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RESEARCH ARTICLE

Cytotoxicity of Compounds Isolated from *Usnea aciculifera*

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Abstract

Atranorin **1**, Usnic acid **2** and D-Arabinitol **3** were isolated from *Usnea aciculifera* by extensive column chromatography. The structures were elucidated based on the comprehensive spectral analysis including FT IR, GC MS, ESI MS, ^{13}C NMR and ^{1}H NMR. The compounds were evaluated for their cytotoxic activity against LN-229 glioblastoma cancer cell line by performing Sulforhodamine B assay (SRB). Usnic acid **2** showed strong cytotoxic activity against LN-229 glioblastoma cancer cell with an IC_{50} value of 3.09 µg/mL.

Keywords: *Usnea aciculifera,* LN-229 glioblastoma cancer cell, Sulforhodamine B assay, Atranorin, Usnic acid and D-Arabinitol

Introduction

Lichens are the peculiar growths that develop when two unlike organisms come together in a mutualistic close association. Lichens are the combination of two organisms, an algae and a fungus, living together in symbiotic association. Lichenologists have estimated that about 2,000 lichen species are found in Nepal [1]. A total of 792 species belonging to 187 genera of lichen have been reported from Nepal, of which 55 species are endemic [2]. Among them *Usnea* commonly known as old man beard is grown all over the world and 23 species of *Usnea* are reported from Nepal [3]. *Usnea aciculifera* is greenish fruticose lichen found on the barks of the trees. The distinguishing features of *U. aciculifera* are the erect to subpendent thallus with isotomic-dichotomous branching, the continuous or irregulary cracked base of thallus, the presence of punctiform soralia and is idiomorphs on the surface of branches, the uninflated branches with a thick axis and compact medulla, the absence of papillae, the absence of soredia, the ceratina type plectenchymatous cortex[4, 5]. In Nepal, lichen is mainly used for medicinal, ritual and spiritual, food, aesthetic and decorative, bedding and ethno-veterinary value. As per ancient records and recent scientific literature, the species of genus *Usnea* have been used as promising traditional medicines, exerting an array of therapeutic properties to relieve sore throats, bronchitis, cold, flu, infection, and indigestion [6]. *Usnea* has also been used as dyes, cosmetics, preservatives, and deodorants. Traditionally in China, *U. aciculifera* was used to treat bladder infection, painful urination, urinary retention, swelling and edema in the heart and kidneys [8].

The chemistry of *Usnea* has been important for researcher because of its wide range of medicinally important secondary metabolites. Compounds

identified from *Usnea* species belong to different class of depsidones, depsides, depsones, lactones, quinines, polyphenolic, polysaccharides, fattyacids, sterols and dibenzofurans [7]. Depsides, benzofuran and depsidones have been isolated from *U. aciculifera* [8]. Herein we reported the isolation of Atranorin **1**, Usnic acid **2** and D-Arabinitol **3** from *U. aciculifera* and its anticancer properties.

Materials and Methods

Chemicals and equipments

The solvents for chromatographic techniques were used after distilling the commercially available solvents. HPLC grade solvents (Fisher Scientific) were used for HPLC purification of compounds. ^{1}H and ^{13}C NMR spectral analysis were carried out in Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. ESI Mass spectra were carried out in Thermo Fisher FINNIGAN LTQ\ESI-LR\BY HQP. ^{13}C and ^{1}H NMR were carried out in Bruker Advance 500. IR Tracer 100 shimazdu was used for IR spectroscopy. Cary 60UV/Vis Agilent spectrometer was used for UV spectroscopy. The agilent 5975 C Mass and 7890 A gas chromatography were used. The column used was HP-5 MS (5% phenylmethylsiloxane 30mX 250µmX 0.25µm). Column chromatography was performed using silica gel (60-120) mesh and Sephadex LH-20 (Sigma). TLC was performed with pre-coated silica gel G-25-UV_{254} plates (E. Merck, Darmstadt, Germany) and detection of compounds were done at UV at 254 and 366 nm, as well as spraying with ceric sulphate sulphuric acid reagent. For purification, the preparative HPLC (Labo ACE LC -5060, Japan Analytical Industry Co. Ltd) with JAIGEL-ODS-AP column was used.

Plant materials

The lichen *Usnea aciculifera* was collected from, Panauti Municipality, Kavre district of Nepal at an altitude of 1900 m, in September and November 2017. The lichens were identified by Central Department of Botany, Tribhuvan University, Kirtipur. *U.* a*ciculifera* was collected by plucking them from the bark of the trees especially from firs. The whole plants were dried in shade for about 15 days and they were grinded into small pieces.

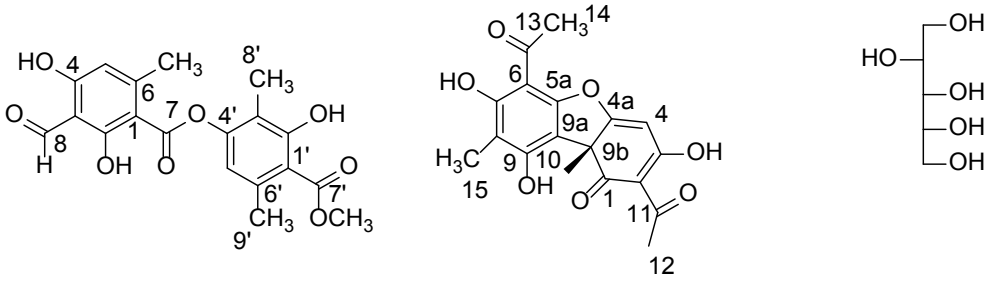
Extraction and isolation

The air dried powder plant material 0.71 kg *Usnea* was extracted sequentially with hexane, acetone and methanol by cold percolation method. Hexane extract (3.18 gm), acetone extract (72.69 gm) and methanol extract (68.56 gm) were obtained after evaporation of solvents in rota evaporator.

The hexane extract (3.18 g) was subjected to silica gel column chromatography. The column was subsequently eluted with *n*-hexane and then gradually with increasing the polarity with acetone. From the column chromatography seven different fractions (I-VII) were obtained after analyzing TLC. From initial fractions (I-III) (polarity between 5-10% acetone in n-hexane) on further silica gel column chromatography, Atranorin **1** was isolated.

The acetone extract (72.68 g) was again dissolved in acetone and filtered by suction pump. The white precipitate (35.18 g) obtained was subjected to silica gel column eluted with n-hexane ethyl acetate solvent system. On gradual increase of polarity in column by adding ethyl acetate, thirteen fractions (I- XIII) were separated after TLC analysis. From fraction II (polarity 40% ethyl acetate in n-hexane) on further silica gel column chromatography, Usnic acid **2** was isolated.

Methanol extract (68.56 g) was subjected to silica gel column chromatography. The column was gradually eluted by dicholoromethane and polarity was increased by adding methanol in the solvent system. From the column, ten fractions (I-X) were compiled after analyzing TLC spots. From fraction VIII (50% methanol/ dichloromethane), brown color precipitated was obtained which was washed with acetone several times to obtain pure UV inactive crystalline compounds. From GC-MS analysis, the compound was confirmed to be D-Arabinitol **3**.



Atranorin **1** Usnic acid **2** D-Arabinitol **3**

Fig1 Compounds isolated from U. aciculifera

Physical and spectroscopic data

Atranorin

White crystalline, ESI MS molecular ion peak at *m/z* 374. ($C_{19}H_{18}O_8$), ^{1}H NMR 500 MHz, $CDCl_3$: δ_H 6.40 (1H, s, H-5), δ_H 10.36 (1H, s, H-8), δ_H 2.69 (3H, s, H-9), δ_H 6.52 (1H, s, H-5'), δ_H 2.54 (3H, s, H-8'), δ_H 2.09 (3H, s, H-9'), δ_H 3.99 (3H, s, 7'-OCH_3); ^{13}C NMR , 125 MHz, $CDCl_3$: 103.0 (C-1), 169.2 (C-2), 108.7 (C-3), 167.6 (C-4), 113.0 (C-5), 152.6 (C-6), 169.8 (C-7), 194.0 (C-8), 25.7 (C-9), 110.4 (C-1'), 163.0 (C-2'), 116.9 (C-3'), 152.1 (C-4'), 116.2 (C-5'), 140.0 (C-6'), 172.3 (C-7'), 9.5 (C-8'), 24.1 (C-9'), 52.5 (7'-OCH_3)

Usnic acid

Yellow crystalline, ESI MS molecular ion peak at *m/z* 344. ($C_{18}H_{16}O_7$), ^{1}H NMR 500 MHz, $CDCl_3$: δ_H 5.97 (1H, s, H-4), 1.75 (3H, s, H-10), 2.67 (3H, s, H-12), 2.66 (3H, s, H-14), 2.09 (3H, s, H-15) and δ_H 13.31, 11.03,(each 1H, s, Ar-OH) ^{13}C NMR 125 MHz, $CDCl_3$: 198.2 (C-1), 105.4 (C-2), 191.9 (C-3), 98.5 (C-4), 179.5 (C-4a), 155.4 (C-5a), 101.7 (C-6), 164.0 (C-7), 109.5 (C-8), 157.7 (C-9), 104.1 (C-9a), 59.2 (C-9b), 28.0 (C-10), 201.9 (C-11), 32.3 (C-12), 200.4 (C-13), 31.4 (C-14), 7.7 (C-15)

D-Arabinitol

Needle shaped brown crystalline, IR (KBr), ν_{max} cm^{-1} : 3381 (OH), 2939 (CH), $C_5H_{12}O_5$

Cytotoxcity activity

Cell culture maintenance

Firstly, LN-229 human brain glioblastoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10 %(v/v) fetal bovine serum, penicillin (50IU/mL) and streptomycin (50 µg/mL) at 37 ºC in a humidified environment (95% air; 5% CO_2). The cell lines were trypsinized and seeded ($5x10^3$ cells/ well) in 96-well cell culture plates and incubated for 24 hrs.

Assessment of cytotoxicity

After 24 hrs incubation, cells were exposed to the compounds dissolved in dimethyl sulfoxide (doses ranging from 6.25 to 50 µg/mL and in triplicate) for 48 hrs and cytotoxicity assessed by Sulforhodamine B (SRB) assay[9]. Concisely, treated cells were washed with phosphate buffer saline (PBS) five times, fixed with trichloroacetic acid (TCA) and then SRB (0.4%) was added to each well and incubated for 20 minutes. Unbound dye was removed by washing with acetic acid and bound dye was solubilized with Tris base (10 Mm; pH 7.5). Further plate shaker was used to shake the plates for 1 hr and finally, absorbance was taken at 540 nm using Synergy™ HT Multimode Micro-plate Reader (Bio Tek, USA).

Statistical analysis

All the experiments in this study were carried out at least three times in triplicate. Data were analyzed using Prism 5.0 (Graph Pad Prism) statistical software package and results (percentage cell viability, IC_{50}) were expressed as mean ± standard deviation (SD).

Results and Discussions

Structure elucidation

Three compounds were isolated from the hexane, acetone and methanol extracts after extensive column chromatography (data presented in experimental

section) Figure 1. Atranorin **1** belonging to depsides was isolated from the hexane extract. It was isolated as a white powder. The ESI MS showed molecular ion peak at m/z 374. The ^{1}H NMR spectrum of Atranorin displayed methyl singlets at δ_H2.09, δ_H2.54, δ_H2.69 and one methoxyl group at δ_H 3.98. The twelve downfield carbon signals at aromatic regions along with the downfield singlets of two aromatic protons at δ_H 6.40 and δ_H 6.51confirms the pentasubstituted two aromatic rings. The downfield quarternary carbon at δ_C 194.0 and highly dishielded proton at δ_H 10.35 showed the presence of aldehyde group. The presence of two carbonyl ester moieties were confirmed by downfield carbon signals at δ_C 169.8 and δ_C 172.3. From comparision of spectral data with reported data, the compound was identified as Atranorin **[**10**]**.

Usnic acid **2** a well known dibenzofuran compound from Usnea species and it was isolated as a yellow crystalline compound from the acetone extract during column chromatography. The ESI MS showed molecular ion peak at *m/z* 344. The ^{13}C NMR of compound **2** showed 18 carbon signals including 4 methyl, 1 methine and 13 quarternary carbons. The downfield carbon signals at δ_C 198.2 (C-1), 201.9 (C-11), 200.4 (C-13) were assigned to carbonyl carbons. The downfield signals at δ_H 5.87 correspond to vinylic proton at C-4. The two methyl singlets of ketones appeared at δ_H 2.67 and 2.66. Other methyl singlets appeared at δ_H 2.09 and 1.75. The carbon signals for aromatic carbon and conjugated unsaturated carbonyl carbon were also observed in downfield region. Comparing the spectral data with reported data, the compound is confirmed to be Usnic acid. [11]

D-Arabinitol **3** is a pentose sugar derivative isolated in good yield from the methanolic extract. The compound was needle crystalline and brown in color. It was UV inactive and after spraying ceric sulphate, dark spot was observed. The structure was confirmed by GC MS. The chromatogram showed one major broad peak at retention time 12.74. The IR spectrum showed broad absorption peak at 3381 cm^{-1} (OH). From comparision of mass fragmentation pattern with NIST library 2008, the compound was confirmed to be D-Arabinitol.

Cytotoxic activity assay

Cytotoxicity of the compound **1** and **2** was evaluated against LN-229 human brain glioblastoma cell line by Sulforhodamine B (SRB) assay. The cytotoxic activities of the two compounds on LN-229 human brain glioblastoma cell line have been summarized in the Figure 2a and 2b and graph is shown in Figure 3. Compounds with IC_{50} value < 5 µg/mL were considered to be potent cytotoxic, while those with IC_{50} value between 5 to 15 µg/mL were considered to be low/moderate and other compounds considered as non-cytotoxic at 48 hrs post-incubation. Usnic acid was found to be cytotoxic to LN-229 human brain glioblastoma cell line with an IC_{50} value of 3.09 µg/mL, however, Atranorin **1** were non-cytotoxic to the cell line with an IC_{50} value of 21.39 µg/mL.

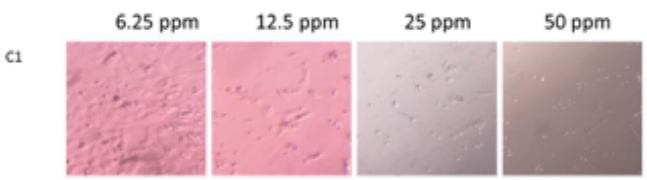


Figure 2b *Morphological changes of the treated cells with Usnic acid*

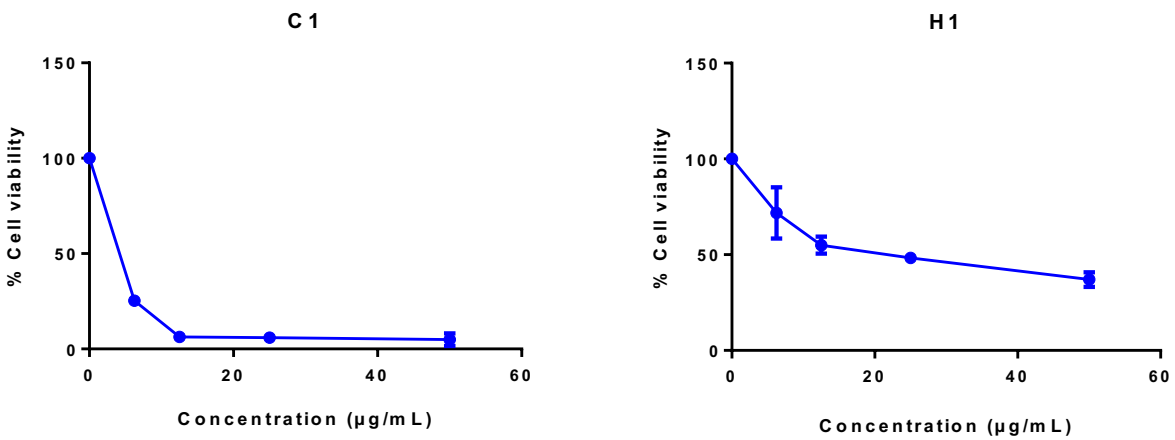


Figure 2a *% of Cell viability at different concentration*

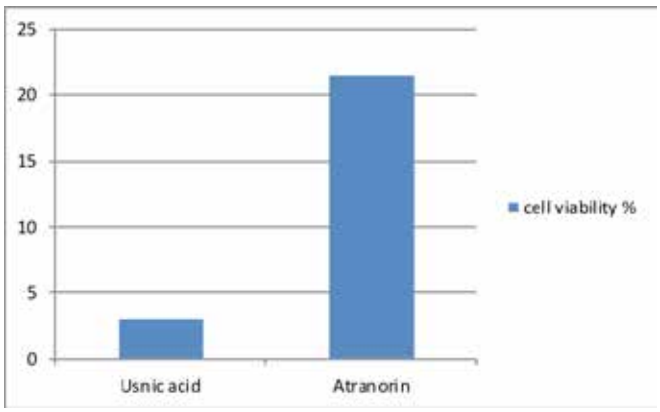


Figure 3. *IC_{50} value Usnic acid and Atranorin against Human brain glioblastoma cell line*

Conclusion

The structures of compounds (**1-3**) were elucidated by detail analysis of various spectroscopic techniques and comparing its spectral data in the literature. Compound **1** showed potent cytotoxicty on evaluating the cytotoxicity of the compound **1** and **2** in LN-229 human brain glioblastoma cell line. In brief, this study demonstrates the potential of *Usnea aciculifera* constituents as anti-cancer agents of plant origin.

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