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Research Paper

Antioxidant Activity Determining Catechol Grouping Flavonol Glycosides from the Flowers of *Aconitum hetrophyllum*

***Pappu Kumar, Chanyal Beena and Verma D. L.**
Dept. of Chemistry, Kumaun University, S. S. J. Campus, Almora, (U. K.), INDIA

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Abstract - *Aconitum hetrophyllum (family, Ranunculaceae) is native to high reaches of Kumaun Himalaya. The extracts derived from the plant, Aconitum hetrophyllum have widely been used as a traditional medicines to cure diseases associate inflammations, cardiovascular, neuro-degenerative and other ailments, microbial, bronchitis and joint pains. Various biological activities, anti-inflammatory, anticarcerogenic, anti-spasmolytic and anti-microbial have been identified from the aqueous extracts of the plant. Although the principle biological active compound, aconite a ditertepenoid alkaloid and its various derivatives have previously been isolated from the plant. Recognizing an enormous traditional medicinal uses of the plant, the aqueous-methanolic extract derived from its flowers was investigated for antioxidative potential and activity bearing flavonol glycosides. The catechol-grouping flavonoids, a highly antioxidant active compounds, were isolated.*

Keywords: *Aconitum hetrophyllum*, flavonoids, antioxidant activity, quercetin.

Introduction

Aconitum, a genus of flowering plants of family Ranunculaceae, comprises 90 species distributed throughout the temperate and sub-temperate regions of the world. Family Ranunculaceae comprises about 20 genera and 300species with cosmopolitan in distribution. *Aconitum hetrophyllum* is herbal constituent of alpine pastures of Central Himalayas, ranging altitude, 3500m to 4000m. The herb has been characterized with the presence of blue and yellow colored spur bearing flowers and the height of the plant is up to 1m to 1.8m. It has been used as traditional medicines to cure various ailments, stomach febrifuge, dyspepsia, abdominal pain, analgesic and diabetes [2]. The genus *Aconitum* is characterized by the presence of diterpenoid alkaloids [1]. Aconite, a diterpenoid alkaloid and a principle biologically active compound, is used as a life saving drug in Ayurvedic and Unani system of medicines. The flavonoids a major group of natural products from genus *Aconitum* following the alkaloids, have previously been isolated and characterized from its various species [4]. It has been established that the curing of various disease from the extracts of traditional medicinal plants have been attributed to the presence of polyphenolic compounds. The flavonol glycosides have previously been identified from the antioxidant bioassay-guided fractionation of aqueous extracts of *Aconitum* herbs and it has also been established that some caffeoyl glycosides of quercetin, a catechol grouping polyphenolics, have been identified to have more antioxidant potential compared to quercetin and dihydroquercetin [3]. Literature survey revealed that the aqueous extracts derived from *Aconitum hetrophyllum* is still awaited to chemical investigation for various biological activities and active flavonoid glycosides.

Material and Methods

Plant material and authentification: *Aconitum hetrophyllum* was collected from the alpine pastures of Kumaun Himalayas ranging altitude, 3500-4000m in the month of August 2010. Its authentification has been carried out from BSI, Dehradun (U.K) and its voucher specimen No, MPH-61, has been deposited in the Botany Department of Kumaun University at S.S.J Campus Almora , Uttarakhand , India.

Extraction and isolation of plant material: 600gms of air dried and powdered flowers were extracted sequentially with 80% MeOH and 50%aq. MeOH by cold percolation methods for six days. The two extracts were combined and reduced in vacuo until only H_2O layer (approx.50ml) remained. It was partitioned successively with n-hexane, diethyl ether and n-BuOH. Each partitioned was examined for antioxidant activity by the standard TLC-DPPH (thin layer chromatography and 2, 2-diphenyl-1-picrylhydrazyl), UV-VIS-DPPH and UV-VIS-ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) methods. Among all

these fractions, n-BuOH exhibited potent antioxidant activity as it reduced intensity of DPPH peak about 80% in UV-VIS spectrophotometer at 518 nm. The residue of n-BuOH soluble was chromatographed on cellulose CC (column chromatography) using 30% HOAc as an eluent. Two dark purple fluorescent bands were observed and each was eluted and collected separately by monitoring under UV light at 360nm. The eluates derived from faster and slower moving bands representing fraction A and B. respectively. The antioxidant activity of each fraction was determined by observing the concentration required to produce 50% quenching of free radical DPPH at 518 nm. The fraction-1 and fraction-2 produced 50% quenching with 14(µg/ml) and 360(µg/ml) concentrations, respectively. The fraction 1, a potent antioxidant activity bearing fraction, was chromatographed on sephadex LH-20 CC using MeOH as an intial eluting solvent followed by 10% MeOH, 20% MeOH, 30% MeOH and 50%MeOH. A total of 30 fractions (each with 50ml) were collected. Each fraction was monitored on cellulose CC (column chromatography) using 15% HOAc as an eluent and ammonical silver nitrate and Naturstoff reagent (NA) as a spraying reagents. All the fractions have been classified into three major fractions resulted after the combination of chromatographically similar fractions 6-10, 16-22 and 26-30 and afforded compounds 1, 2 and 3, respectively.

Results and Discussion

Compound 1 gave positive colour reactions with $FeCl_3$, Mg^+ HCl and α-naphthol indicating a flavonoid glycoside. The dark purple fluorescent spot of 1 turned to yellow green with NH_3 and orange with NA reagents in presence of UV light, supporting an aromatic system of the compound exhibited free hydroxyl groups at positions 5, 3' and 4' [5]. Complete acid hydrolysis of 1 with HCl gave quercetin caffeic acid and glucose and were identified by comparing with their standards on PC using three different solvent system, 15% HOAc, BAW (4:1:5) and BEW (4::2.2). LC-MS (negative mode /deprotonated molecule) gave a molecular ion at m/e 949 $(M-H)^-$ and other prominent ions were observed at m/e 787, m/e 463 and m/e 301 resulted after abstraction of caffeic acid and three moles of glucose from quercetin.

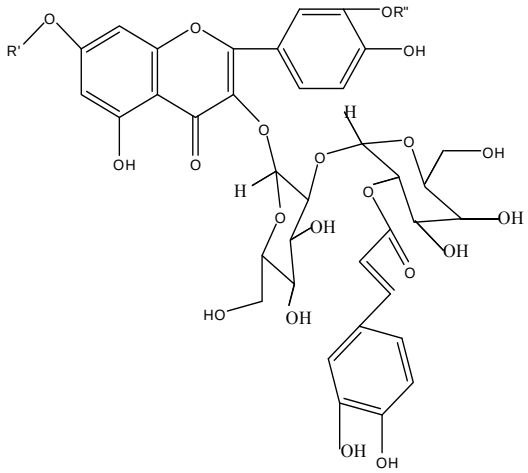

Figure -1

Compounds:

	R'	R"
1.	glucose	H
2.	glucose	CH_3
3.	Rhamnose	H

Molecular formula of 1 was established as $C_{42}H_{46}O_{25}$ on the basis of HRESIMS. Alkali hydrolysis of 1with $NaHCO_3$ gave a dark purple fluorescent compound 1(a) and caffeic acid (CoPC). The compound 1(a) exhibited molecular formula, $C_{33}H_{38}O_{22}$ (LC-MS), $(M-H)^-$at 787 and prominent ions observed at m/e 625and m/e 301. The (1a) was identified as quercetin -3-O-ß-D-glycopyranosyl (1→2)-glucopyranoside-7-O-ß-D-glucopyranoside on the basis of 'HNMR studies (DMSO-d_6, 400MHZ).

'HNMR of 1(a) showed five aromatic signals at £6.41 (1H(d) 2.0Hz), £6.74(1H,d,2.0), £6.80(1H,d,8.5Hz), £7.51(1H,d,2.0Hz) and £7.69 (1H,d,2.0 and 8.5Hz) representing, H-6, H-8, H-3', H-2' and H-6', respectively of quercetin. Two anomeric proton signals appeared at £5.80 (d, 7.0Hz) and £5.16(d, 7.5Hz) were attributed to two glucose moieties (ß-cofiguration) attached directly to quercetin nucleus at C-3 and C-7, position. A high field anomeric proton signal appeared at £4.67 (d, 7.6 Hz) represent at glucose moiety linked to primary glucose (C-3) by 1→2 interglycosidic linkage [6].

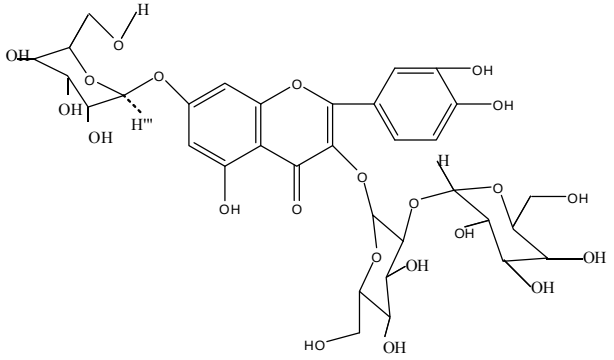

Figure -1(a)

The compound 1, which produced caffeic acid and 1(a) after alkali hydrolysis was analysed by 'HNMR (DMSo-d6, 400MHz).'HNMR of 1 showed two sets of ABX one AB system.The downfield ABX system comprises proton signals at £6.80(1H,d,8.5Hz) £7.51(1H,d,2.0Hz) and £7.69 (1H,dd,J=2.0 and 8.5Hz) for H-5, H-2' and H-6', respectively of B-ring protons of quercetin and high field ABX system forming protons appeared at £6.70 (1H,d, J=8.5 Hz), £6.88 (1H,dd,8.5, 2.0Hz) and £7.0 (2.0Hz) were representing to H-5"', H-6"' and H-2"', respectively of caffeoyl group. The A ring of quercetin is characterized by the presence of two meta coupled doublets appeared at £6.40 (1H,d,2.0Hz) and £6.74 (1H,d,2.0Hz) broad signals appeared at £6.23 (1H,d,16.2Hz) and £7.41 (1H,d,16.2Hz) were assignable to α and ß protons, respectively of cinnamoyl double bond. On comprising the proton shifts of t-glucose moieties of 1 and 1(a) the down field shift of anomeric proton (1"') and 2"' of compound 1(a) than to the

corresponding proton signals of 1 indicating caffeoyl group substitutes the 2"' hydroxyl group of t-glucose. Thus, compound 1 was identified as quercetin -3-O-ß-(2"-trans–caffeoyl)-ß-glucopyranosyl (1→2)-ß.glucopyranoside-7-O-ß-D-glucopyranoside.

Compound 2 appeared as a dark –purple fluorescent on PC and turned to yellow with NH_3 and orange with NA in presence of UV light (360nm), indicating a flavonoid with free hydroxyl groups at 5-3' and 4'-positions (Mabry et al.,1970; Markham, 1982). The methanolic solution of 2 gave positive tests to $Fecl_3$, $K_4Fe(CN)_6$, Mg+HCl and α –naphthol, idicating a flavonoid glycoside. LC-FABMS of 1 (in a negative mode) gave a molecular ion at m/e 933$(M-H)^-$ and other prominent ions observed at m/e 787(m/e 933-rhamnose)$^-$, m/e 301 (m/e 787- (glu-gluco-caffeoyl)$^-$supporting the release of two moles of glucose, one each caffeoyl and rhamnose from quercetin, complete acid hydrolysis of 2 with 2NHCl afforded quercetin (CoPC),caffeic acid (CoPC), glucose (CoPC) and rhamnose (CoPC). Alkali hydrolysis of 2 with $NaHCO_3$ gave caffeic acid (CoPC) and a dark purple fluorescent compound on PC under UV light and it was referred to as 2(a). FABMS(-) gave a molecular ion at m/e 771 $(M-H)^-$ and other prominent ions observed at m/e 625(m/e 771-rhamnose)$^-$ and at m/e 301 (m/e 625-(glucose – glucose)$^-$ indicating the release of two molecule of glucose and one rhamnose from quercetin further , H_2O_2 oxidation of 2(a)afforded a disaccharide, sophrose (CoPC) and a yellow fluorescent compound under UV light , referred to as 2(a-1). FABMS (-) of 2(a-1) gave a molecular ion at m/e 447$(M-H)^-$ and other prominent peak observed at m/e 301(m/e 447-rhamnose)$^-$ supporting the release of rhamnose from quercetin. The chromatographic behavior, UV, 'HNMR and MS data of 2(a-1) were found similar to those reported to quercetin -7-O-α-rhamnopyranoside (CoPC). Thus, 2(a) was identified as quercetin-3-O-ß-glucosyl (1→2)-ß-glucopyranoside-7-O-α-L-rhamnopyranoside. The structure 2(a) was further supported by 'HNMR (DMSO-d_6, 400MHz) studies.

The 'HNMR of 2(a) shoed five aromatic proton signals at £6.40 (1H, d 2.0Hz), £6.74(1H, d, 2.0Hz), £6.80 (1H, d, 8.5Hz), £7.51 (1H,D,2.0Hz) and £ 7.70 (1H, dd, 2.0 and 8.5 Hz) were found similar to the corresponding proton signals, H-6, H-8, H-5, H-2' and H-6' of aglycone quercetin. The aliphatic region of 2(a) was characterized by the presence three anomeric proton signals, appeared at £5.75 (d, 7.2Hz), £7.40 (d, 1.5 Hz) and 4.65 (d, 7.5 Hz) were attributed to p-glucose (C-3), p.rhamnose (C-7) and t-glucose (C-2) respectively. The t-glucose linked to C-2" of p-glucose by inter –glycosidic linkage (1→2) (Altoona and Haasnoot, 1970). A highly downfield doublet appeared at £1.30 (3H, d, J=6.5Hz) was characterized to methyl protons of rhamnose sugar. Thus, the compound 2(a) was finally confirmed as quercetin -3-O-ß-glucosyl (1→2)-ß-glucopyranoside -7-O-α-L-rhamnopyranoside.

Enzymatic hydrolysis of 2 with α-rhamnosidase gave rhamnose sugar and dark purple fluorescent compound, 2(b). The 2(b) on alkali hydrolysis produced caffeic acid (CoPC) and quercetin-3-O-sophoroside 2(c). Thus, it has been concluded that the caffeoyl group is linked to sophrose sugar. On comparing the 'HNMR spectra of 2 with 2(a), the downfield shift of the proton linked to C-2" position of t-glucose was observed to that of its corresponding proton of the compound 2(a). Thus, it has been established that the caffeoyl group of t-glucose. Thus, compound 2. Was identified as quercetin -3-O-(2"-Trans caffeoyl)-glucosyul (1→2)-ß-D-glucopyranoside-7-O-£-L-rhamnopyranoside.

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