Flavonol Glycosides of *Cheilanthes anceps* Roxb.

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Abstract: *Cheilanthes anceps* Roxb, a member of Silva-back group of fern of the family, Sinopteridaceae, is a fern constituent of pine forest of Central Himalaya, ranging altitude from 2000m to 3000m. It has been recognized as a traditional medicine by the tribal inhabitants of Central Himalaya. The aqueous extract of the fern has been used for curing number of human ailments like cough, bronchitis, diabetes, inflammatory and healing wounds. The aqueous-ethanolic extract of fern fronds of *C. anceps* was concentrated and partitioned with CH_2Cl_2 and n-BuOH. The flavonoid positive fraction derived from 30% HOAc fractionation of BuOH soluble gave antioxidative activity against the methanolic solution of free radical, DPPH by the standard thin layer autobiography and UV-VIS spectrophotometer assay. Two flavonol glycosides, kaempferol-3-O-α-L-rhamnopyranosyl (1→2)-α-L-rhamnopyranosyl (1→6)-β-D-galactopyranoside and quercetin-3-O-α-L-rhamnopyranosyl (1→2)-α-L-rhamno pyranosyl(1→6)-β-D-galactopyranoside, were isolated and identified from antioxidative activity guided fraction and named as compound [1] and [2] respectively. The compound [2] was found to be more active than the compound [1] using quercetin as a reference substance. Both the compounds [1] and [2] showed lower activity than reference substance, quercetin. [Journal of American Science 2009;5(4):183-188]. (ISSN: 1545-1003).

Key words: Ferns, *Cheilanthes anceps,* Roxb, Central Himalaya

1. Introduction

Genus *Cheilanthes,* a group of Silva-back ferns of the family, Psinopteridaceae, is widely distributed in temperate and humid environs of Central Himalaya ranging altitude from 2000m to 3000m. Eighteen species of genus *Cheilanthes* have been reported from the hills of Kumaun Himalaya of newly created state Uttarakhand. Some *Cheilanthes* species, native of Himalaya have been recognised as a traditional medicine by some ethnic groups of the region (Pande, 1992). Various extracts derived from *Cheilanthes* species have been identified for antimicrobial activities (Nickell, 1959; Bhakuni *et al.*, 1969). *Cheilanthes anceps,* an abundant fern constituent of the pine forests of Central Himalaya, has been identified as a traditional medicine by the local Kumaun people of the region and aqueous extract has been used for curing some human ailments like cold, cough, asthma, bronchitis, diabetes, inflammatory and heeling wounds (Banerjee and Sen, 1980). Similar studies on different kind of medicinal plants have been reported by Verma and his co-workers (Khetwal and Verma, 1983, 1984, 1986, 1990; Khetwal *et al*., 1985, 1986; Mishra, 2009a, Mishra and Verma, 2009b and Mishra and Verma, 2009c).

Naturally occurring flavonoidal compounds have a vital role as antioxidants (Oleszek, 2002). These compounds have widely been used to cure diseases related to oxidative stress like, diabetes, stoke, arthritis, cancer, cardiovascular and inflammatory (Kapiszewska *et al.*, 2005). These natural antioxidants are an integral constituent of angiosperm food and fodder plants. The objective of present chemical investigation is to investigate new natural products responsible for antioxidative activity from non-flowering and not a usual food and fodder plants like ferns. Attributing a traditional medicinal uses of *C. anceps* for curing number of ailments, the present communication revealed the isolation and characterisation of flavonol glycosides. *C. anceps* is rich source of flavonoids producing general and a number of methylated flavonols, flavonol-O-glycosides of quercetin and kaempferol have been reported in the literature (Erdtman *et al.*, 1966; Salatino and Prado, 1998). *C. anceps* has neither been reported for biological activities and nor been reported for active secondary metabolites.

2. Material and method

Fern fronds of *Cheilanthes anceps* Blanford was collected from 3000m height of pindari glaciers route of Kumaun Himalaya and the authentification of the species was made by Prof. P. C. Pande, Botany Department of Kumaun University, S. S. J. Campus, Almora (Uttarakhand). Its vouch. Specimen No.13 has

been deposited in the Chemistry Department of Kumaun University at SSJ Campus, Almora, Uttarakhand, India.

About 1kg air dried and powdered fronds of *Cheilanthes anceps* was extracted sequentially with 70% aqueous ethanol and 50% aqueous ethanol by cold percolation method for six days. The two were combined and concentrated under reduced pressure until only a small H_2O layer (approx. 50ml) remained. It was partitioned with CH_2Cl_2 and BuOH successively. The BuOH fraction was adsorbed on cellulose CC (Merck) and it was eluted initially with H_2O then increasing polarity with HOAc. On eluting CC with 30% HOAc, three dark fluorescent bands were observed on CC and each was eluted and collected separately by monitoring with UV light. The eluents derived from faster, middle and slower moving bands represent Frac-I, Frac-II and Frac-III, respectively.

3. Antioxidative screening of Frac-I, Frac-II and Frac-III

Each fraction was evaporated to dryness under reduced pressure at 70^0C. The residue of each fraction was dissolve in MeOH and evaluated for antioxidative activity against DPPH free radical solution with UV-VIS spectrophotometer and the quenching of fluorescence was measured at 515nm. Besides the spectrophotometer evaluation, the thin layer autobiographic methods using SiO_2 TLC plate of the fraction developed with suitable solvent and sprayed with methanolic solution of DPPH. The methanolic solution of Frac-I afforded active spots (yellow spots in purple background) while Frac-II and Frac-III did not produce any active spot.

Frac-I, an antioxidative active fraction was adsorbed on Whatman No.3 PC and fractionated with BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as a developing solvent. On inspecting PC under UV light, two dark purple fluorescent bands were observed and each was cut and eluted with 70% EtOH. The eluate of faster moving component which produced two antioxidative active spot on TLC was separated by Sephadex LH-20 CC using 40% MeOH as an eluent. Two compounds [1] and [2] were isolated. The compound [2] was found more antioxidative compound to compound [1] using quercetin as a reference substance.

4. Result and discussion

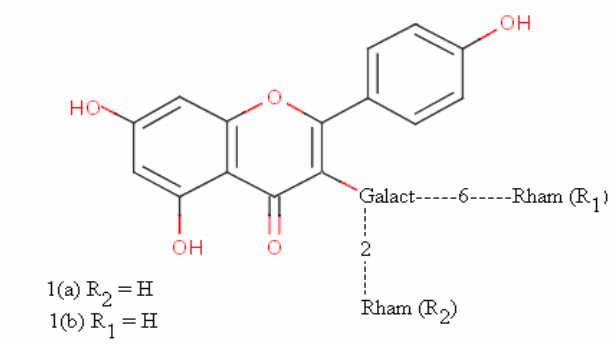
Compound [1], a grey-yellow amorphous solid, gave positive results in Molish and Mg+HCl test. It appeared as a dark purple fluorescent spot on PC under UV light and changed to yellow-green after fuming with NH_3 vapours, indicating the presence of free hydroxyl groups at C-4'and C-5. When cellulose TLC of the compound was sprayed with methanolic solution of Naturstoffreagenz A (NA) reagent, the spot turned to yellow, indicating the presence of a free hydroxyl group at C-4' but absence of ortho-di-hydroxyl group in the B-ring. Its UV spectrum in MeOH showed characteristic absorption at 268nm (band II) and 361nm (band I), indicting 3-O-substituted flavonol skeleton (Markham, 1982) and analysis with the usual flavonoid shift reagents, NaOMe (282, 396); $AlCl_3$ (267, 305, 345); $AlCl_3$/HCl (267, 305, 344); NaOAc (270, 351); NaOAc/H_3BO_3 (270, 310, 314) and $ZrOCl_2$+citric acid (282, 396), suggesting the presence of free hydroxyl groups at positions, C-4', C-5 and C-7 (Markham, 1982). The mono-saccharides obtained after complete acid hydrolysis were identified as glucose and rhamnose by paper chromatographic comparison with their standards.

FABMS (-ve) of the compound [1] gave a molecular ions at m/z 739 $[M-H]^-$ calculated for $C_{33}H_{40}O_{19}$ and prominent ions observed at m/z 447 [m/z 739-2x rham]$^-$ and m/z 285 [m/z 447-galac]$^-$ supporting the release of two molecules of rhamnose and one molecules of galactose from kaempferol. H_2O_2 oxidation of compound gave kaempferol (CoPC) and a tri-saccharide sugar on PC at Rf 16 in BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) which on partial acid hydrolysis released rhamnose first then glucose. Partial acid hydrolysis of compound [1] with 0.1N-HCl gave three dark purple fluorescent compounds on PC at Rf, 48, 46 and 56 in BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer), representing compounds [1(a)], [1(b)] and [1(c)] respectively. These three constituents were isolated by RPPC using BAW as a developing solvent followed by their final purification on Sephadex LH-20 CC.

The compound [1(a)] and [1(b)] were identified as a kaempferol -3- O- di-glycoside by their chromatographic behaviour and UV spectral data in MeOH and with diagnostic shift reagents (Mabry *et al.*, 1970; Markham and Mabry, 1975). The structure of [1(a)] and [1(b)] were identified as a kaempferol -3-O-α-L-rhamnopyranosyl (1→6)-β-D-galactoside and kaempferol-3-O– α – L - rhamnopyranosyl (1→2) -β-D-galactoside, respectively by their ^{1}HNMR studies in (DMSO-d_6 and 400MHz), table [1] and by comparison of the physico-chemical data with those of authentic samples on the reported values in the literature (Yasukawa and Takido, 1987; Cui *et al.*, 1993).

Table 1. ^{1}HNMR of compound [1(a)] and [1(b)] in DMSO-d_6 (400MHz).

Shift (Multiplicity) of [1(a)]	Shift (Multiplicity) of [1(b)]	Identification
6.15 (1H, d, J=2.0 Hz)	6.16 (1H, d, J=2.0 Hz)	H-6
6.36 (1H, d, J=2.0 Hz)	6.38 (1H, d, J=2.0 Hz)	H-8
6.83 (2H, d, J=8.5 Hz)	6.87 (2H, d, J=8.5 Hz)	H-3', 5'
8.04 (2H, d, J=8.5 Hz)	8.04 (2H, d, J= 8.5 Hz)	H-2', 6'
12.60 (1H,s)	12.60 (1H,S)	5-OH
5.33 (1H, d, J=7.4 Hz)	5.66 (1H, d, J=7.5 Hz)	H-1''
4.48 (1H, d, J=1.5 Hz)	5.10 (1H, d, J=7.5 Hz)	H-1'''
2.90-4.10 (m)	2.95-4.00 (m)	Remaining protons of sugar moieties
1.10 (3H, d, J=6.0 Hz)	1.15 (1H, d, J=8.3 Hz)	6''-OCH_3



The compound [1(c)] was identified as kaempferol-3-O-β-D-galactoside by comparison on PC with its standard. The similar product was also identified from enzymatic hydrolysis of compound [1] with α-rhamnosidase. On the basis of chromatographic behaviour, UV spectral data, FABMS and hydrolytic methods, the compound [1] was identified as kaempferol-3-O-(2, 6-di-O-α-L-rhamnopyranosyl -β-D- galactopyranoside).

Finally, the structure of compound [1] was confirmed by ^{1}HNMR studies in (DMSO-d_6 and 400MHz): ^{1}HNMR of compound [1], table (2), showed two ortho coupled symmetrical doublets appeared at δ 6.20 (1H, d, J=2.0 Hz)) and δ 6.42 (1H, d, J=2.0 Hz) representing for H-6 and H-8 respectively of A-ring and two ortho coupled symmetrical doublets appeared at δ 6.87 (2H, d, J=8.5 Hz) and δ 8.10 (2H, d, J=8.5 Hz) assignable to H-2', 5' and H-2', 6' respectively of B-ring. A broad singlet appeared at δ 12.62 represent chelated 5-OH of A-ring. In aliphatic region , an anomeric proton signal appeared at δ 5.68 (1H, d, J=7.5 Hz) attributed to a galactose (β-configuration) sugar moiety directly attached to aromatic ring and two high field anomeric proton singlets appeared at δ 5.12 (1H, S) and δ 4.46 (1H, S) were attributed to two rhamnosyl moieties (α-configuration) linked to the 2'' and 6'' positions respectively of 3-O-galactosyl moiety (Overend, 1972; Altona and Haasnoot, 1980). The rhamnosyl methyls appeared as doublets at δ 0.90 (3H, d, J=6.0 Hz) and δ 1.12 (3H, d, J=6.0 Hz).The remaining sugar protons were observed in the range δ 3.0-4.0.

Table 2. ^{1}HNMR spectra of compound [1] in DMSO-d_6 (400MHz)

Shift (δ)	Multiplicity	Identification
6.20	1H, d, J=2.0 Hz)	H-6
6.42	1H, d, J=2.0 Hz)	H-8
6.87	2H, d, J=8.5 Hz)	H-3', 5'
8.10	2H, d, J= 8.5 Hz)	H-2', 6'
5.68	1H, d, J=7.5 Hz	H-1''
5.12	1H, d, J=7.5 Hz	H-1'''
4.46	1H,s	H-1''''
0.90	3H, d, J=6.0 Hz	6'''-CH_3
1.12	3H, d, J=6.0 Hz	6''''-OCH_3
3.00-4.00	(m)	Remaining protons of sugar moieties

On the basis of ^{1}HNMR studies, the compound [1] was identified as kaempferol-3-O-α- L- rhamnopyranosyl (1 → 2) - α – L - rhamnopyranosyl (1 → 6) – β – D - galactopyranoside.

^{13}CNMR data chemical shift of sugar unit with those of kaempferol-3-O-galactoside showed glycosylation shift for C-2" by 5.9 ppm and C-6" by 5.5 ppm in the residual galactose unit. The signals at δ 76.2 and at δ 66.3 were attributed to C-2" and C-6" of inner galactose linked to two molecules of rhamnose as rhamnosyl (1→2) – β – D - galactopyranoside and rhamnosyl (1→6)-β-D- galactopyranoside respectively (Markham *et al.*, 1978).

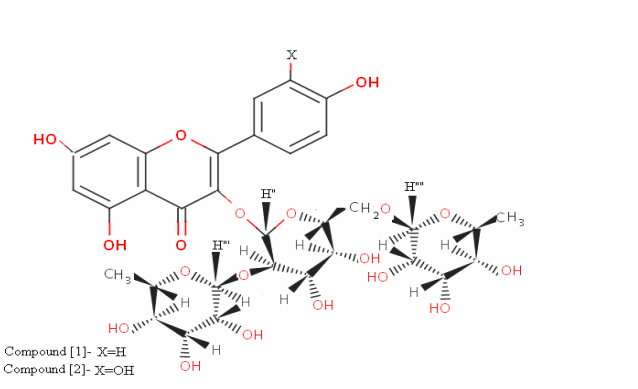
Compound [2] gave positive reactions with Mg+HCl and α-naphthol. Complete acid hydrolysis of [2] with 2N-HCl gave quercetin (CoPC), galactose (CoPC) and rhamnose (CoPC). FABMS (-ve) of the compound [2] gave a molecular ions at m/z 755 $[M-H]^{-}$ calculated for $C_{33}H_{40}O_{20}$ and prominent ions were observed at m/z 463 $[m/z\ 755\text{-}2x\ rham]^{-}$ and m/z 301$[m/z\ 463\text{-}galac]^{-}$ supporting the abstraction of two molecules of rhamnose and one molecule of galactose from quercetin.

H_2O_2 oxidation of compound [2] afforded quercetin (CoPC) and a tri-saccharide, 2, 6-di-rhamnpside of galactose which was identified by comparing with its standard on paper chromatogram. Enzymatic hydrolysis of compound [2] with α-rhamnosidase gave quercetin-3-O-β-D-galactoside CoPC) and rhamnose (CoPC). Partial acid hydrolysis of compound [2] with 0.1N-HCl gave three compounds, quercetin – 3 - robinobioside, quercetin – 3 – α – L – rhamnopyranosyl (1 → 2) – β – D - galactoside and quercetin – 3 – O – β – D - galactopyranoside were identified by comparison of the physico-chemical data with those of authentic samples or the reported values in the literature (Yasukawa *et al.*, 1989; Nawwar *et al.*, 1989).

Finally, the structure of compound [2] was confirmed by ^{1}HNMR studies in (DMSO-d_6 and 400MHz) in table (3): ^{1}HNMR data of compound [2] in sugar region were found similar to the corresponding sugar region of compound [1]. Thus, the compound [2] was identified as quercetin-3-O-α-L-rhamnopyranosyl (1→2)-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside.

Table 3. ^{1}HNMR spectra of compound [2] in (DMSO-d_6, 400MHz)

Shift (δ)	Multiplicity	Identification Protons
6.24	1H, d, J=2.0 Hz	H – 6
6.43	1H, d, J=2.0 Hz	H – 8
6.84	1H, d, J=8.5 Hz	H – 5'
7.56	1H, d, J=2.0 Hz	H – 2'
7.64	1H, dd, J=2.0and 8.5 Hz	H – 6'
12.60	1H (S)	5-OH
5.65	1H, d, J=7.5Hz	H – 1"
5.10	1H, d, J=1.0 Hz	H – 1"'
4.45	1H (S)	H – 1""
0.92	3H, d, J=6.0 Hz	CH_3 at 6"'
1.10	3H, d, J=6.0 Hz	CH_3 at 6""
3.0-4.0	(m)	Remaining protons of sugar moieties



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