Antioxidative properties of flavonoids from *Cheilanthes anceps* Swartz.

Sanyukta Chowdhary [a], D. L. Verma [b], Rachana Pande [b] and Harish Kumar[c*]
[a] Department of Botany, Kumaun University, S. S. J. Campus, Almora-263601, India.
[b] Department of Chemistry, Kumaun University, S. S. J. Campus Almora-263601, India
[c*] Department of Botany and Microbiology, Gurukul Kangri University, Haridwar-249404, India
Email[*]: harish2129@gmail.com, hellosanyukta28@gmail.com

Abstract: Antioxidative guided chromatographic fractionation of BuOH fraction from aqueous-ethanolic extract of fern fronds of *Cheilanthus anceps* gave flavonol glycosides, Quercetin-3-0-δ–L-rhamnopyranosyl(1→2)-β-D-glucopyranoside-7-O-β-D-glucopyranoside, Kaempferol-3-O- δ-L-rhamnopyranosyl (1→2)-β -D-glucopyranoside-7 O-β -D-glucopyranoside, Quercetin-3-O-β -D-glucosyl (1→2)-β -D-galactoside-7-O-β -D-glucoside, Quercetin-3-methyl ether-5-O-glucoside, Quercetin-3-O-glucoside and Kaempferol-3-O-glucoside. Of these flavonol-glycosides, the glycosides of Quercetin showed prominent antioxidative activity compared to Kaempferol glycosides. [Journal of American Science 2010; 6(5):203-207]. (ISSN: 1545-1003).

Keywords: *Cheilanthes anceps*, Flavonol glycosides, Antioxidative activity.

1. Introduction

Cheilanthes Swartz, a genus of fern's family psinopteridaceae and a group of leptosporangiate ferns of flora of filicinae, comprises 180 species with cosmopolitan in distribution. Fifteen species of *Cheilanthes* have been reported from sub-tropical and temperate Kumaun Himalayas (Pandey et al., 2002). Members of genus *Cheilanthes*, commonly known as silva back ferns, have widely been used as traditional medicines by the local inhabitants of Kumaun Himalayas. *Cheilanthes anceps* Blanford, a common fern constituent of sun facing hills and pine forest areas of Kumaun hills, nearly an altitude ranging from 1000 to 2000m, is characterized morphologically to bear 20-30 cm long and 10-12 cm broad lanceolate fronds. Various species of *Cheilanthes* have been recognized as a traditional medicine and extracts derived from them have been screened for various biological activities (Chopra et al., 1958; Banerjee and Sen, 1980; Lal et al., 1994). Flavonoids, a highly diversified group of natural products and a dietary health promoting substances, have widely been reported from Cheilanthoideae of filicinae group of ferns (Wollenweber et al., 1997). *Cheilanthes* have been characterized for the presence of methoxylated flavones, flavonols and glycosides of Kaempferol and Quercetin (Salatino and Prado, 1998).

Cheilanthes anceps, a fern native to low reaches of Central Himalaya and a constituent of Pine forests of the region, has been used as traditional medicines by the tribal folk of Kumaun Himalaya, and has widely been recommended as medicines to cure cough, asthma, tuberculosis and joint pain. Literature survey revealed that the fern is still awaited for chemical investigation. Present chemical investigation revealed the presence of mono and 3, 7-di-O-glycosides of Kaempferol and Quercetin from antioxidative guided chromatographic fractionation of aqueous-ethanolic extracts of *Cheilanthes anceps.*

2. Materials and Methods

The fresh fronds of *Cheilanthes anceps* were collected from Kumaun hills, altitude ranging from 2500m to 3,000m. The species was identified and deposited (Herbarium specimen No.18) in the Department of Chemistry, Kumaun University campus, Almora (UK).

Extraction and Isolation of flavonoidal compounds

About 2.5 kg of air dried and powered fronds of *Cheilanthes anceps* was extracted sequentially with 80% and 50% EtOH by cold percolation methods. Two extracts were combined and concentrated under reduced pressure at 60ºC in Rota evaporator. The residue was partitioned between dichloromethane and H_2O (1:1). After separating lower dichloromethane layer, the upper H_2O layer was further partitioned with BuOH. The BuOH fraction was concentrated under reduced pressure and residue was adsorbed on cellulose (Merck grade) CC and eluted initially with H_2O then increasing polarity with AcOH. On eluting column with 20% aq. AcOH three dark purple fluorescent bands observed with UV light (360 nm) and each was eluted and collected separately. 2DPC examination of the eluents of faster moving, middle and slower moving bands, using BAW (n-BuOH-AcOH-H20, 4:1:5, V/V, upper layer) and 30% HOAc as a developing solvent system, afforded three, five and two flavonoidal positive

constituents respectively. Each fraction was resolved into pure components by RPPC using BAW (n-BuOH-AcOH-H2O, 4:1:5, V/V, upper layer) as a developing solvent. The pure compounds obtained were finally passed over sephadex LH-20 using MeOH: H_2O (1:1) for final purification.

3. Results and Discussion

Three flavonol glycosides 1, 2 and 3 were isolated from FRAC –1, an eluent derived from faster moving dark purple UV fluorescent band. Compound **4**, a dull blue fluorescent, was isolated from the eluents of middle band. Compounds **5** and **6** were isolated from the eluent of slower moving band (FRAC-III). Compounds **2** and **3** were identified as Quercetin-3, 7-di O-glycosides and compound **1** was identified as a Kaempferol-3, 7-di O-glycoside on the basis of chromatographic behavior and UV spectral methods (Mabry et al., 1970; Markham et al., 1975). Complete acid hydrolysis of compounds **1**, **2** and **3** gave two aglycones which exhibited a dull yellow colour under UV light indicating a release of sugar moiety from the 3-position. H_2O_2 oxidation of these compounds gave two monoglycoside, which exhibited bright yellow fluorescent colour with and without the presence of NH_3 vapours indicated the Flavonol-3, 7-dioglycoside nature (Willians and Grayer, 2004).

Compound 1, showed a $[M + H]^+$ peak m/2 779 $[M + Na]^+$ in FABMS, 757 $[M + H]^+$, and other prominent ions at 611 $[(M + H)-146]^+$, 449 $[(M + H)-308]^+$ and 287 $[(M + H)-470]^+$, supported the 3,7-dioglycoside of Kaempferol and successive elimination of rhamnose and two hexoses respectively. Further complete acid hydrolysis of the compound afforded Kaempferol (CoPC), Glucose (CoPC) and Rhamnose (CoPC). H_2O_2 oxidation of the compound gave Kaempferol-7-O-β-D-glucoside and a disaccharide sugar on PC at Rf 14 BAW solvent. Complete acid hydrolysis of the compound **1** gave Kaempferol (CoPC), Glucose and Rhamnose (CoPC). Compound **1** appeared as a purple fluorescent spot on a paper chromatogram under UV light and changed to yellow – green with ammonia, indicating the presence of free 5- and 4`-hydroxyl groups. When a cellulose TLC plate was sprayed with Naturstoffreagenz (A) reagent, the spot turned yellow indicating a 4`-hydroxyl but no ortho-dihydroxy group in the B-ring. Compound **1** exhibited UV maxima in methanol at 265 (band II) and 354 (band I) and shifts obtained with diagnostic reagents : NaOMe, 245, 270, 350 sh, 390 (it) ; $AlCl_3$, 275, 302, 356, 400 ; $AlCl_3$ + HCl : 275, 300, 355, 400 ; NaOAc : 267, 360, 405 sh ; and NaOAc + H_3BO_3: 266, 320 sh, 353 indicated the presence of free hydroxyls at C-5 and C-4`.

^{1}HNMR of the compound (DMSO-d_6, 400MHz) gave two meta coupled doublets at δ 6.40 and δ 6.77, each with J=20 Hz, indicated the presence of H-6 and H-8 of 7-O-substituted flavone moiety. Two symmetrical ortho coupled doublets each with J=8.5 Hz at 6.84 and 8.02 represent p-di substituted β-ring for H-3`, 5` and H-2`, 6`. Three anomeric protons signals observed at δ 5.62 (d, J=7.5 Hz), δ 5.06 (1 H,d,J=7.5 Hz) and a broad singlet at 5.03 (1H, brS) assignable to the glucose moieties at C-3, C-7 and terminal rhamnose unit respectively. An overlapped multiples for remaining protons of three sugar units observed between 3, 15 – 4.0. A downfield broad singlet observed at 12.50 represents 5-OH (chelated). A high field doublet at 0.80 (3H, d, J=6.5 Hz) for rhamnose-CH_3 group (**Table 1**).

Table 1: ^{1}HNMR of Compound 1

Shift (δ)	Multiplicity, J=Hz	Identification
6.40	1H, d, 2.0	H-6
6.77	1H, d, 2.0	H-8
6.84	2H, d, 8.5	H-3` and 5`
8.02	2H, d, 8.5	H-2` and H-6`
5.62	1H, d, 7.5	glucose anomirz (C-3)
5.06	1H, d, 7.5	glucose anomeric at C-7
5.03	1H, br.S	rhamnosyl terminal sugar
3.15-4.0	m	remaing protons of rhamnose + two glucose moieties
0.80	3H, d, J=6.5	-CH3 rhamnose
12.50	5 (br)	5 –OH

The recognizable down-field shift (Δδ=0.4 ppm) which was detected on comparing the chemical shifts of the anomeric glucoside proton signals with those of Kaempferol-3-glucoside proved that the terminal α-rhamnosyl moiety is attached to C-2 of the primary β-glucoside moiety (Altona and Haasnoot, 1980). Thus, the compound **1** was identified as Kaempferol-3-O-β-(rhamnosyl 1→2) glucoside-7-O-β-D-glucoside.

Compound 2, Chromatographic isolation of the compound gave yellow amorphous powder, which exhibited a molecular ion in FABS, m/e at773 $[M + H]^+$ consistent with a molecular formula $C_{33}H_{40}O_{21}$. The signals found at m/e 627 $[(M + H) – 146]^+$, 465 $[(M + H) – 308]^+$ and 303 $[(M + H) – 470]^+$ indicated successive elimination of rhamnose and two hexoses respectively. These finding supported a

3, 7-glycosylated flavonol structure. Chromatographic behavior and UV spectral data of the compound in MeOH at (λmax, nm) 256 (band I) and shifts obtained with diagnostic reagents, NaOMe, 269, 400; $AlCl_3$, 275, 330sh, 433; $AlCl_3$ + HCl, 269, 300sh, 360sh, 400, NaOAc, 262, 408 and NaOAc + H_3BO_3, 260, 378, suggested a flavonol triglycoside with a free hydroxyls at C-5, C-3` and C-4` [7-8]. ^{1}HNMR of the compound (in DMSO-d_6, 400MHz) gave five aromatic signals at (δ) 6.40 (1H, d, J=2Hz), 6.75 (1H, d, J=2Hz), 6.80 (1H, d, J=7.5Hz), 7.51 (1H,d, J=2.0 Hz) and 7.75 (1H, dd, J=2.0 and 7.5 Hz) for H-6, H-8, H-5`, H-2` and H-6` respectively. Three anomeric proton signals appeared at 5.62 (1H, J=7.5 Hz), 5.06 (1H, d, J=7.5) and 5.03 (1H, S) identified for C-3 (glucose), C-7 (glucosyl) and a terminal rhamnosyl respectively. Complete acid hydrolysis of the compound gave Quercetin (CoPC), Glucose (CoPC) and Rhamnose (CoPC). The recognizable downfield shift (Δδ=0.3 ppm) which was detected on comparing the chemical shifts of the anomeric glucoside proton that the terminal α-rhamnosyl moiety is attached to C-2 of the inner β-D-glucoside moiety [10]. The ^{1}HNMR of compound **2** in sugar region was found similar to the compound **1**. Thus, the compound **2** was identified as Quercetin 3-O-β (rhamnosyl 1→2 glucoside) 7-O-β-D-glucoside.

Compound 3, Chromatographically isolated grey amorphous powder gave a molecular ion in FAB-MS, m/e at 811 $(M + Na)^+$ and other prominent

ions, 465 $[M + H - 324)^+$ and m/e 303 $[M + H - 486]^+$, indicated successive elimination one and two hexose moiety respectively. These findings supported a 3,7di-glycosylated flavonol structure. Chromatographic behaviour and UV spectral data of the compound which were found similar to compound **2** suggested a flavonol triglycoside with a free hydroxyls at C-5, C-3` and C-4` [7-8]. Complete acid hydrolysis of compound gave quercetin, glucose and galactose. ^{1}HNMR of compound (DMSO-d6, 400 MHz) gave five aromatic protons signals at (δ) 640 (1H, d, J=2.0 Hz), 6.74 (1H, d, J=2.0 Hz), 6.85 (1H, d, J=8.5 Hz), 7.56 (1H, d, J=2.0 Hz) and 7.65 (1H, dd, J=8.5 and 2.0 Hz) assignable to H-6, H-8, H-5`, H-2` and H-6` respectively. Three aliphatic anomeric proton signals appeared at 4.57 (1H, d, J=7.5 Hz), 5.07 (1H, d, J=8.0 Hz) and 5.64 (1H, d, J=7.5 Hz) representing for terminal glucose, C-7- glucose and C-3 primary galactose respectively. The recognizable down field shift of (Δδ=1.17) which was detected on comparing the chemical shifts of the anomeric galactoside proton signals in the spectrum of 3 with those of Quercetin-3-galactoside proved that the terminal glucosyl moiety is attached to C-3 of the inner β-galactoside moiety. Consequently 3 was identified as Quercetin-3-O-β-glucosyl (1-2) glactoside-7-O-β-D-gluoside.

Compound 4, FABMS of the compound gave a molecular ion m/e at 477 $[M - H]^-$ calculated for $C_{22}H_{22}O_{12}$. It appeared as a dull blue florescent spot on paper chromatogram under UV light and changed to yellow-green with ammonia, indicating the presence of free 5- and 4`-hydroxyl groups. When a cellulose TLC plate was sprayed with Naturstoffreagenz (NA), the spot turned orange

indicating the presence of ortho-dihydroxyl group in the B-ring. ^{1}HNMR of the compound (in DMSO-d6, 400 MHz) gave two meta coupled doublets at 6.45 (1H, d, J=2.0 Hz) assignable to H-6 and H-8 of A-ring. An ABX system for three B-ring protons observed at 6.89 (1H, d, J=8.5 Hz), 7.43 (1H, dd, J=2.0 and 8.5 Hz) and 7.53 (1H, d, J=2.0 Hz) identified for H-5`, H-6` and H-2` respectively. The dull blue florescence of compound on PC under UV light and down field shift of C-6 and C-8 protons of A-ring, indicating either algence of 5-OH group or 5-OH group is substituted. Further, two prominent ions in MS, m/e at 461 (M-CH_3-H) and 301 (M-176-H) showing the attachment of sugar moiety at C-5 position of flavones. ^{1}HNMR spectra (**Table. 2**) indicated the attachment of glucose unit at C-5 position (Mabry et al., 1970).

Table 2: ^{1}HNMR (DMSO-d_6) 400 MHz of COMPOUND 4

Shift (δ)	Multiplicity, J=Hz	Identification of protons
6.45	1H, d, 2.0	H-6
6.75	1H, d, 2.0	H-8
6.89	1H, d, 8.5	H-5
7.43	1H, dd, 8.5, 2.0	H-6
7.53	1H, d, 2.0	H-8
4.76	1H, d, 7.5	H-1``
3.2-4	6H, m	glucosyl
3.90	3H (s)	protons –OCH_3

In aliphatic region of ^{1}HNMR spectra 4, gave a doublet at δ 4.76 (1H, J=7.5 Hz) indicated β-configuration in pyranose form of glucose (Altona and Haasnoot, 1980). A three proton sharp singlet observed at 3.90 for OCH_3 group attached at C-3 position. Except anomeric proton, remaining five protons of glucose appeared as a multiplet between

3.2 to 4.0. Complete acid hydrolysis of compound 4 gave a dark purple florescent aglycone on PC and a dull brown spot of sugar at Rf 23 (BAW) after spraying with benzidine reagent. The sugar component was identified as glucose by comparing with its standard on PC. The structure of aglycone was identified as follows:

The aglycone, representing structure 4(a) was isolated from acid hydrolysed mixture of 4 by RPPC using 30% HOAc as a developing solvent. It crystallized as deep yellow fine needles, mp, 282. The MS of 4(a) exhibited a molecular ion at m/e 316 $[M]^+$ (100%), 315 (70%), 301 $[M –CH3)^+$, 287 $(M – HCO)^+$, 153 $(A + H)^+$ (23%), 144 (10%), 137 $[B2]^+$ (21%) and 121 [B1 – COMe] (10%). Cellulose TLC of aglycone when sprayed with methanolic solution of Naturstoffreagenz reagent, the dark purple fluorescence of compound turned to orange, indicating the presence of ortho-dihydroxyl compound in the B-ring. ^{1}HNMR of 4(a) gave two meta coupled doublets at 6.24 (1H, J=2.0 Hz) and 6.48 (1H, J=2.0) assignable to H-6 and H-8 of A-ring. A three protons ABX system was observed at 7.00 (1H, d, J=8.5), 7.59 (1H, dd, 2.0 and 8.5 Hz) and 7.24 (1H, d, J=2.0 Hz) representing H-5`, H-6` and H-2` respectively. A three proton singlet observed at δ 3.90 attached at C-3 position. A high field singlet observed at 12.80 indicated presence of chelated 5 – OH in the A-ring. The compound 4(a) was hydrolyzed with H1 in presence of base, form a dull yellow fluorescent compound on PC under UV light (CoPC). It was identified as Quercetin. Thus, 4(a) was identified as Quercetin-3-OCH_3. On the basis of UV, ^{1}HNMR, MS and hydrolytic methods, the compound 4 was identified as Quercetin-3-OCH_3-5-O-β-D-glucoside.

Compund 5, FABMS (NBA) of compound gave a molecular ion at m/e 447 $[M – H]^-$ and a prominent ion observed at 285 $[M – 162 – H)^-$ showing loss of glucose moiety from aglycone, kaempferol. ^{1}HNMR (DMSO –d6, 400 MHz) of 5 gave four doubles at 6.20 (1H, d, J=2.0 Hz), 6.42 (1H, d, J=2.0 Hz), 6.86 (2H, d, J=8.5 Hz) and 8.03 (2H, d, J=8.5 Hz) assignable to H-6, H-8, H-3`,5` and H-2`, 6` respectively of flavonoid nucleus. A doublet observed at 5.20 (J=7.5 Hz) represent anomeric proton of glucose and β-configuration of pyranose form of sugar (Altona and Haasnoot, 1980). Complete acid hydrolysis of compound with 2NHCl gave glucose (CoPC) and kaempferol (CoPC). On the basis of above spectral evidences the 5 was identified as Kaempferol-3-O-β-D-glucoside.

Compound 6, FABMS (NBA) of 6 gave a molecular ion m/e, 463 $[M – H]^-$, and other prominent ion at 301 $[M – 162 – H]^-$ showing loss of glucose moiety from aglycone, quercetin. It has also been supported by the acid hydrolysis of compound with 2NHCl as it produced quercetin (CoPC) and glucose (CoPC). ^{1}HNMR of the compound in aglycone region gave two meta coupled doublets each with J=2.0 Hz at 6.20 and 6.40 for H-6 and H-8 of phloroglucosiol type A-ring and ABX system for three protons at 6.86 (1H, d, J=8.5 Hz), 7.58 (1H, dd, 2.0 and 8.5 Hz) and 8.01 (1H, d, J=2.0 Hz) assignable to H-2`, H-6` and H-2` respectively of B-ring. The ^{1}HNMR of compound in sugar region was found similar to the compound **5**. The compound **6** was identified as Quercetin-3-O-β-D-glucoside.

Antioxidative activity of flavonoidal compounds isolated from BuOH fraction

Six flavonol glycosides 1, 2, 3, 4, 5 and 6 were isolated from FRAC-1, FRAC-2 and FRAC-3 derived from 20% HOAc cellulose CC fractionation of BuOH fraction of aqueous-ethanolic extract of *Cheilanthus anceps*. Methanolic solution of each isolate was examined for antioxidative activity by the standard method of thin layer autobiography using SiO_2 as an adsorbant and methanolic solution of DPPH (0.02%, 2,2 diphenyl-1 (2,4,6-trinitrohydrazide) as a spraying reagent (Cuendet et al., 2000; 2001; Chacha et al., 2005). Thin layer autobiography of compound **1** to **6** revealed that the compounds **2**, **3**, **4** and **6** gave active spots as they produce yellow spots against purple background.

Finally the antioxidative activity of compounds **2**, **3**, **4** and **6** confirmed by UV-VIS spectrophotometer. 30µl methanolic solution of each isolate and 200µl of MeOH were added to 50µl of a 0.02% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30mins. and the percentage of activity was calculated. The decreasing sequence of activity among the active compounds was found in the order 6>3>2>4. The compounds 1 and 5, the glycosides of Kaempferol, did not show any activity while the glycoside derivatives of Quercetin were found to be active. Among the active glycosides of Quercetin, the compound 6, a Quercetin-3-O-β-D-glucoside was identified as a prominent antioxidative compound. It has further been established that Quercetin-3-O-monoglycosides are comparatively more active than Qurecetin-3-O-oligosaccharides and Quercetin-3,7-dioglycosids. Quercetin, a non glycosidic flavonol derivative and a widely distributed naturally occurring pigment of various food and fodder plants, has a more pronounced antioxidative activity compared to its glycosides (Yamashita and Kawanishi, 2000; Rietjens et al., 2005; Cantero et al., 2006). It was concluded that

Quercetin attached with more sugar moieties is less antioxidative.

Acknowledgements:

Authors are grateful to the Head, Botany Department Kumaun University, S.S.J. Campus, Almora to carry out this work.

Corresponding Author:
Harish Kumar
Department of Botany and Microbiology
Gurukul Kangri University
Haridwar, Uttarakhand 249404, India
E-mail: harish2129@gmail.com

4. References

[1] Pandey PC, Pandey HC. Pteridology in Western Himalaya. Bishan Singh Mahendrapal Singh, Dehradun, Uttarakhand India. 2002. p. 49-53.

[2] Chopra RN, Chopra IC, Handa KL, Kapoor LD. Medicinal Ferns. In: Chopra's Indigenous Drugs of India. UN Dhar and Sons, Calcutta. 1958. 2nd edition. p.60.

[3] Lal B, Vats SK, Singh RSD Gupta AP. International congress on Ethnobiology, Lucknow, U.P., India.1994, 17-21 Nov. p. 143.

[4] Banerjee RD, Sen SP. Economic Botany1980; *34*(3): 284-298.

[5] Wollenweber E, Dorr M, Fritz H, Papendieck S, Yatskievych G, Roitman JN Zeitschritt fűr Naturfor chung 1997; *52*: 301.

[6] Salatino MLF, Prado J. Biochemical Systematics and Ecology 1998; *26*: 761-769.

[7] Mabry TJ, Markham KR, Thomas MB. The Systematic Identification of Flavonoids. Springer, New York. 1970. p. 11.

[8] Markham KP, Mabry TJ. In: JB Harborne, TJ Mabry & H Mabry. The flavonoids. Chipman and Hall, London. Chapter 2. 1975. p. 46-63

[9] Willians CA, Grayer RJ. Nat. prod. Rep 2004; *21*:539-373.

[10] Altona C, Haasnoot CAG. Org. Magn. Reson 1980; *13*: 417.

[11] Cuendet M, Potterat O, Salvi A, Testa B, Hostettmann K. Phytochemistry 2000; *54*: 871-874.

[12] Cuendet M, Potterat O, Hostettmann K. Phytochemistry 2001; *56*: 631-636.

[13] Chacha M, Bojase-Moleta S, Majinda RRT. Phytochemistry 2005; *66*: 99-104.

[14] Yamashita N, Kawanishi S. Free Radical Research 2000; *33*:623-633.

[15] Cantero S, Campanella C, Mateos S, Cortes F. Topoisomerase II inhibition and high yield of endoreduplication induced by flavonoids, luteolin and Quercetin; 2006. p. 321-325.

[16] Rietjens IMCM, Boersma MG, Woude HVD, Jeurissen SMF, Schutte ME, Alink GM. Mut. Research 2005; *574*: 124-138.

16/3/2010