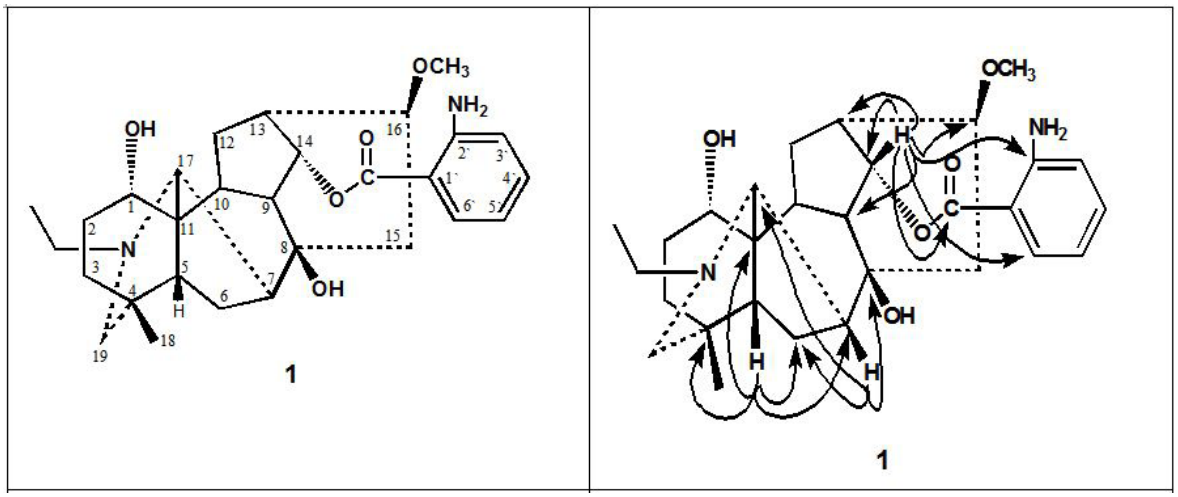

Figure 1: Key HMBC interactions in compound 1

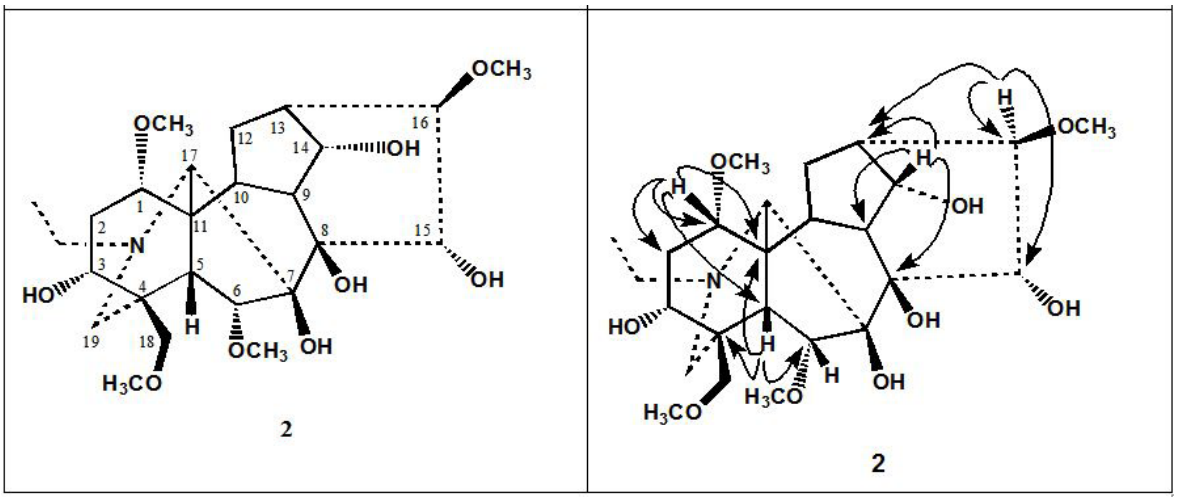

Figure 2: Key HMBC interactions in compound 2

Position	Compound (1)			Compound (2)		
	δ_H (*J* in Hz)	δ_C	Multiplicity	δ_H (*J* in Hz)	δ_C	Multiplicity
1	3.67, t, 6.0	69.2	CH	4.76, *t*, 6.32	83.0	CH
2	2.32, 2.26, *m*	29.1	CH_2	2.52, 2.45, *m*	29.2	CH_2
3	1.22, 1.30, *m*	33.7	CH_2	3.67, *m*	71.5	CH
4	-	38.0	C	-	43.1	C
5	1.78, br *s*	46.7	CH	2.15, br *s*	48.3	CH
6	2.50, 1.05, *m*	21.2	CH_2	5.78, *d*, 5.38	82.0	CH
7	3.30, br *s*	48.1	CH	-	78.9	C
8	-	72.9	C	-	77.4	C
9	2.50, *m*	49.2	CH	2.66, *m*	41.5	CH
10	2.32, *m*	45.3	CH	2.15, *m*	47.5	CH
11	-	50.1	C	-	50.1	C
12	2.42, 2.50, *m*	28.6	CH_2	2.15, 2.22, *m*	36.8	CH_2
13	2.63, *m*	45.8	CH	2.52, *m*	49.0	CH
14	4.56, br *s*	71.0	CH	4.22, *t*, 4.7	81.3	CH
15	2.05, 1.95, *m*	44.1	CH_2	4.28, *d*, 5.9	78.5	CH
16	3.40, *t*, 8.4	81.3	CH	3.12, *t*, 8.1	90.6	CH
17	2.55, br *s*	62.5	CH	3.61, br *s*	61.2	CH
18	0.84, *s*	21.8	CH_3	3.67, 3.59	77.4	CH_2
19	2.63, 2.32	60.5	CH_2	3.93, 2.76	49.0	CH_2
1`	-	131.6	C			
2`	-	150.2	C	3.34, *s*	55.5	OCH_3
3`	6.84, *d*, 8.5	116.0	CH	3.29, *s*	59.1	OCH_3
4`	7.10, *d*, 8.4	131.8	CH	3.47, *s*	57.9	OCH_3
5`	6.70, *d*, 8.4	116.0	CH	3.65, *s*	59.1	OCH_3
6`	7.38, *d*, 2.4	130.5	CH			
N- CH_2 | CH_3	2.91, 2.81, *m* 1.07, *t*, 7.0	44.1 19.2	CH_2 CH_3	2.66, 2.80, *m* 1.07, *t*, 7.0	53.0 12.8	CH_2 CH_3
C=0	-	172.9	C			

Table 1: ^{1}H and ^{13}C data of compound 1 and compound 2 ($CDCl_3$)

Heterophylline-B (2) was obtained as a white amorphous powder, and was assigned the molecular formula $C_{25}H_{41}NO_9$ on the basis of HREI-MS *(m/z* 499.5942, calcd. 499.5886). The IR spectrum of compound 2 showed absorption bands at 3492 (hydroxy), 1083 (simple ether bonds). The mass fragmentation of compound 2 is characteristic of alkaloids with a lycoctonine skeleton. The base peak was that of the (M^+- 31) ion which indicated the presence of an α-methoxy group at C–1 [16].

The ^{1}H-NMR spectrum of compound 2 exhibited signals for *N*-ethyl, four methoxy groups and several methine protons with geminal oxygen substituents. In the down field region of the spectrum two triplets of one proton at δ 4.22 (*J* = 4.7 Hz) and 4.76 (*J* = 6.32 Hz) was assigned to H-14 and H-1. Similarly, in the down field region a doublet of one proton at *δ* 5.78 (*J* = 5.38 Hz) was assigned to the H-6 geminal to methoxy group. While a multiplet of one proton at δ 3.67 was assigned to H-3 geminal to hydroxyl group. Triplet of three protons at *δ* 1.07 (*J* = 7.0 Hz) was due to the methyl of N-ethyl group. Similarly, a doublet of one proton at δ 4.28 (*J* = 5.9 Hz), was assigned to the H-15, geminal to hydroxy group. A broad singlet of one proton at *δ* 2.15 was assigned to the H-5. The ^{13}C-NMR spectrum (BB, DEPT) (Table 1), showed twenty five signals, including five methyls, five methylene, eleven methine, and four quaternary carbons. The ^{1}H- ^{13}C correlation was determined by the HMQC spectrum, while the long range ^{1}H- ^{13}C connectivities were obtained through HMBC technique (Figure 2). The H-5 (*δ* 2.15) showed correlation with C-4 (*δ* 43.1), C-11 (*δ* 50.1), and C-6 (*δ* 82.0), whereas H-14 (*δ* 4.22), showed correlation with C-13 (*δ* 49.0), C-14 (*δ* 81.3), C-9 (*δ* 41.5) and C-8 (*δ* 77.4). Similarly, H-16 (*δ* 3.12) exhibited interaction with C-16 (*δ* 90.6), C-15 (*δ* 78.5) and C-13 (*δ* 49.0), while H-17 (*δ* 3.61) showed interaction with C-10 (*δ* 47.5), C-11 (*δ* 50.1), and C-5 (*δ* 48.3), whereas, H-1 (*δ* 4.76) exhibited interactions with C-17 (*δ* 61.2), C-2 (*δ* 29.2), C-5 (*δ* 48.3) and C-11 (δ 50.1). Thus on the basis of above spectral data, the structure of compound 2 was deduced as heterophylline-B. Compound 3 i.e. condelphine was a known compound (isolated first time from *A. heterophyllum*), and characterized by comparing its spectroscopic data with those reported in literature [9].

All the compounds were assayed for their antibacterial and antifungal potential by hole-diffusion method followed by determination of their corresponding minimum inhibitory concentration (MIC) values against tested fungal and bacterial strains (Table 2 and 3) [17,18]. MIC of standard drugs and test compounds was determined against test isolates by broth micro-dilution technique. Compound 1 showed significant (MIC = 1.3 µg/mL) antibacterial activity against *Escherichia coli*. Compound 2 exhibited significant antibacterial activity against *S. aureus* and *P. aeruginosa* having MIC values for these bacteria 2.1 µg/mL and 2.4 µg/mL respectively, the compound 3 showed moderate antibacterial activity only against *P. aeruginosa* (MIC = 7.6 µg/mL). In case of antifungal activity, compound 1 exhibited significant antifungal activity (MIC = 3.4 µg/mL) against *T. longifusus*. The compound 2 showed significant antifungal activity ((MIC = 2.7 µg/mL) against *M. canis*. Compound 3 presented moderate antifungal activity against *T. longifusus* (Table 3).

Compd.	i		ii		iii		iv		v		vi	
	a (mean ± S.E.M)	b	a (mean± S.E.M)	b	a	b	a (mean ± S.E.M)	b	a (mean ± S.E.M)	b	a (mean ± S.E.M)	b
1	20.45±0.89	1.3	-	-	-	-	12.67±0.68	17	-	-	9.32±2.99	13
2	12.56±1.34	8	-	-	-	-	21.30±0.80	2.1	16.45±1.77	2.4	7.81±0.88	28
3	9.78±0.99	34	-	-	-	-	-	-	15.71±0.56	7.6	-	-
imp	24.90±2.21	0.19	23.44±0.77	0.24	28	0.13	27.64±0.99	0.17	20.24±2.52	0.21	26.67±0.35	0.18

i) *Escherichia coli* (ATCC 25922); ii) *Bacillus subtilus* (ATCC 6633); iii) *Shigella flexenari* (clinical isolate);iv) *Staphylococcus aureus* (ATCC 25923); v) *Pseudomonas aeruginosa* (ATCC 27853); vi) *Salmonella typhi* (ATCC 19430); a) zone of inhibition (mm); b) MIC (µg/ml); Imi: Imipenum

Table 2: Antibacterial activity of Compounds 1-3

Compd.	i		ii		iii		iv		v		vi	
	a (mean ± S.E.M)	b	a (mean± S.E.M)	b	a	b	a (mean ± S.E.M)	b	a (mean ± S.E.M)	b	a (mean ± S.E.M)	b
1	73.40±0.80	3.4	-	-	-	-	23.60±2.64	37	-	-	-	-
2	32.50±1.30	-	-	-	-	-	81.0±1.66	2.7	-	-	-	-
3	51.53±2.55	17	-	-	-	-	-	-	-	-	-	-
Mic	13.20±1.20	1.4	29.98±0.67	1.8	ND	ND	29.12±1.66	1.8	25	2	26.67±0.35	2
Amp	ND	ND	ND	ND	12.50±0.90	1.5	ND	ND	ND	ND	ND	ND

i) *Trichophyton longifusus* (clinical isolate); ii) *Candida albicans* (ATCC 2091); iii) *Aspergillus flavus* (ATCC 32611); iv) *Mycosporumcanis* (ATCC 11622); v) *Fusarium solani* (ATCC 11712); vi) *Candida glaberata* (ATCC 90030); a) % Inhibition ; b) MIC (µg/ml); Mic: Miconazole; Amp: Amphotericin-B; ND: not done

Table 3: Antifungal activity of Compounds 1-3

Experimental

General Experimental

Optical rotations were measured on a JASCO DIP 360 polarimeter. IR spectra were recorded on a JASCO 302-A spectrophotometer. EI-MS and HREI-MS were recorded on JMS HX 110 with data system and on JMS-DA 500 mass spectrometers. The ^{1}H and ^{13}C NMR spectrums were recorded on Bruker NMR spectrometers operating at 400 MHz, (100 and 125 MHz for ^{13}C). The chemical shifts values are reported in ppm (δ) units and the coupling constants (*J*) are given in Hz.

Chromatographic conditions

For TLC, precoated aluminium sheets (silica gel 60F-254, E. Merck) were used. Visualization of the TLC plates was achieved under UV at 254 and 366 nm and by spraying with Dragendorff's reagent. Solvent system; "n-hexane-acetone-diethylamine 8:2:10 drops", was used.

Plant Material

The roots (3 kg, dry wt) of *Aconitum heterophyllum* Wall. (Ranunculaceae), were collected from Swat, Khyber Pakhtunkhwa, Pakistan, at an elevation of 2000 m in August 2005 and identified by Prof. Mehboob ur Rahman, Department of Botany, Jahanzeb Post Graduate College, Saidu Sharif, Swat, Khyber Pakhtunkhwa, Pakistan. The voucher specimen (HA-014) is deposited in the herbarium of the botany department.

Extraction and Isolation

Roots (3 Kg) of *A. heterophyllum* were made into small pieces and then grinded through a common grinder machine and then subjected to cold-extraction with n-hexane (3 x 8 L) followed by extraction with 80% EtOH (3 x 10 L) at room temperature for 7 days (3-times). The filtrate was evaporated in *vacuo* to yield 60 g of residue. The residue was acidified to pH 1.5 by 0.5 NH_2SO_4 and extracted with CH_2Cl_2 (3 x 2 L) collected alkaloidal mixture (18 g). The acidic aqueous solution was basified (pH 8-10) by using 10 % KOH and extracted with CH_2Cl_2 (5 x 2 L) to yield 13.8 g of crude basic fraction. The crude basic fraction was further fractionated

on silica gel 60 F_{254} columns (260 g) and five combined fractions were obtained. On repeated flash column chromatography using solvent system *n*-hexane-acetone-diethylamine (9:1:10 drops per 100 ml) Heterophylline-A (1), Heterophylline-B (2), along with a known alkaloid condelphine were obtained.

Heterophylline-A (1): White amorphous powder (8 mg), mp 168-170 °C; [α] ^{30}D - 28.1o (c 1.0, MeOH); IR υ_{max} $CHCl_3$, 3492 (OH groups), 1700 (carbonyl), 1600, 1280, 1250, and 750 cm^{-1} (aromatic ring), 1083 (simple ether bonds); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.56 (1H, *br. s*, H-14), 3.67 (1H, *t*, *J* = 6.0 Hz, H-1), 3.40 (1H, *t*, *J* = 8.4 Hz, H-16), 2.55 (1H, *br. s*, H-17), 2.63 (1H, *m*, H-13), 2.50 (1H, *m*, H-9), 3.30 (1H, *br*, *s*, H-7), 1.78 (1H, *br. s*, H-5), 2.32 (1H, *m*, H-10), 5.64 (2H, *br. s*, NH_2), 6.84, 7.10, 6.70 and 7.38 (each 1H, aromatic proton), 1.07 (3H, *t*, *J* = 7.0 Hz, NCH_2CH_3) and ^{13}C-NMR (Table 1). EI-Ms *m/z:* 496 $[M]^+$, 359 [M – aminobenzoate] (6.5), 330 (78.0), 315 (4.2), 284.9 (5.4), 104 (11.8), 90.9 (18.4), 58 (100). HREI-MS (M^+ *m/z*, 496.6382).

Heterophylline-B (2): White amorphous powder (11 mg), mp 145-147 °C; [α] ^{30}D - 48 (c 0.2, $CHCl_3$); IR υ_{max} CHCl3, 3492 (OH groups), 1089 (simple ether bonds); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.78 (1H, d, J = 5.38 Hz, H-6), 4.67 (1H, t, J = 6.32 Hz, H-1), 4.22 (1H, t, J= 4.7 Hz, H-14), 4.28 (1H, d, J = 5.9 Hz, H-15), 3.67 (1H, m, H-3), 3.12 (1H, t, J = 8.1 Hz, H-16), 3.61 (1H, br. S, H-17), 3.34 (3H, s, OCH_3), 3.29 (3H, s, OCH_3), 3.47 (3H, s, OCH_3), 3.65 (3H, s, OCH_3), 1.07 (3H, t, J = 7.0 Hz, NCH_2CH_3); ^{3}C-NMR (Table-1). EI-MS *m/z*: 499 $[M]^+$ (3.2), 467 [M – OCH_3] (100), 449 (14.7), 432 (4.3), 100.9 (14.1), 84.8 (32.2), 58 (47). HREI-MS (M^+ m/z, 499.5942).

Antibacterial Activity

Antibacterial activity was performed on six bacteria reference strains. These were *E. coli* ATCC 25922, *B. subtilis* ATCC 6633, S. flexeneri (clinical isolate), *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *S. typhi* ATCC 19430. These were maintained on an agar slant at 4 °C and were activated at 37 °C for 24 h on nutrient agar (NA) prior to screening. Anti-bacterial activity was carried out by Agar well diffusion method [19]. The drug *Imipenem,* a broad-spectrum *β*-lactam antibiotic, was used as a positive control (standard drug). As a negative control, DMSO was used. Each assay was repeated three times and then means diameter was calculated. The results of these experiments are summarized in Table 2.

Antifungal Activity

Antifungal screening was performed on six fungi reference strains. Fungal strains include *T. longifusus* (clinical isolate), *C. albicans* ATCC 2091, *A. flavus* ATCC 32611, M. canis ATCC 11622, *F. solani* 11712 and *C. glaberata* ATCC 90030. They were maintained on an agar slant at 4 °C. The strains were activated at 37 °C for 24 h on Sabouraud Dextrose Agar (SDA) prior to screening. Agar tube dilution protocol was followed for antifungal activity [20]. Amphotericin-B is used as standard drug for *A. flavus* while miconazole was used as standard drug for rest of fungal strains. The results of these experiments are summarized in Table 3.

Acknowledgment

Higher Education Commission, of Pakistan is hereby acknowledge for providing financial support under the NRPU research project no 203536.

References

1. Ma CM, Kully M, Khan JK, Hattori M, Daneshtalab M (2007) Synthesis of chlorogenic acid derivatives with promising antifungal activity. Bioorg Med Chem 15: 6830-3.

2. Cowan MM (1999) Plant products as antimicrobial agents. Clin Microbiol Rev 12: 564-82.

3. Benn MH, Jacyno JM (1984) In Alkaloids: Chemical and biological perspectives. Wiley, New York, USA 1.

4. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342: 1007-11.

5. Jacyno JM, Harwood JS, Lin NH, Campbell JE, Sullivan JP, et al. (1996) Lycaconitine revisited: partial synthesis and neuronal nicotinic acetylcholine receptor affinities. J Nat Prod 59: 707-9.

6. Bisset NG (1981) Arrow poisons in China. Part II. Aconitum--botany, chemistry, and pharmacology. J Ethnopharmacol 4: 247-336.

7. Mhaskar KS, Blatter E, Caius JF, Vaidya Asha Ram (2000) Indian medicinal plants: their usage in Ayurveda and Unani Medicines 1: 28.

8. T Baytop (1984) Therapy with Medicinal Plants in Turkey. Istanbul University Press, Istanbul.

9. Chen M, Przyborowski M, Berthiaume F (2009) Stem cells for skin tissue engineering and wound healing. Crit Rev Biomed Eng 37: 399-421.

10. Pelletier SW, Aneja R, Gopinath KW (1968) The alkaloids of aconitum heterophyllum wall.: Isolation and characterization. Phytochemistry 7: 625-35.

11. Jacobs WA, Craig LC (1942) The aconite alkaloids Ix. The isolation of two new alkaloids from Aconitum heterophyllum, heteratisine and hetisine. 143: 605-609.

12. Ahmad M, Ahmad W, Ahmad M, Zeeshan M, Obaidullah, et al. (2008) Norditerpenoid alkaloids from the roots of Aconitum heterophyllum Wall with antibacterial activity. J Enzyme Inhib Med Chem 23: 1018-22.

13. Nisar M, Obaidullah, Ahmad M, Wadood N, Lodhi MA, et al. (2009) New diterpenoid alkaloids from Aconitum heterophyllum Wall: Selective butyrylcholinestrase inhibitors. J Enzyme Inhib Med Chem 24: 47-51.

14. Sultankhodzhaev MN, Yunusov MS, Younusov SY (1973) Khim Prir Soedin 19: 157.

15. Yang TR, Hao XJ, Chow J (1979) Acta Botanica Yunnanica 1: 41-5.

16. Nishanov A, Tashkhodzhaev B, Usupova I, Sultankhodzhaev MN (1992) Alkaloids of Aconitum kirinense. Structure of akirine. 28: 466-9.`

17. Berghe VA, Vlietinck AJ (1991) Screening methods for antibacterial and antiviral agents from higher plants. Method Plant Biochem 6: 47-68.

18. Tereschuk ML, Riera MV, Castro GR, Abdala LR (1997) Antimicrobial activity of flavonoids from leaves of Tagetes minuta. J Ethnopharmacol 56: 227-32.

19. Atta-ur-Rahman M, Thomson W (1999) Manual of bioassay techniques for natural product research.

20. Paxton JD (1991) Assay for antifungal activity In: Methods in Plant Biochemistry. Academic Press: London, UK.