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Bioassay Guided Phyto-chemical Investigation of *Bergenia ciliata (Haw) Sternb*: A Rocky Himalayan Medicinal Plant of Nepal

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Abstract: *Bergenia ciliata (Haw) Sternb.* (Saxifragaceae) is a promising, but rare and vulnerable Himalayan medicinal herb of Nepal. It grows on moist crevices of rocks and bounders. Commonly, it is known as pashanved (pasan = rock stone, ved = piercing) or rock foil. It grows between the rocks possessing lithotropic property. A decoction of rhizomes and roots of this species is used in the Ayurveda, Unani and folk systems of medicine for the treatment of ulcers, fevers, tumors, eye sores and lungs, liver, heart, urinary diseases. Due to over-exploitation for medicinal value, it is in the process of extinction. So conservation by cultivation is highly recommended. The present study was carried out on bio-guided fractionation of rhizomes powder of *B. ciliata (Sternb)* which is known to possess several pharmacological properties. It afforded the bioactive natural product bergenin (Isocoumarin, 3%) from ethyl acetate fraction. In addition, other four compounds namely afzelechin (2.5%), leucocyanidin, β-sitosterol and β-sitosterol glycoside were isolated from ethyl acetate fraction. co-TLC/2D-TLC, HRMS, LC-MS, NMR, IR methods were employed to identify these compounds.

Keywords: *Bergenia ciliata,* Saxifragaceae, Rhizomes, Chemical Constituents, Bergenin, Afzelechin, Leucocyanidin, β-sitosterol Glycoside

1. Introduction

B. ciliata (Haw) sternb is an evergreen perennial herb growing to 0.31 meter (1 ft) - 1.0 meter (3.2 ft) by 0.5 meter (1.8ft) found in temperate Himalayas in range 400 to 4300 meter up to the frontier of vegetation. It is frost resisting and shade loving plant grows in extremely hard condition in moist stony slopes, debris, crevices, rock builders and forest shade. It is stunted creeping herb with a stout and cylindrical root stock called rhizomes (which are buff and brown outside, pinkish brown inside), stalked leaves, circular, obovate or elliptic 5-35 cm long freshly denticulate, bright green and densely ciliated at margins, sparsely hairy to glabrous on both surfaces. Its leaves are edible (pakora), used as tonic and also as plates in picnic parties and agricultural fields. It flowers and fruits in between February to August. Its beautiful pinkish white or purple flowers of cymose panicles and rounded capsules with entire bristly margins have ornamental (decorative) value as good luck (in Phool sangrum). This plant possesses urolithotropic, anti-calciphication, styptic, astriangent, anti-hepatotoxic, anti-inflammatory, anti-pyretic, antiviral, antimalarial, anti-hypertensive activity and cytoprotective effects [1], antibacterial, anti-stress, anticancer, anti-diabetic, analgesic and diuretic properties [2]. So it has strong abilities for curing respiratory-cough, pulmonary-heart, livers and urinary diseases alleviating bleeding, but intensifying immunity. Its rhizome (in the form of powder, paste or juice) is widely used in Ayurveda, Unani and other traditional/cultural system of medicines for renal disorders, oxidative stress, fever,

asthma and inflammation [3]. There is a good demand of rhizomes of this plant species in various pharmaceutical companies as it solubilizes stones situated around kidney, bladder and urinary tract [4]. Chemical constituents of this plant consists of many different active parameters like wax, gallicin, gallic acid, galloylepicatechin, tannins, tannic acid, mucilage, glycosides, catechin, flavonoids and other [5]. As there is no record of the research on this plant of Nepalese origin, the demonstrated significant medicinal properties prompted us to undertake phytochemical investigation. We here in report the isolation and structural elucidation of active principles.

2. Materials and Methods

The fresh rhizomes were randomly collected neglecting altitudinal variation from Dhaulagiri zone of 2000-4300m height during August, 2005. The voucher specimen BS-134 was authenticated at the national Herbarium and plant laboratory, Godawari, Nepal.

The air dried powdered rhizomes (1.0kg) of the plant were successively extracted with methanol (3× 1000ml) at 60°C using Soxhlet apparatus. The concentrated alcoholic extract (250gm) was diluted with cold distilled water (1000ml) and defatted with petroleum ether (1.5 × 1000ml) and petrol extract was concentrated to afford petrol extract (18gm). After defatting it was successively fractionated with chloroform (1.5 × 1000ml), ethyl acetate (1.5 × 1000ml) and methanol (1.5 × 1000ml). These extracts were concentrated under reduced pressure in Rota evaporator of Buchi type to yield chloroform extract (25gm), viscous ethyl acetate (100gm) and methanol (30gm). Brine shrimp bioassay procedure [6] and zone of inhibition test from agar cup plate method [7] (*Staphylococcus aureus and Salmonella enterica*) of these extracts were performed. Ethyl acetate extract (LC_{50} = 1500µg/ml, high inhibition rate) found more bio active so it was chosen and fractionated by column chromatography thereafter repeated column chromatography in different solvent system of increasing polarity using pressure as a positive force. TLC, co-TLC, 2D-TLC, Prep TLC were also done during collection and identification of eluents, finally five pure compounds were isolated.

Melting points were determined on hot stage apparatus using the capillary method and are uncorrected. IR Spectra were recorded in KBr discs and nujol on a Perkin-Elmer spectrophotometer. NMR Spectra were recorded in $CDCl_3$ at 300 MHz on a Brunker instrument using TMS as internal standard. NMR values are in δ scales. EIMS spectra were scanned at 70 ev on a Joel D-300 instrument. Silica gel (60-120 mesh) and silica gel G were used for performing column chromatography, TLC and Prep TLC respectively. These compounds were identified on the basis of the comparison of their physical and spectral data with literature values as well as their Mass, IR and NMR spectra. The purity of these compounds was checked on TLC Silica gel UV_{254} recoated plates. Final compounds/fractions were checked by using Agilent 1100 series LC-MS and a low-resonance electrospray model (ESI) with UV detection at 25nm. High resolution mass spectra (HRMS) were recorded on an Agilent ESI-TOF (time of flight) using ESI (electrospray ionization) mass spectrometer in positive-ion mode.

3. Results and Discussions

(2S, 3R, 4R, 4aS, 10bS)-3, 4, 8, 10-tetrahydroxy-2-(hydroxymethyl)-9-methoxy-3, 4, 4a, 10b-tetrahydropyrano [3, 2-c] isochromen-6(2H)-one, /Bergenin (Isocoumarin).

Brine shrimp bioactive ethyl acetate fraction on repeated column chromatography gave a white crystalline prismatic compound whose LC_{50} value was 83µg/ml. This compound can be considered as a potent bioactive in bioassay as evident from important therapeutic properties like anti-cancer, anti-inflammatory, anti-microbial, anti-oxidant, anti-arrhythmic, anti-arthritis, analgesic properties with the healing component of Alzheimer's disease[8].

Isolated yield was 3% (0.6% reported in *B. ligulata*). The structure was elucidated as below. The compound MP 137-139°C, R_f 0.25 (1: 9, MeOH: $CHCl_3$ v/v), HRMS (ESI) calculated for $C_{14}H_{17}O_9$ ($[M+H]^+$) 329.0828 and found: 329.0824 corresponding to the exact molecular formula. It gave the positive test of isocoumarin.

IR absorption at 3400 cm^{-1} and 1695 cm^{-1} were indicative of presence of respective –OH and COOR. The values at 1612, 1528, 1464 cm^{-1} are aromatic groups. NMR spectrum pattern was consistent with isocoumarin. The singlet at δ 3.89 was assigned as methoxy group attached to C-9. The singlet at δ 6.5 are exchangeable proton with D_2O corresponding to 3, 4, 8, 10 & 16 C-OH group as conformed by integration. Similarly the singlet at δ 7.65 was an aromatic proton. The doublet at δ 5.18 (J = 10.1 Hz) and triplet at δ 4.55 (J = 9.8 Hz) suggested 6a and 5a proton with their *trans* configuration. The respective triplet at δ 4.15 (J = 8.6 Hz) and δ 4.40 (J = 8.6 Hz) showed C-3 and C-4 protons were also in *trans* orientation. The multiplet at δ 2.84 showed C-2 proton. The two protons at δ 3.30- 3.45 were C-16 protons.^{13}C-NMR values ranging from δ 60.3 - 164.4 indicated the presence of 14 carbons. The value at δ 60.3 was methoxy group and δ 164.4 was assigned as ester (COOR) group. These spectral evidences suggested the compound to be isocoumarin. Furthermore the structure was supported by mass fragmentation pattern, 208, $C_{10}H_8O_5^{+\cdot}$ as base peak and others like 152, 165, 180, 195, 222, 237 to be consistent with the reported structural features. ^{1}H-^{1}H COSY, HMBC and HMQC were also utilized for identification. To further verify the structure, two derivatives were prepared. Simple acylation reaction in presence acetic anhydride and pyridine at 0°C for 16 hr provided the penta-O-acetyl bergenin as a white solid with HRMS calculated as 561.3523 while methylation in presence of methyl iodide and potassium carbonate resulted

tri-methoxy bergenin as a colored mass with HRMS found as 357.1137. Progress of the reaction was monitored via LC-MS. Therefore, the compound was identified as bergenin [9].

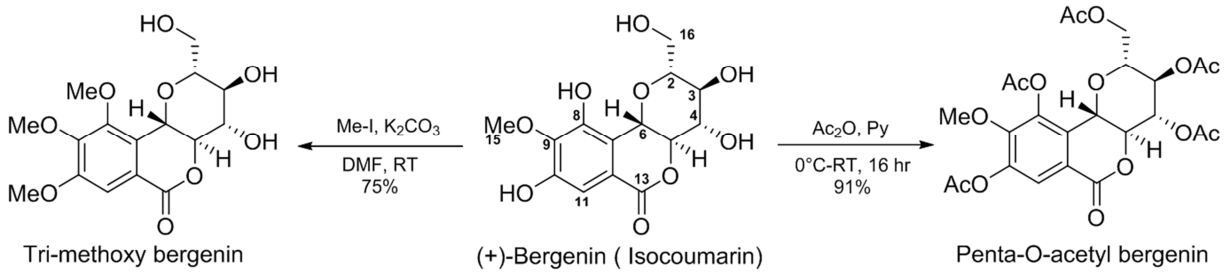

Figure 1. *Bergenin and its two different derivatives.*

2, 3 trans, 2R, 3S Flavan 3-OL, /(+) Afzelechin

It was isolated as pale crystalline solid with M P 220-222°C, R_f 0.32 (1: 9, MeOH: $CHCl_3$) and recrystallized from ethyl acetate. The yield was 2.5%. HRMS (ESI) calculated for flavan tetrol ($[M+H]^+$): 275.0875 and found 275.0879 corresponding to the molecular formula $C_{15}H_{14}O_5$. IR spectra on KBr showed phenolic –OH at 3410 cm^{-1} aromatic C = C at 1600 cm^{-1} & 1510 cm^{-1} and aromatic C-O at 1241 cm^{-1}. The ^{1}H-NMR was consistent with 3- flavanol, a catechin. The doublet at δ 7.31 (J = 8.5Hz) showed H-2' and 6'. Similar doublet at δ 6.77 (J = 8.5Hz) were H-3' & 5'. The doublet at δ 5.91 & δ 5.94 with (J = 2.4 Hz) and are 6-H & 8-H of long range coupling. The singlet at δ 4.86 was H-2 and multiplet at δ 4.17 was H-3. The doublet of doublet at δ 2.73-2.87 (J = 3.17Hz) was H-4. The mass fragmentation patterns were strongly supported the proposed skeleton. The ^{13}C-NMR showed the presence of 15 carbons in the molecule. Three different chemical derivatives were prepared utilizing enhanced directing effect of two phenolic –OH groups in electrophilic aromatic substitution. Bromo, Iodo, and formyl group were substituted at 7 position and characterized from NMR comparison, LC-MS analysis and HRMS of the final product (353.9925, 400.9835 and 303.0814) respectively. Thus, the compound was identified as (+)afzelechin [10].

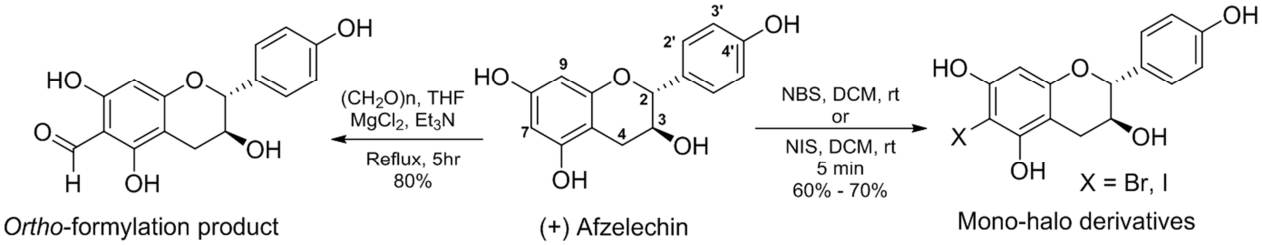

Figure 2. *Chemoselective formylation and halogenation of Afzelechin.*

2-(3, 4 –dihydroxyphenyl)-3, 4, 5, 7-chromane tetrol, Flavan 3, 4-diols, /Hexahydroxyflavan, (Leucocyanidin)

The compound was pale yellow amorphous powder with R_f 0.37 (1: 9, MeOH: $CHCl_3$). It gives the positive test of anthocyanidin. The yield was 130mg (0.0013%). HRMS (ESI) calculated for this cyanidin is ($[M+H]^+$): 307.0773 and found: 307.0769 corresponding to the molecular formula $C_{15}H_{14}O_7$. The IR spectra showed the presence of –OH at 3410 cm^{-1}, other peaks at 1080, 1055, 1028, 890 cm^{-1} were due to 8-pyranone form. The ^{1}H-NMR values at δ 4.9 & δ 4.04 (J = 10.3 Hz) showed that 2-H and 3-H are in *trans* configuration. The values at δ 5.9 (J = 2.1Hz) showed the 6 and 8 protons. The C-3 & C-4 δ 5.1 protons (J = 6.3 Hz) showed their *cis* orientation. The six –OH (4-phenolics and 2-alcoholic) groups protons are exchangeable with D_2O are at δ 6.5 its integration showed the no of protons. Remaining 2', 5' & 6'-H are conformed from the coupling constants at ^5J = 1.1 Hz & ^4J = 1.85 Hz. Thirteen different carbon were observed from ^{13}C-NMR spectrum. In addition, the obtained penta-nitrated and tetra-O-methylated leucocyanidin analogs from chemical modification suggested that the compound is leucocyanidin [11].

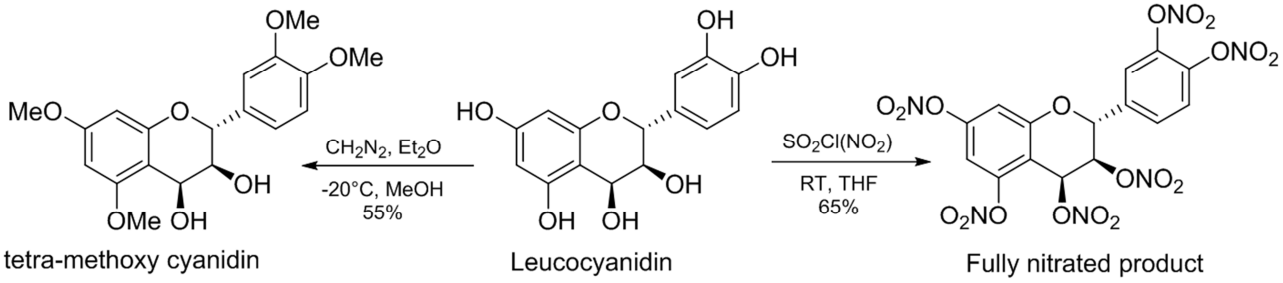

Figure 3. *Two different molecules from Leucocyanidin.*

β-Sitosterol

It was isolated as white crystalline, MP 136°C, R_f 0.32 (1: 9, ethyl acetate: hexane). It gave positive Libermann-Burchard test indicating the compound to be sterol. HRMS (ESI) calculated for this steroid is ($[M+H]^+$): 415.3895, found: 415.3888 corresponding to the molecular formula $C_{29}H_{50}O$. The β-Sitosterol was also confirmed by co-TLC/2D-TLC and LC-MS analysis with the sample already isolated in our laboratory.

All NMR, IR and mass spectral patterns were identical with that of reported β-Sitosterol [12].

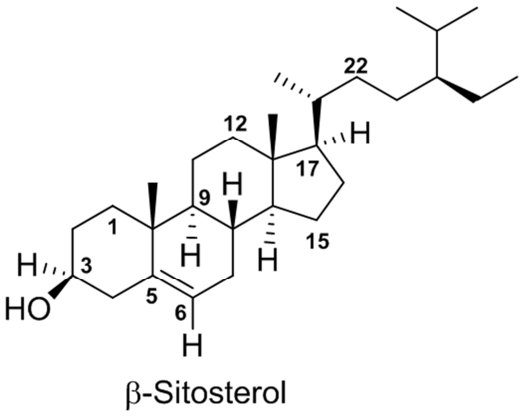

Figure 4. *Structure of an isolated steroid.*

β-Sitosterol 3-O- β-D glucopyranoside

It was white powder, MP above 280°C, R_f 0.66 (1: 4, methanol: chloroform). HRMS (ESI) calculated for this steroid is ($[M+H]^+$): 577.4423, found: 577.4418 corresponding to the molecular formula $C_{35}H_{60}O_6$. The co-spot/2D-TLC and LC-MS with already authenticated glycoside in lab also confirmed it.

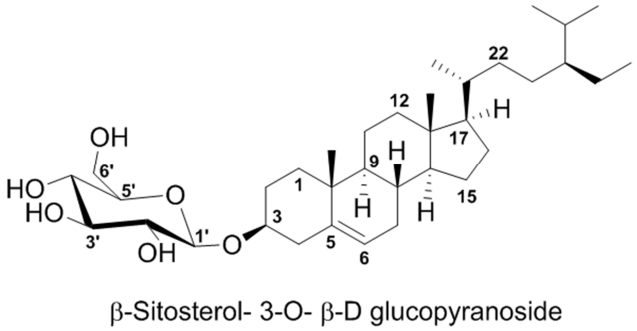

Figure 5. *Structure of a glycoside.*

^{1}H NMR spectrum pattern was similar to that of β-sitosterol with some additional peaks relating to a carbohydrate moiety. The multiplet at δ 4.27 Hz was assigned for the proton of C-3. Its de-shielding may be due to attachment of β-O glucoside moiety at C-3 carbon. The proton signals at δ 3.96 Hz, 4.03 Hz, 4.27 Hz, 4.52 Hz, 4.53 Hz and 5.02 Hz in the de-shielded region were assigned for respective C-5′, C-2′, C-3′, C-4′, C-6′, and C-1′ protons of glucoside moiety. ^{13}C-NMR pattern was also similar to β-sitosterol with six more peaks confirming the glucose ring. Furthermore, the structure was supported by ^{1}H-^{1}H COSY, HMBC and HMQC. All these spectral values were found to be consistent with β-Sitosterol glucoside reported previously [12].

4. Conclusions

It can be stated that the *B. ciliata* of Nepalese origin showed interesting composition of bergenin and (+) afzelechin with medicinal and ornamental applications in traditional and folk medicine. So, it served as the valuable source of these two compounds which could potentially be used in medicinal chemistry program to discover the potent drug molecule. Research work showed that the ethyl acetate extract/fraction exhibit extreme bioactivity. It is also found that the active principles are potent in bioassay. To the best of our knowledge the above five compounds were isolated from this Himalayan plant of Nepalese origin for the first time. Further work is necessary in terms of chemical and pharmacological aspect. We have obtained different mixtures which are still to be determined.

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