

An Assessment of Ethnomedicinal Use, Chemical Constituents Analysis and Bioactivity Evaluation on High Altitude Medicinal Plant *Delphinium brunonianum* of Manang District

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Abstract

The medicinal plant *Delphinium brunonianum* collected from Manang district, Nepal has been selected for its study on ethnomedicinal uses, phytochemical investigation and bioactivity evaluation. This plant is found to be used extensively by the community for the treatment of fever, headache, stomachache and poison removal. From phytochemical investigation, four known compounds namely β -amyrin (**1**), β -sitosterol (**2**), β -sitosterol glucoside (**3**) and anthriscifoldine (**4**) have been isolated from methanol extract of *D. brunonianum*. Compound **1** is triterpene, **2** and **3** are sterols, whereas, **4** is a diterpenoid alkaloid. The structures of all compounds were determined with modern spectroscopic techniques including 1D- and 2D- Nuclear Magnetic Resonance techniques and comparison with literature data. The extract and all compounds exhibited antibacterial properties with respect to the zone of inhibition and minimum inhibitory concentration against *Bacillus subtilis*, *staphylococcus aureus*, *Pseudomonas aureginous*, *Escherchia coli* and *Salmonella flexinarie*. Compound **4** was found to be more active than other compounds, and even than the standards ampicillin and gentamicin in most of the tested bacteria.

Key words: *Delphinium brunonianum*, phytochemical investigation, spectroscopic tools, antibacterial property, zone of inhibition

Introduction

Plants have been used as medicines from time immemorial. Early men relied on plants for food, medicine, clothing and shelter. Natural products, since the very beginning, have served as a template for the development of many important classes of drugs (Swain, 1972). Plants are remarkable in their ability to produce a vast array of diverse metabolites varying in structural complexity and biological activity. Plants continue to retain their historical significance as an important source of novel compounds, useful directly as medicinal agents, as

model compounds for synthetic or semi-synthetic structure modifications and optimization, and as biochemical and pharmacological probes (Balandrin *et al.* 1993). Today, natural products and their derivatives still represent over 50% of all drugs in clinical use, with higher plant-derived natural products representing *ca.* 25% of the total. It has been reported that about a quarter of all the prescription drugs from community pharmacies in the world still contain plant extracts or active principles of plant origin (Rahman and Choudhary, 2001). Most of the wild floras of Nepal are rich in medicinal and aromatic properties like antibacterial,

antiviral, antihelminthic, anticancer, sedative, laxative, cardiotoxic, diuretic and others. They are important sources of bio-molecules, with application for the manufacture of pharmaceuticals and cosmeceuticals (Heinrich & Gibbons 2001).

Delphinium is a genus of about 300 species of perennial flowering plants. Several phytochemical studies have been conducted in the genus *Delphinium* and more than 200 diterpenoid alkaloidal compounds have so far been isolated. Several new C(19)-diterpenoid alkaloids such as anthriscifolcines A-C, nudicaulamine, delbruninol, blacknine, winkleriline etc. and C(18)-diterpenoid alkaloids such as anthriscifolcines F and G etc. were obtained from different *Delphinium* species (Song et al., 2009). Their structures were elucidated based on the interpretation of NMR and high-resolution ESI MS data, and chemical transformation

A large number of high altitude medicinal plants are available at Manang district, out of which *Delphinium brunonianum* is one of the most popularly used herb for the treatment of different ailments. This herb is not only popular among the communities of the district, but also have great demand throughout Nepal and have good international market as well. It is distributed in Pamir, Afghanistan, the Himalayan region (Kashmir to Nepal) and South East Tibet at the altitude range of 3500 to 6000 m (Ghimire 1999). Exploration of ethnomedicinal uses, chemical constituent analysis and biological activity on such important medicinal herb in institutional level is very important.

Evaluation of biological activity on medicinal plants have important role on discovery of rational drugs. Based upon the traditional uses of the plant and its utilization pattern by the communities, different biological assays used to be conducted to justify the ethnomedicinal uses by scientific investigation. Out of several bench-top bioassays, antibacterial activity evaluation is one of the important assays which deal about the ability of medicinal plant to inhibit different bacteria. Based upon the ethnomedicinal uses of *D. brunonianum* by the communities of Manang on different bacterial infections and availability of bacteria on Western Regional Hospital (WRH), Pokhara, some common pathogenic bacteria such as *Escherchia coli*, *staphylococcus aureus*,

Pseudomonas aureginous, *Salmonella flexinarie* and *Bacillus subtilis* were used to test the antimicrobial activity of *D. brunonianum*.

Methodology

General site survey

All the *D. brunonianum* richness area of Manang district was identified through the help of official records of DFO, Manang and reconnaissance survey of concern VDCs of the district. From the general site survey, Alukharka of Dharapani VDC which is rich in *D. brunonianum* was selected.

Social data collection

For social data collection, semi-structured questionnaire household survey and published and unpublished secondary data was applied to gather the indigenous knowledge of *D. brunonianum* in the selected sites.

Collection of plant material, shade drying and grinding

The rhizome of *D. brunonianum* was collected during the field visit of Manang District. It was chopped in to small pieces and shade dried until the moisture content removed from the pieces. It was then ground into fine powder for extraction.

Preparation of herbarium

Herbarium specimen was prepared following standard botanical procedure for *D. brunonianum*. The herbarium was identified by Prof. Dr. Krishna Kumar Shrestha, Department Head, Central Department of Botany, Kirtipur and deposited at the same Department.

General experimental conditions

Melting points were determined on a Yanaco MP-S3 micro melting point apparatus and are uncorrected. Specific rotation $[\alpha]_D$ (CHCl₃, *c* in g/mL) was determined by using a JASCO digital polarimeter (model DIP-3600). Infrared spectra were recorded on a JASCO FT/IR-410 spectrophotometer. UV spectra were measured on a Spectronic Unicam spectrophotometer. HRESI MS were recorded on a APEX III (Bruker Daltonik) 7 Tesla (ESI-FT-ICR-MS). EI-MS spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorokerosene as the reference substance for

HREI MS. The ^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively, on a Bruker AMX 500 NMR spectrometer. Methyl, methylene and methine carbons were distinguished by DEPT experiments. Chemical shifts were reported in δ (ppm) using TMS as internal standard and coupling constants (J) were measured in Hz.

Column chromatography was performed on a column (silica gel 60, 70-230 and 240-300 mesh sizes, E. Merck). Pre-coated silica gel TLC plates (E. Merck, F₂₅₄) were used to check the purity of compounds. TLC plates were viewed under ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Ceric sulfate and Dragendorff's spraying reagents were used for the staining of non alkaloidal and alkaloidal compounds, respectively on TLC.

Extraction, fractionation and isolation of compounds

The powdered rhizome (500 g) was extracted with methanol by using the Soxhlet apparatus. The extract was concentrated under vacuum by using rotatory evaporator to obtain the crude extract (56 g), which was then dissolved in methanol (Scheme-1). The methanol soluble fraction (53.0 g) was subjected to a column chromatography on silica gel and eluted with a gradient system of pet. ether, chloroform and methanol, which yielded eight major fractions (**S1-S8**).

The fraction **S4** (2.2 g), obtained on elution with 40% chloroform/pet. ether was loaded on to a flash silica gel column. It was eluted with 35% chloroform/65% pet. ether to yield two major sub-fractions (**1S4**, and **2S4**). Elution of the sub-fraction **2S4** (250.5 mg) with 40% dichloromethane/60% pet. ether yielded compound **1** (60.5 mg). The fraction **S7** (1.8 g) on elution with 5% methanol/95% chloroform on a flash column chromatography over silica gel yielded compounds **2** (40.2 mg) and **3** (20.2 mg). Similarly, the fraction **S6** (1.2 g) when eluted with 1% methanol/99% chloroform on a flash chromatography over silica gel yielded compound **4** (17.2 mg).

β -Amyrin (1)

Colorless needle shaped crystals, *R_f*: 0.44 (CHCl₃/pet. ether in 60:40), M. P.: 188-190° C [Reported 193-195° C], $[\alpha]_{\text{D}25}$: 103° (*c* = 0.06, MeOH) [Reported 88° (CHCl₃)], IR (CHCl₃) ν_{max} cm⁻¹: 3150 (OH), 1663 (C=C), EI MS *m/z* (rel. int. %): 426 (M⁺, 63), 411 (16), 408 (6), 393 (8), 218 (100), 207 (16), 203 (32), 191 (17), 189 (59), 175 (15), 161 (11), 135 (30), 133 (15), 123 (17), 121 (27), 119 (14), HREI MS *m/z* :

426.3854 (calcd for C₃₀H₅₀O, 426.3861), $^1\text{H-NMR}$, δ (CDCl₃, 500 Hz): 0.74 (3H, s, Me-24), 0.77 (3H, s, Me-28), 0.83 (3H, s, Me-25), 0.92 (3H, s, Me-23), 0.95 (6H, s, Me-29, Me-30), 0.97 (3H, s, Me-26), 1.02 (3H, s, Me-27), 3.22 (1H, m, H-3), 5.24 (1H, br d, *J*_{12,11} = 6.3 Hz, H-12).

β -Sitosterol (2)

White amorphous solid, *R_f*: 0.53 (MeOH/ CHCl₃ in 8:92), M. P.: 142-144° C [Reported 142-144° C], IR (CHCl₃) ν_{max} cm⁻¹: 3402 (OH), 2901 (CH), EI MS *m/z* (rel. int. %): 414 (M⁺, 100), 399 (15), 329 (19), 303 (925), 273 (20), 213 (18), 95 (21), HREI MS: *m/z* 414.3857 (calcd for C₂₉H₅₀O, 414.3850), $^1\text{H-NMR}$, δ (500 MHz, CDCl₃): 0.65 (3H, s, Me-18), 0.79 (3H, d, *J*_{27,25} = 6.8 Hz, Me-27), 0.82 (3H, t, *J*_{29,28} = 7.0 Hz, Me-29), 0.83 (3H, d, *J*_{26,25} = 6.8 Hz, Me-26), 0.90 (3H, d, *J*_{21,20} = 6.2 Hz, Me-21), 0.98 (3H, s, Me-19), 3.51 (1H, m, H-3), 5.33 (1H, m, H-6).

β -Sitosterol glucoside (3)

Colorless needle shaped crystals, *R_f*: 0.48 (MeOH/ CHCl₃ in 12:88), M. P.: 273-274° C [Reported 270-272° C], IR (CHCl₃) ν_{max} cm⁻¹: 3400 (OH), 2893 (CH), 1630 (C=C), EI MS *m/z* (rel. int. %): 414 (M⁺, 100), 399 (15), 329 (19), 303 (25), 273 (20), 213 (18), 95 (21), FAB MS: *m/z* 573 (M-H)⁺, $^1\text{H-NMR}$, δ (500 MHz, CDCl₃+MeOD): 0.67 (3H, d, *J*_{27,25} = 6.4 Hz, Me-27), 0.69 (3H, t, *J*_{29,28} = 6.6 Hz, Me-29), 0.71 (3H, s, Me-18), 0.73 (3H, d, *J*_{26,25} = 6.8 Hz, Me-26), 0.80 (3H, d, *J*_{21,20} = 6.3 Hz, Me-21), 0.87 (3H, s, Me-19), 3.43 (1H, m, H-3), 4.28 (d, *J*_{1',2'} = 7.7 Hz, H-1'), 5.24 (1H, m, H-6).

Anthriscifoldine C (4)

White amorphous powder, *R_f*: 0.48 (MeOH/CHCl₃/DEA in 12:87:1), IR (KBr) ν_{max} cm⁻¹: 3387, 2872, 1740, 1245, 1087, HR ESI MS *m/z*: 492.2940 [M+H]⁺, Calcd for C₂₇H₄₂NO₇, 492.2961, $^1\text{H-NMR}$, δ (500 MHz, CDCl₃): 1.04 (3H, t, *J* = 7.2 Hz, H-22), 2.09 (3H, s, OAc), 3.13, 3.07 (ABq, *J* = 8.8 Hz, H-18), 3.36, 3.26, 3.25 (each 3H, s, 3 x OCH₃), 4.85 (1H, t, *J* = 4.8 Hz, H-14b), 5.01, 4.94 (each 1H, s, OCH₂O), $^{13}\text{C-NMR}$, δ (125 MHz, CDCl₃): 83.5 (C-1), 26.1 (C-2), 31.9 (C-3), 38.0 (C-4), 43.3 (C-5), 31.8 (C-6), 91.2 (C-7), 80.8 (C-8), 47.1 (C-9), 36.6 (C-10), 50.7 (C-11), 27.2 (C-12), 44.0 (C-13), 75.2 (C-14), 33.1 (C-15), 81.7 (C-16), 62.0 (C-17), 78.8 (C-18), 52.1 (C-19), 50.7 (C-21), 14.2 (C-22), 55.7 (1-OCH₃), 56.3 (16-OCH₃),

59.4 (18-OCH₃), 92.9 (OCH₂O), 171.9 (C=O of OAc), 21.2 (CH₃ of OAc).

Antibacterial assay

Antimicrobial assay of extracts, fractions, sub-fractions and compounds was performed on different microbes such as *Escherichia coli*, *staphylococcus aureus*, *Pseudomonas aureginous*, *Salmonella flexinarie* and *Bacillus subtilis* with respect to the measurement of Zone of Inhibition (ZOI) and Minimum Inhibitory Concentration (MIC).

The ZOI and MIC values determination was performed as explained by Devkota et al. (2000) and Devkota and Dutta (2001). Briefly, ZOI was measured by agar well diffusion technique in which 6 mm diameter well was prepared in Muller Hinton Agar (MHA) plate containing inoculums of different bacteria. Appropriate concentration of extracts along with the standard drugs were added to the well and incubated at 37°C for 24 hours. The ZOI was the area on the plates where the growth of bacteria is inhibited by the plant extracts. Similarly, MIC value was determined by two fold serial dilution technique and incubated at 37°C for 24 hours. The incubated material was then be swabbed to the MHA plates and result observed after 24 hours incubation at 37°C.

Comparison with the action of antibiotics

Whether the antimicrobial activity of medicinal plant is strong, moderate or weak can be find out by comparison between the effect of the plant extract or compounds and the standard drug on particular bacteria. For this, plant extract, compound and standard drug were tested for their zone of inhibition, to find out whether their antimicrobial activities were strong, weak or medium. The antibiotics selected during this study were ampicillin and gentamycin.

Results and Discussion

Ethnomedicinal uses of *D. brunonianum*

The utilizing pattern of *D. brunonianum* by the community of Dharapani VDC of Manang district was acquired by using house hold survey and key informant interview. From the study, *D. brunonianum* was found to be popularly used by the community for the treatment of fever, headache, stomachache, cough and removal of poison. In case of fever and cough, the dry and powdered rhizome is taken with water, a teaspoonful twice in a day after taking food. When a person is suffering from stomachache, headache and poison, either the fresh

rhizomes are chewed twice to thrice in a day or taken its powder by mixing with sugar and water twice in a day for 3-4 days.

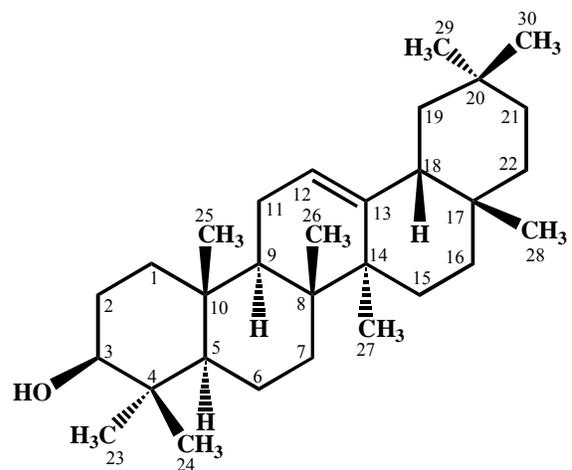
The community of the study area was found to be happy to have different medicinal plants in their area and are able to cure diseases by using those plants. Previously, most of the community members weret involved to collect those valuable plants and marketed. But, now a days they are not involving in such activities, however, according to them, many people from other districts such as Gorkha, Lamjung and Kaski involved to collect those medicinal plants in illegal way. The community want to give message to policy makers of Governmental and Non-governmental organization that there should be some program related to conservation of such valuable medicinal plants in their natural habitat.

Phytochemical investigation of *D. brunonianum*

The phytochemical investigation on secondary metabolites from the fractions of *D. brunonianum* yielded following four known compounds 1-4.

β-Amyrin (1)

Compound 1 was obtained as colorless needle shaped crystals. The HREI MS showed the M⁺ peak at *m/z* 426.3854 supporting the formula C₃₀H₅₀O (calcd 426.3861). The IR spectrum indicated the presence of hydroxyl (3150 cm⁻¹) and olefinic (1663 cm⁻¹) functionalities in compound 1.



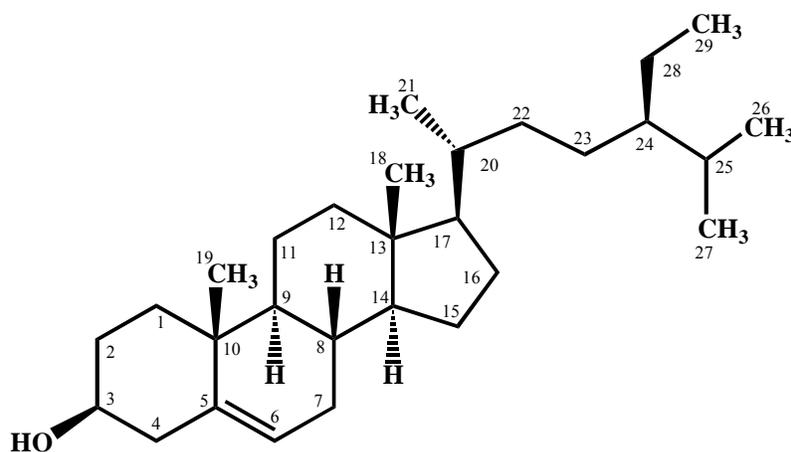
β-Amyrin (1)

The ¹H-NMR spectrum of compound **1** showed upfield signals for eight tertiary methyl groups, characteristic of a triterpene skeleton (Bahuguna and Jangwan, 1987). A multiplet at δ 3.22 was assigned to the C-3 proton geminal to a hydroxyl group, while a broad doublet resonating at δ 5.24 ($J_{12,11} = 6.3$ Hz) was due to the C-12 olefinic proton. The EI MS fragmentation was characteristic of an Δ_{12} -unsaturated oleanane type triterpenes, largely resulting from a retro-Diels-Alder's cleavage (Budzikiewicz *et al.*, 1963).

The above spectral data was in agreement with the reported values for β -amyrin (Ulubelen and Topcu, 1987). This is the first report of its isolation from this plant, although this compound was previously obtained from several other plant species (Ahmad and Rahman, 1994).

β -Sitosterol (2)

Compound **2** was isolated as a white amorphous material. The HREI MS showed the molecular ion at m/z 414.3857 in agreement with the formula $C_{29}H_{50}O$ (calcd 414.3850) corresponding to five degrees of unsaturation.



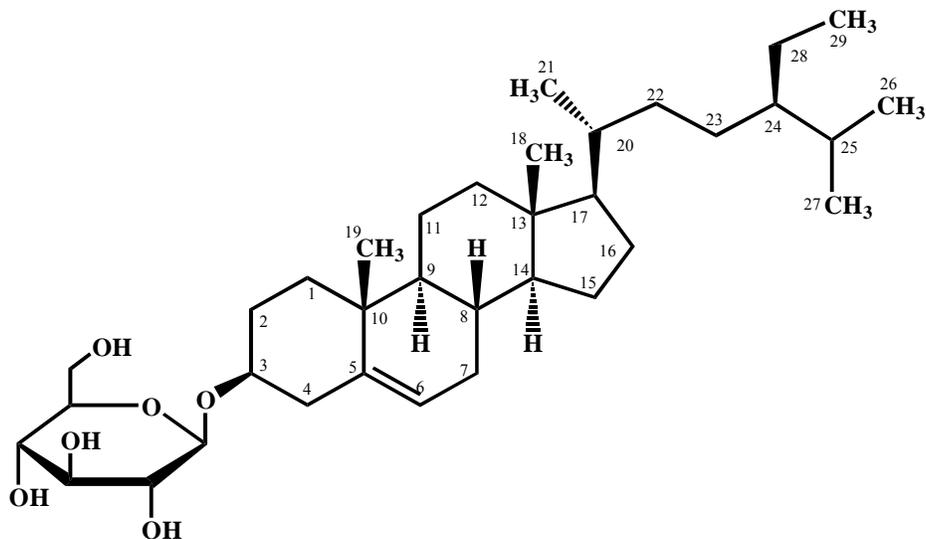
β -Sitosterol (2)

The ¹H-NMR spectrum of compound **2** was characteristic of a steroidal system. Two singlets resonating at δ 0.65 and 0.98 were due to the C-18 and C-19 quaternary methyl protons, respectively. Three doublets appearing at δ 0.90 (d, $J_{21,20} = 6.2$ Hz), 0.83 (d, $J_{26,25} = 6.8$ Hz) and 0.79 (d, $J_{27,25} = 6.8$ Hz) were due to C-21, C-26, and C-27 secondary methyl protons, respectively. A triplet at δ 0.82 ($J_{29,28} = 7.0$ Hz) was due to the primary C-29 methyl protons. A multiplet resonating at δ 3.51 was consistent with the H-3 α , geminal to the 3 β -hydroxy functionality. A multiplet at δ 5.33 was assigned to the C-6 olefinic proton. A comparative study of its spectroscopy data with the literature revealed that compound **2** is the

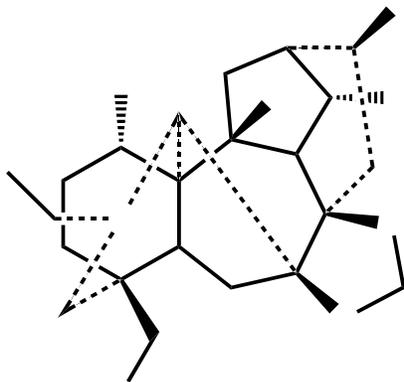
well known phytosterol β -sitosterol, previously isolated from a large number of plant species (Heble, 1967).

β -Sitosterol glucoside (3)

Compound **3** was isolated as a crystalline solid by column chromatography of the sub-fraction **S7**. The FAB MS (-ve) of **3** showed the molecular ion at m/z 573. A comparative study of the spectroscopic data of compound **3** with the reported data revealed it to be β -sitosterol glucoside (Monaco and Previtera, 1984) which was further confirmed by TLC comparison with authentic sample.

 β -Sitosterol glucoside (3)***Anthriscifoline C (4)***

Compound **4** was isolated as a white amorphous material. The HR ESI MS showed the molecular ion at m/z 492.2940 $[M+H]^+$ in agreement with the formula $C_{27}H_{42}NO_7$ (calcd 492.2961).



The detail study of 1H -NMR spectra in $CDCl_3$ indicated compound **4** as a lycotonine-type C19-diterpenoid alkaloid. Its 1H - and ^{13}C -NMR data showed distinctive signals at δ_H 1.02 (3H, t, $J = 7.2$

Hz) and δ_C 14.2 for an *N*-ethyl group; δ_H 3.36, 3.26, 3.25 (each 3H, s) for three methoxy groups; δ_H 4.93, 5.01 (each 1H, s) and δ_C 92.9 for a methylenedioxy group; and δ_H 2.09 (3H, s) with δ_C 21.2 q, 171.9 s for an acetoxy group. A comparative study of its spectroscopy data with the literature revealed that compound **4** is the well known compound, previously isolated from *Delphenium* species (Song *et al.* 2009) is the first report from *D. brunonianum*.

Antibacterial activity evaluation of *D. brunonianum*

During the research period, the antibacterial activity on crude extracts and compounds **1-4** that has been obtained from *D. brunonianum* was determined by measuring ZOI and MIC determination. The test organisms were *B. subtilis*, *S. aureus*, *P. aereginosa*, *E. coli* and *S. flexinarie*. The ZOI was measured by well diffusion method whereas MIC was determined by two fold serial dilution technique. The result of ZOI measurement is presented in Table 2. The extract and compounds displayed moderate to good antibacterial properties on the tested bacteria in which compound **4** showed comparatively higher activity than other compounds. The higher activity of compound **4** might be due to its alkaloidal nature as alkaloids shows diverse biological activities.

Table 2. ZOI of crude extract and compounds **1-4** from *D. brunonianum*

Extracts, fractions and compounds ^a	ZOI (mm)				
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>P. aureginosa</i>	<i>E. coli</i>	<i>S. flexinariae</i>
Crude extract	12.5	11.2	15.3	16.7	18.8
1	10.1	9.7	7.4	9.2	8.7
2	9.7	8.5	8.8	9.4	13.1
3	8.5	12.3	10.6	9.5	10.1
4	24.0	21.2	18.4	23.4	24.2
Ampicillin ^b	29.0	26.0	n.t.	n.t.	n.t.
Gentamycin ^b	n.t.	n.t.	29.0	31.0	27.0

^aTested at 100.0 mg/mL for extracts and fractions and 5.0 mg/mL for pure compounds.

^bStandard drugs in this assay.

n.t.: not tested

The MIC value (Table 3) of compounds **1-3** was found to be less than standard drugs, whereas, compound **4** exhibited higher MIC value than the standard ampicillin on *B. subtilis* and *S. aureus*. Similarly, compound **4** also showed higher MIC

values than the standard gentamicin against *E. coli* and *S. flexinariae*. The higher activity of compound **4** might be due to its alkaloidal nature as alkaloids shows diverse biological activities.

Table 3. Minimum inhibitory concentration values of compounds **1-4** from *D. brunonianum*

Compounds	MIC (µM)				
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>P. aureginosa</i>	<i>E. coli</i>	<i>S. flexinariae</i>
1	405.4	405.4	308.4	512.4	424.5
2	588.2	348.2	548.2	886.2	548.2
3	446.8	358.4	446.8	646.4	358.4
4	64.4	24.4	24.4	80.2	31.0
ampicillin ^a	89.7	5.6	n.t. ^b	179.4	89.7
gentamicin ^a	n.t. ^b	n.t. ^b	5.2	n.t. ^b	n.t. ^b

^aPositive controls used in the assay

^bn.t. = not tested

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