

Antiviral Activity and Constituents of the Nepalese Medicinal Plant *Astilbe rivularis*

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Abstract: During the screening of Nepalese ethnomedicinal plants for antiviral activities, *Astilbe rivularis* BUCH.-HAM., Saxifragaceae, was identified as a promising species. Bioassay-guided fractionation led to the isolation of arbutin, bergenin and a bergenin derivative. The structures were established by NMR studies. Except bergenin, the two compounds were found in this plant for the first time. A dimer of bergenin has not been described as a natural product before. The compounds showed *in vitro* antiviral activity against herpes simplex virus type-1 in non cytotoxic concentrations.

Keywords: *Astilbe rivularis*; antiviral; arbutin; bergenin; dimer of bergenin.

1. Plant Source

Astilbe rivularis Buch.-Ham. ex D. Don (Saxifragaceae) locally known as THULO AUSADHI (in Nepalese language) or (in Gurung language, Manang district) is distributed throughout Nepal at an BHADHANGOO altitude between 2000-3600 meter on moist rocky hills. It is a shrubby plant propagated by rootstocks. The juice of the plant is applied traditionally to sprains and muscular

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swelling, the rhizome is valued in curing diarrhoea, dysentery, peptic ulcer, headache, hemorrhages, prolapse of uterus and to improve fertility [1].

The rhizomes of *Astilbe rivularis* were collected from Kavre district in December 2005 and authenticated by comparison with herbarium specimens at the Central Department of Botany, Tribhuvan University. A voucher specimen (Ar/Sax 005) is deposited at Research Center for Applied Science and Technology, RECAST, Tribhuvan University.

2. Previous Studies

Phytochemical investigation of *A. rivularis* revealed the presence of flavonoids, terpenoids and bergenin [2,3,4] but the active principles present in *Astilbe rivularis* are still unknown.

In our screening study, the methanol extract of the rhizome of *A. rivularis* showed potent anti-herpes viral activity with IC_{50} values of $<6.25 \mu\text{g/mL}$ without cytotoxicity to the Vero cells with CC_{50} values of $67 \mu\text{g/mL}$ [5].

3. Present Study

Extraction and purification: In an attempt to determine the compounds responsible for anti-herpes viral activity, a phytochemical investigation of this extract was initiated. The dried rhizome of *Astilbe rivularis* (1 kg) was powdered and extracted successively with hexane, dichloromethane and methanol in a soxhlet extractor. The methanol extract after removal of the solvent (300 g) was partitioned between ethylacetate and water to get respective extracts (EtOAc 70 g, $IC_{50} < 6.25 \mu\text{g/mL}$; water 180 g).

The active EtOAc fraction (25 g) was chromatographed over Sephadex LH 20 eluting with 50% aqueous ethanol. The fractions were combined according to their TLC profile into five pools A1 (8.30 g), A2 (7.74 g), A3 (4.22 g), A4 (2.74 g) and A5 (2.76 g). Finally the column was eluted with ethanol and acetone. The activity was accumulated in fractions A2 ($IC_{50} > 25 \mu\text{g/mL}$), A3 ($IC_{50} 12.5 \mu\text{g/mL}$) and A4 ($IC_{50} < 6.25 \mu\text{g/mL}$).

Fraction A3 (4 g) was further chromatographed on polyamide column eluting with water, 50% aqueous methanol and methanol. Altogether nine fractions were collected. Fraction A3-6 (375 mg, $IC_{50} < 12.5 \mu\text{g/mL}$) was further chromatographed on polyamide column eluting with 50% aqueous methanol. Three active fractions (A3-6-I, A3-6-II and A3-6-III, $IC_{50} 12.5 \mu\text{g/mL}$) were collected.

Fraction A3-6-III (50 mg) was purified by semi-preparative HPLC (HPLC system: Shimadzu system controller SCL-10Avp; column: Phenomenex C_6 -Phenyl) using an isocratic solvent of 80% water in CH_3CN to get compound **1** (6 mg, t_R 11 min) and a crude fraction (23 mg). The latter was further purified by HPLC using a gradient of 85% water in CH_3CN to 80% water in CH_3CN for 20 minute to collect compound **2** (2 mg t_R 14.0 min).

Fraction A3-6-II (30 mg) was purified by HPLC in an isocratic solvent system of 80% water in CH_3CN to isolate compound **3** (10 mg, t_R 14.7 min).

NMR spectra were recorded on an AVANCE-II spectrometer (Bruker Rheinstetten, Germany) at 600.27MHz ($^1\text{H-NMR}$) and 150.95MHz ($^{13}\text{C-NMR}$). Chemical shifts are given in ppm relative to the residual solvent signal of CD_3OD (^1H : 3.31ppm; ^{13}C : 49.15ppm). All spectra were recorded at a nominal temperature of 300K.

Mass spectral analysis was performed using a microTOF high resolution mass spectrometer (Bruker Daltonik Bremen, Germany). All spectra were recorded in negative ESI mode using nitrogen as dry and nebulizer gas. The capillary voltage was set to -150V and the hexapole RF to 150V. The TOF detector voltage was set to 2175V. The ESI source was operated at 180°C.

Compound **1** was identified as arbutin by comparison of spectral data with literature [6]. NMR spectra showed typical signals of a glucose moiety at 3.9, 3.7 and 3.4 ppm. The aromatic protons of the hydroquinone residue were found at 6.70 and 6.95 ppm. The mass spectrum showed at m/z 271.0804 the molecular mass of the (m-H)- peak corresponding to a molecular mass $C_{12}H_{16}O_7$. The spectra of compound **2** showed typical NMR spectral signals for bergenin [7, 8]. Compound **3** showed very similar signals to the NMR data of bergenin. One striking difference was the absence of the aromatic signal at 7.06 ppm. Other signals were almost not shifted compared to bergenin. The mass spectral analysis showed a negative ion at m/z 653.1327 corresponding to a molecular formula of $C_{28}H_{30}O_{18}$. By comparison with the literature data, compound **3** was identified as the dimer of bergenin [9]. This compound was previously described as a product of biotransformation via oxidation of bergenin by fungal enzymes.

Antiviral tests; Vero cells (cell bank of the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany) were used as host cells and maintained in MEM supplemented with 5% FCS (GIBCO, Paisley, UK). The exponentially growing cells were harvested and seeded at 60,000/well in a 96 well microtiter plate and incubated for 24 h at 37 °C with 5% carbondioxide in a humidified chamber so as to form confluent monolayers. Herpes simplex virus type 1 (HSV-1, strain KOS) was obtained from the strain collection of the Consiliar and Reference Center for Alpha Herpes Virus Infection, Institute of Virology and Antiviral Therapy, University Jena, Germany, and propagated in Vero cells. The virus infected cells were frozen and thawed and the virus suspension was titrated on Vero cells and stored at -70 °C. Before antiviral tests the cellular toxicity of test samples on Vero cells was assessed by dye uptake method using neutral red in 96-well tissue culture plates as described before [5].

Confluent monolayer of Vero cells were treated with 100 μ l two fold serial dilutions of test samples in four replicates for 30 minutes. After that Vero cells were infected with 30 TCID₅₀ of HSV-1 and incubated for 72 h at 37 °C. TCID₅₀ (tissue culture infectious dose) is the virus dose that leads to the infection of 50% of the cells. The virus suspension and dilution medium without samples were added, respectively, to the cell cultures to serve as the virus control and cell control. After incubation the supernatant was replaced by 200 μ L neutral red solution (0.005 %) and the cells were incubated for 3 h at 37 °C. After removal of the supernatant the dye incorporated by viable cells was eluted with 100 μ L ethanol / water / glacial acetic acid solution (50:50:1) by shaking for 15 minutes. The absorbance was measured at 540 nm and the percentage protection was calculated by the following formula:

$$(\text{OD}_T)_V - (\text{OD}_C)_V / (\text{OD}_C)_M - (\text{OD}_C)_V \times 100 (\%).$$

$(\text{OD}_T)_V$, $(\text{OD}_C)_V$ and $(\text{OD}_C)_M$ correspond to absorbance in virus infected cells with test compounds, virus infected cells without test compounds and the mock infected control (assay without viruses) respectively. Acyclovir was used as reference compound.

Among the isolated compounds bergenin had the highest antiviral activity with an IC₅₀ value of < 6.25 μ g/mL. The IC₅₀ values of arbutin and the dimer of bergenin were 100 and 25 μ g/mL respectively. The IC₅₀ of the reference compound acyclovir was 0.7 μ g/mL.

Arbutin was previously isolated from many Ericaceae but also from some Saxifragaceae, e.g. from the leaves and the rhizomes of *Bergenia* sp. [8]. Its antiviral activity is weak. But it is well known for its antibacterial activity possibly caused by the metabolite hydroquinone. Herbal drugs containing arbutin, e.g. prepared from the leaves of *Arctostaphylos uvae-ursi* (Ericaceae), are used for the treatment of urinary infections.

Bergenin occurs in several plants mainly belonging to Ericaceae and Saxifragaceae. Among the genus *Astilbe* it was isolated e.g. from the rhizomes of *Astilbe chinensis* (Maxim.) Franch. et Sav. [10] and *A. thunbergii* (Sieb. et Zucc.) Miq. [11]. It is known e.g. for its anti-tussive and anti-inflammatory [12] activities.

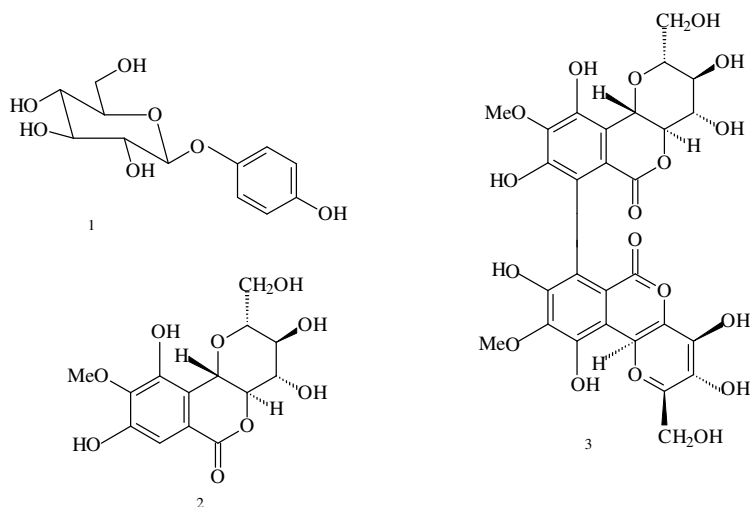


Figure 1. Compounds isolated from the rhizomes of *Astilbe rivularis*.

The dimer of bergenin was previously described only as a biotransformation product synthesized in the mycelium of the basidiomycete *Pleurotus ostreatus* after adding bergenin to the culture medium [9]. To the best of our knowledge this derivative was isolated for the first time as a natural product. It can not be excluded that oxidative reactions during the extraction process promote the dimerization of bergenin. Another possible source of compound **3** could be a biotransformation of bergenin by rhizome associated fungi. The chemical analysis of the other active fractions and subfractions is in progress. The results showed that the not often phytochemically investigated Nepalese plant *Astilbe rivularis* showed the presence of antiviral constituents. Thus, the ethnomedicinal use of *A. rivularis* is justified.

Acknowledgements

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