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NOTE

Chemical constituents of *Anisodus luridus* roots and their antimicrobial screening

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Abstract : The weak base and strong base fractions were separated from methanolic extract of roots of *Anisodus luridus*. Chromatographic resolution of both fractions yielded compounds 1-5 which were characterized as β-sitosterol (1), esculetin (2), β-sitosterol-β-D-glucoside (3), apohyoscyamine (4) and hyoscyamine (5) with the help of spectroscopic analysis. Four compounds, 1-4 were isolated from *A. luridus* for the first time. The weak base, strong base and isolated constituents were screened against six microbes viz. *E. coli*, *S. aureus*, *E. faecalis*, *C. albicans*, *C. tropicalis* and *C. krusei*. Weak base, strong base and isolated pure compounds exhibited their potential against these microbes.

Keywords : *Anisodus luridus*, esculetin, hyoscyamine, apohyoscyamine, antimicrobial.

Introduction

Anisodus luridus (Link et Otto) is found mainly in the Himalayan region (Nepal, Bhutan, India and China) at the altitude range of 3200 to 4200 metres[1]. Flowers of *A. luridus* (AL) have been traditionally used with milk to cure cold and cough. Crushed dried flowers are mixed with tobacco and smoked to cure gingivitis and toothache. Seeds of this plant are used to eliminate toothache. The seeds are ignited and a pungent hot smoke is inhaled to treat wounds inside the nose[2]. Roots and seeds are also traditionally used for plague. Seeds are also used as anthelmintic, in sinusitis and colic pain[3].

A. luridus roots are rich in alkaloids. Only few alkaloids hyoscyamine, cuskohygrine and scopolamine have been isolated previously[4,5]. Tropane alkaloids such as hyoscyamine and scopolamine are widely used as anticholinergic agents that act on the parasympathetic nervous system[6]. However, phytochemistry of this plant is not much studied because of its geographical distribution (Himalayan range).

The present study is aimed at the isolation, characterization and antimicrobial screening of major constituents of *A. luridus* roots. This is the first report of the antibacterial and antifungal activity of AL roots.

Results and discussion

Methanolic extract of roots of *A. luridus* was prepared and separated into weak base (WB) and strong base (SB) fraction. Five compounds were isolated from these two fractions. These were identified as β-sitosterol (**1**), esculetin (**2**), β-sitosterol-β-D-glucoside (**3**), apohyoscyamine (**4**) and hyoscyamine (**5**). Structures of these compounds **1-5** were established with the help of their spectroscopic analysis (UV, IR, MS, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY and HMBC). The spectral data of the compounds were also compared to those reported in literature[7–11]. The important HMBC correlations of apohyoscyamine are indicated in Fig. 1.

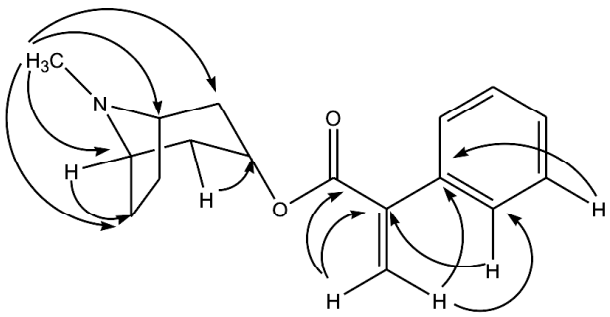


Fig. 1. Important HMBC correlations in apohyoscyamine (**4**).

WB, SB and isolated compounds (except **4**, due to paucity of sample) were tested for their antimicrobial activity and results are expressed as zone of inhibition (Table 1). WB exhibited strong antimicrobial activity (7–16 mm) against all the microbes tested and maximum efficacy was observed against *S. aureus* and *C. krusie*. were not quite effective at low concentrations as the MIC varied from 3.125–25.0 mg/mL. However, compound **1** and **5** were found to be inhibitory even at 0.312 mg/mL against *S. aureus* and *E. coli* respectively. Compound **2** was found to be effective at 0.625 mg/mL against both *S. aureus* and *E. faecalis*, however it showed MIC 1.25

Table 1. Antimicrobial activity (zone of inhibition) of weak base (WB), strong base (SB) and isolated compounds from roots of *A. luridus* against different bacterial and fungal strains

	Antimicrobial activity expressed as zone of inhibition					
	Bacterial Strains			Fungal Strains		
Fractions/ Constituents	*E. coli* (ATCC 35218)	*S. aureus* (ATCC 25323)	*E. faecalis* (Clinical isolate)	*C. albicans* (ATCC 90028)	*C. tropicalis* (ATCC 750)	*C. krusei* (ATCC 6258)
WB (100 mg/mL)	10.20 ± 0.25	15.40 ± 1.17	07.17 ± 0.24	10.18 ± 0.44	11.18 ± 0.16	12.56 ± 0.34
SB (100 mg/mL)	08.45 ± 1.15	17.38 ± 0.76	–	09.34 ± 0.52	10.30 ± 1.15	12.12 ± 0.19
1 (5 mg/mL)	10.9 ± 0.54	11.17 ± 0.45	10.17 ± 0.28	9.10 ± 0.46	–	–
2 (5 mg/mL)	–	10.95 ± 0.62	9.40 ± 0.31	10.46 ± 0.64	9.25 ± 0.43	9.85 ± 0.38
3 (5 mg/mL)	9.09 ± 0.38	–	–	–	–	–
5 (5 mg/mL)	11.33 ± 0.57	–	–	11.32 ± 0.15	–	10.55 ± 0.17
Ciprofloxacin (20 µg/disc)	33.46 ± 0.80	30.93 ± 0.49	28.86 ± 0.46	–	–	–
Amphotericin B (5 µg/disc)	–	–	–	15.66 ± 0.55	16.80 ± 0.55	15.93 ± 0.75
DMSO (Solvent)	–	–	–	–	–	–

SB showed the antimicrobial activity (8–18 mm) against all the microbes used except *E. faecalis* and optimum effect was found against *S. aureus* and *C. krusie* as zone of inhibition was calculated 17.38 mm and 12.12 mm respectively. Compound **1** was found to be effective against all the bacterial strains and only one *Candida* strain whereas compound **2** was found to be effective against all the microbes used except *E. coli*. Compounds **3** exhibited antimicrobial activity only against *E. coli*. Compound **5** was found to be effective against *E. coli, C. albicans* and *C. krusei* exhibiting the zone of inhibition 11.33, 11.32 and 10.55 mm respectively.

The MIC (minimum inhibitory concentration) values were also calculated for WB, SB as well as isolated pure compounds (Table 2) which suggest that base fractions mg/mL against *Candida* strains. Compound **3** exhibited MIC 1.25 mg/mL against *E. coli* only.

Experimental

General experimental procedures :

Column chromatography was carried out over silica gel (60–120 mesh; Merck) and silica gel G (Merck) for separation and purification. Thin layer chromatography (TLC) plates were prepared using silica gel G (Merck). Spots on TLC were monitored by spraying Dragendorff's, Liebermann-Burchardt and phosphomolybdic acid reagents. Melting points were obtained with digital melting point apparatus Innovative™ DTC-72 and are uncorrected. IR spectra were measured with a Perkin-Elmer spectrophotometer. ^{1}H, ^{13}C and 2D NMR were measured with a

Table 2. Antimicrobial activity (MIC, mg/mL) of weak base (WB), strong base (SB) and isolated compounds from roots of *A. luridus* against different bacterial and fungal strains

	Antimicrobial activity expressed as zone of inhibition					
	Bacterial strains			Fungal strains		
Fractions/ Constituents	*E. coli* (ATCC 35218)	*S. aureus* (ATCC 25323)	*E. faecalis* (Clinical isolate)	*C. albicans* (ATCC 90028)	*C. tropicalis* (ATCC 750)	*C. krusei* (ATCC 6258)
WB	12.5	3.125	12.5	12.5	12.5	6.25
SB	25	3.125	–	25	12.5	12.5
1	0.625	0.312	0.625	2.5	–	–
2	-	0.625	0.625	1.25	1.25	1.25
3	1.25	–	–	–	–	–
5	0.312	–	–	0.625	-	0.625
Ciprofloxacin (20 μg/disc)	6.25	6.25	3.2	–	–	–
Amphotericin B (5 μg/disc)	–	–	–	0.5	0.5	0.5
DMSO (Solvent)	–	–	–	–	–	–

Bruker DRX500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C); in $CDCl_3$; δ (H) in ppm, rel. to Me_4Si, *J* in Hz; δ (C) in ppm referenced to the solvent $CDCl_3$.

Plant material :

The roots of *A. luridus* (Link et Otto) were collected from the Mustang district of Nepal in October, 2011. Plant specimen was identified by Professor S. D. Dubey, Department of Dravyaguna, Banaras Hindu University, Varanasi, India and a sample is kept in the department.

Extraction and isolation :

The roots of *A. luridus* (6.2 kg) were dried, coarsely powdered and defatted with *n*-hexane. Defatted plant material was subjected to methanolic extraction (8 × 6 L, 36 h each) using Soxhlet apparatus. The solvent was evaporated to dryness and methanolic extract (753 g) was obtained. The methanolic extract was treated with 7% aq. citric acid and stirred for 24 h. Then, it was extracted with chloroform to obtain weak base fraction (20 g). The aqueous extract was basified with NH_4OH and extracted with chloroform to obtain strong base fraction (354 g). WB (20 g) was subjected to column chromatography (silica gel 60–120 mesh). Column was eluted (flow rate 5 mL/min) using different solvents in order of increasing polarity viz. *n*-hexane (50 mL each, 0.4 L), benzene (400 mL each, 2.4 L), chloroform (400 mL each, 2.4 L), ethyl acetate (one fraction of 50 mL and 3 fractions of 400 mL each, 1.25 L) and methanol (50 mL each, 0.2 L). *n*-Hexane and methanol fractions were discarded. Fractions 10–12 eluted in benzene exhibited the homogeneity on TLC plate and yielded compound **1** (12 mg) which was crystallized in $CHCl_3$: MeOH (1 : 3). Fractions 16–18 eluted in chloroform yielded compound **2** (110 mg). Fractions 21–22 eluted in EtOAc yielded compound **3** (2 mg). SB was subjected to column chromatography using silica gel G. Eluents were used in order of their increasing polarity viz. chloroform and/or methanol. Fractions 3–4 eluted from chloroform : methanol (99 : 1) showed two spots on TLC. Since, one spot is more intense than the other; sample was concentrated and kept in $CHCl_3$: MeOH (3 : 1) for crystallization. After crystallization, compound **4** (4.5 mg) was obtained in pure state. Fractions 14–16 yielded another compound **5** (140 mg) which was crystallized from benzene. The compounds **1**-**5** exhibited single spots on TLC.

Screening of antimicrobial activity :

Test microorganism :

A total of three bacterial strains viz. *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 25323), *Enterococcus faecalis* (clinical isolate) and three fungal strains viz. *Candida albicans* (ATCC 90028), *Candida tropicalis* (ATCC 750) and *Candida krusie* (ATCC 6258) were used for antimicrobial assay of WB, SB and isolated pure compounds. All cultures were obtained from American Type Culture Collection (ATCC) and preserved in

the Microbiology Department of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. The fresh bacterial/fungal broth cultures were prepared in normal saline before the screening procedure.

Antimicrobial susceptibility test :

Muller Hinton agar (MHA)/potato dextrose agar (PDA) plates were prepared by pouring 15 mL of molten media into sterile petri-plates. MHA and PDA plates were used for antibacterial and antifungal assay respectively. The paper disc diffusion method was used for antimicrobial activity[12]. Briefly, 1.0 mL of an 18 h culture of bacteria/fungi was adjusted to 0.5 McFarland standards in sterile saline to achieve a concentration of 10^7 CFU/mL. This suspension was spread over the surface of MHA/PDA agar plates. The WB and SB were used in a concentration of 100 mg/mL and isolated compounds (except compound **4**, due to paucity of the sample) were used in a concentration of 5 mg/mL. These dilutions were put on different 6 mm sterile disc of Whatman filter paper no. 1. The disc was then placed on the surface of medium and the base fractions and isolated compounds were allowed to diffuse for 5 min and then plates were kept for incubation at 37 °C for 24 h. Standard disc (diameter : 6 mm) of a Ciprofloxacin (20 μg/disc), a broad spectrum antibiotic, was used in antibacterial study whereas amphotericin B (5 μg/disc) was used as standard in antifungal study to compare the results. At the end of incubation, inhibition zone was examined around the disc which, if present, was measured with transparent ruler in millimeters. This study was performed in triplicate.

Determination of minimum inhibitory concentration (MIC) :

MIC is defined as the lowest concentration of the compound which inhibits the visible growth of microorganism[13]. Micro-dilution method was used in determination of MIC using serially diluted (2 folds) base fractions according to the National Committee for Clinical Laboratory Standards (NCCLS)[14]. Different concentrations of the bases (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL... etc.) and isolated pure compounds (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL... etc.) were serially diluted in micro-titre plates. Specifically, 0.1 mL of standardized inoculum (1–2 × 10^7 CFU/mL) was added in each tube along with liquid media. The micro-titre plates were incubated aerobically at 37 °C for 24 h for bacterial culture while fungal inoculum was incubated at 25 °C for 34 h. The lowest concentration (the highest dilution) of the base or pure compound was regarded as MIC at which no visible bacterial growth (no turbidity) was observed when compared with the control.

Statistical analysis :

Data analysis for antimicrobial activity was carried out and results are expressed as the mean ± S.E.M. of 3 independent experiments.

Esculetin **(2)** : $C_{10}H_8O_4$, white feathery crystals (MeOH), m.p. 197–200 °C; UV (λ_{max}) : 345, 298, 253, 228; IR spectrum (KBr, ν cm^{-1}) : 3337 (OH), 1703 (C=O); ^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.60 (1H, d, *J* 9.45 Hz, H-4), 6.91 (1H, s, H-8), 6.84 (1H, s, H-5), 6.26 (1H, d, *J* 9.45 Hz, H-3), 3.95 (3H, s, -OC*H*$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) : δ 161.44 (C-2), 150.26 (C-6), 149.73 (C-9), 144.03 (C-7), 143.29 (C-4), 113.38 (C-3), 111.49 (C-10), 107.51 (C-5), 103.18 (C-8), 56.41 (-O*C*H$_3$).

Apohyoscyamine **(4)** : $C_{17}H_{21}NO_2$, whitish orange crystals ($CHCl_3$), m.p. 60–62 °C; ^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.38–7.32 (5H, m, Ar-H), 6.38 (1H, d, *J* 1.0 Hz, H-10a), 5.89 (1H, d, *J* 1.0 Hz, H-10b), 5.28 (1H, t, H-3), 3.67 (1H, s, H-5), 3.04 (1H, br s, H-1), 2.69 (3H, s, -NC*H*$_3$), 2.08–1.99 (8H, br m, 4 × CH_2); ^{13}C NMR (125 MHz, $CDCl_3$) : δ 165.2 (C-8), 141.4 (C-9), 136.6 (C-11), 128.3 (C-12, C-13, C-15, C-16), 127.8 (C-10, C-14), 65.3 (C-3), 61.8 (C-5), 34.3 (C-2), 24.4 (C-6).

Hyoscyamine **(5)** : $C_{17}H_{23}NO_3$, colourless crystals (C_6H_6), m.p. 104–106 °C; UV (λ_{max}) : 258, 220; IR spectrum (KBr, ν cm^{-1}) : 3085, 2943, 2834 (-OH), 1724 (ester carbonyl), 1601 (aromatic); ^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.34 (2H, m, Ar-H), 7.29 (1H, m, Ar-H), 7.27 (2H, m, Ar-H), 5.03 (1H, t, H-3), 4.17 (1H, dd, *J* 8.6, 10.8 Hz, H-10a), 3.82 (1H, dd, *J* 5.3, 10.8 Hz, H-10b), 3.79 (1H, dd, *J* 5.3, 8.6 Hz, H-9), 3.08 (1H, t, H-5), 2.97 (1H, t, H-1), 2.23 (3H, s, -NC*H*$_3$), 2.2–1.18 (8H, br m, 4 × C*H*$_2$); ^{13}C NMR (125 MHz, $CDCl_3$) : δ 172.2 (C-8), 135.7 (C-11), 128.9 (C-12, C-16), 128.1 (C-13, C-15), 127.7 (C-14), 67.9 (C-3), 64.1 (C-10), 59.7 (C-5), 59.6 (C-1), 54.4 (C-9), 40.0 (C-17), 36.1 (C-4), 35.9 (C-2), 25.3 (C-7), 24.8 (C-6).

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References

1. M. B. Gewali, "Aspects of traditional medicine in Nepal", Institute of Natural Medicine, University of Toyama, Japan, 2008, 77.
2. S. K. Ghimire, I. B. Sapkota, B. R. Oli and R. R. Parajuli, "Non-timber forest products of Nepal Himalaya", 1st ed., WWF, Nepal, 2008, 142.
3. U. Quattrocchi, "CRC World Dictionary of medicinal and poisonous plants : common names, scientific names, eponyms, synonyms and etymology", 1st ed., CRC Press, United States, 2012.
4. K. Jovankovics, *Herb. Hung.*, 1966, **5**, 41.
5. M. S. Rabinovich and R. A. Konovalova, *Zhurnal Obshchei Khimii*, 1946, **16**, 2121.
6. G. Y. Kai, L. Li, Y. Jiang, X. Yan, Y. Zhang, X. Lu and P. Liao, *Biotechnol. Appl. Biochem.*, 2009, **54**, 177.
7. P. Ghosh, A. Ghosh, P. Chakraborty and B. Saha, *J. Indian Chem. Soc.*, 2014, **91**, 309.
8. X. Zhang, P. Geoffroy, M. Miesch, D. J. David, F. Raul, D. A. Werner and E. Marchioni, *Steroids,* 2005, **70,** 886.
9. N. B. Begmatov, A. Yili, K. A. Eshbakova and H. A. Aisa, *Chem. Nat. Compd.*, 2014, **50**, 541.
10. F. S. Sariaslani and J. P. Rosazza, *Appl. Environ. Microbiol.*, 1983, **46**, 468.
11. A. El-Shazly, A. Tei, L. Witte, M. El-Domiaty and M. Wink, *Z. Naturforsch.*, 1997, **52c**, 729.
12. M. Gangwar, D. Kumar, R. Tilak, T. D. Singh, S. K. Singh, R. K. Goel and G. Nath, *J. Pharm. Res.*, 2011, **4**, 4214.
13. I. Wiegand, K. Hilpert and R. E. W. Hancock, *Nat. Protoc.,* 2008, **3**, 163.
14. Wayne Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically : approved standards, 5th ed., NCCLS document M7-A5. National Committee for Clinical Laboratory Standards (NCCLS), PA, USA, 2000.