Quercetin Glycosides From Antioxidative Active Aqueous Ethanolic Extract Of Lepidium Ruderale Linn

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Abstract: *Lepidium* Linn, (fam. Brassicaceae) Comprises 150 Species with Cosmopolitan in distribution. *Lepidium i*s a rich source of vegetable producing traditional medicinal plants of Kumaun Hills. *Lepidium ruderale* Linn, Commonly known as Ban Halam in Kumaun Hills, is a food, fodder and Medicinal Plant of the region. It has been used to cure cough, asthma, piles, depurative, skin diseases and rheumatism by the tribal inhabitants of central Himalaya. Glucosinolates, a class of naturally occurring thioglycosides and a prominent constituents of various members of *Lepidium*, have been identified for a wide range of biological activities, antifungal, antibacterial and insecticidal. *Lepidium ruderale* Linn, is a small herbaceous weed with 30 to 45 cm in height, leaves of the plant have been used as a vegetable by local inhabitants, of kumaun hills and is a good fodder for sheeps and goats. *Lepidium ruderale* has been screened for various biological activities, antifungal, antibacterial and antiviral

[Jyoti Agarwal And D.L. Verma. **Quercetin Glycosides From Antioxidative Active Aqueous Ethanolic Extract Of Lepidium Ruderale Linn.** Academia Arena, 2011;3(3):25-33] (ISSN 1553-992X). http://www.sciencepub.net.

Keywords: Quercetin; Glycosides; Antioxidative; Aqueous; Ethanolic; Lepidium Ruderale Linn

INTRODUCTION

Lepidium Linn, (fam. Brassicaceae) Comprises 150 Species with Cosmopolitan in distribution. *Lepidium i*s a rich source of vegetable producing traditional medicinal plants of Kumaun Hills *(Chopra et al, 1956). Lepidium ruderale* Linn, Commonly known as Ban Halam in Kumaun Hills, is a food, fodder and Medicinal Plant of the region. It has been used to cure cough, asthma, piles, depurative, skin diseases and rheumatism by the tribal inhabitants of central Himalaya (Chopra et al, 1956). Glucosinolates, a class of naturally occurring thioglycosides and a prominent constituents of various members of *Lepidium*, have been identified for a wide range of biological activities, antifungal, antibacterial and insecticidal (Fahey et al, 2001, burrow et al, 2007). *Lepidium ruderale* Linn, is a small herbaceous weed with 30 to 45 cm in height, leaves of the plant have been used as a vegetable by local inhabitants, of kumaun hills and is a good fodder for sheeps and goats. *Lepidium ruderale* has been screened for various biological activities, antifungal, antibacterial and antiviral (Katar and Akulyan, 1971). The Benzyl glucosinolate, the principle constituents of *Lepidium ruderale*, showed anti- amoebic activity (Acevedo et al, 1993).

It has been recognized that various traditional Medicinal uses of the plants have been attributed to the presence of flavonoids present in their extracts. (Kuehnau, 1976). Flavonoids, a class of heterocylic polyphenolic compounds, and a major constituents of various food and fodder plants, have been identified as a potential antioxidative and enzymes inhibition substances (Jovanovic et al; 1994, Shui and Peng, 2004). The antioxidant ability of flavonoids resides mainly in their tendency to donate hydrogen atoms and thereby scavenge the free radicals generated during lipid peroxidation. The antioxidative potential of the flavonoids have been associated for curing various disease related to oxidative stress like inflammatory, LDL, HDL, Cancer, neuro-degenerative, and cataract (Bor's et al, 1990). Hydroxyl and methoxyl substitutions in the flavonoids play a vital role in the antioxidative potential determination (Arora et al 1997). It has been observed that an antioxidant potential of flavonoids increases with introducing ortho-dihydroxyl group (catechol grouping) in the B-ring (Rice-Evans and Miller, 1996). Quercetin, a catechol grouping flavonol and the most abundant constituent in fruits and vegetables, has a higher antioxidative potential than vitamins C anl E and find enormous biological activities (Arora et al, 1997). Present chemical investigation reveals the presence of catechol grouping, particularly quercetin glycosides from anti-oxidative

activity guided fractionation of aqueous-ethanolic extract of *Lepidium ruderale*. Although, quercetin and some uncharacterized aglycones have previously been isolated from various members of *Lepidium* (Fursa et al 1970, Justeen, 2000). Literature survey revealed that flavonol glycosides are still awaited to investigation from antioxidative active fraction of *Lepidium ruderale.*

MATERIAL AND METHODS

1. AUTHENTIFICATION OF PLANT SPECIES:

Aerial parts, leaves, petiole and inflorescence of the plant, *Lepidium ruderale* was collected from kumaun hills ranging altitude, 1600 m to 1800 m in the month of July 2009. Its herbarium specimen, No. 201 has been deposited in the Department of plant chemistry, Kumaun University, Campus Almora and its authentification has been made from BSI Dehradun (Uttarakhand) and finally by Prof. Y.S. Pangati, Emeritus Professor, Department of Botany, Kumaun University, Nainital (Uttarakhand).

2. METHODOLOGY:

(A) Extraction of plant species; 3 kg air dried and powdered aerial parts of *Lepidium ruderale* was extracted sequentially with 70% aq. EtOH and 50% aq. EtOH by cold percolation method for six days. The two extracts were filtered and combined.The combined extract was concentrated under reduced pressure until only H_2O layer (approx. 50 ml) remained. It was partitioned with CH_2Cl_2 : H_2O (1:1). After separating CH_2Cl_2 layer, H_2O layer was further partitioned with n-BuOH.

(B) The n-BuOH soluble was chromatographed on cellulose (Merck) cc using 40% HOAc as a developing solvents,Three fluorescent bands, a blue between faster and slower moving dark purple, were observed on CC and each was eluted and collected separately in order to decreasing mobilities by monitoring with UV (360 nm) light and representing FRAC-I, FRAC-II and FRAC-III, respectively.

(C) The methanolic solution of the chromatographically isolated fractions, FRAC-I, II and III were evaluated separately for antioxidative activity against the free radical DPPH in UV-VIS spectrophotometer at 518 nm and quercetin has been used a standard.

(D) Identification of catechol grouping flavonoids from FRAC-I, II and III using 2 DPC and spraying regents;

I. 2 DPC examination of FRAC-l:

FRAC-l, an elute of faster moving dark purple fluorescent band derived from 40% HOAc fractionation of n-BuOH soluble on celluose CC, gave four dark purple fluorescent spots on PC at RF 48, 42, 40 and 39 in BAW (4:1:5, upper layer) and marked as spot-1, spot-2, spot-3 and spot-4, respectively. The dried and developed 2 DPC was sprayed with ammonical solution of $AgNO_3$ and methanolic solution of 1% NA (Naturstoffreagenz-A) reagents to assign the presence of total catechol grouping flavonoids. Spot-2, 3 and 4 gave positive tests for catechol groping flavonoids while the spot-1, a faster moving component, did not give positive test.

II. 2 DPC Examination of FRAC-II.

FRAC-II, a blue green fluorescent and a medium polarity fraction produced on cellulose CC between faster and slower moving dark purple fluorescent bands after fractionation of n-BuOH soluble with 30% HOAc, gave three blue green fluorescent spots on 2 DPC. Non of these spots gave positive tests for flavonoids.

III. 2 DPC Examination of FRAC-III

FRAC-III an elute of slower moving dark purple fluorescent band derived from 40% HOAc fractionation of n-BuOH soluble on cellulose CC gave four spots on 2DPC at Rf 69, 62, 60, and 58 in BAW (4:1:5, v/v, upper layer) and marked as spot-5, spot-6, spot-7 and spot-8, respectively. After spraying dried and developed 2DPC with ammonical silver nitrate and NA reagent, the spot-6, and spot-7 gave positive and spot-5 and spot-8 gave negative tests for catechol-grouping flavonoids.

(E) Isolation of catechol grouping flavonoids from FRAC-I;

FRAC-I, a three catechol grouping flavonoids bearing fraction, was chromatographed on whatman NO.3PC and repeatedly developed three times, with BAW (n-BuOH-AcOH-H_2O, 4:1:5, v/v, upper layer). Two dark purple fluorescent bands were separated on PC under UV light (360 nm) and each was eluted and collected separately. An elute derived from faster moving band gave

only one spot and it was identified as spot No.1 as evidenced from CoPC with FRAC-I. An elute derived from slower moving band comprising three spots on PC under UV light and were identified as spot-2, spot-3 and spot-4 by CoPC with FRAC-I. An aqueous ethanolic elute of slower moving band was evaporated to dryness under reduced pressure and it was chromatographed on sephadex LH-20 CC using MeOH $-H_2O$ (1:1) as an eluent. Three dark purple fluorescent bands were observed on CC and each was eluted and collected separately in order to their decreasing mobilities. Three compounds, representing structures, 5, 4 and 3. were isolated from faster, middle and slower moving bands, respectively.

(F) Isolation of catechol grouping flavonoids from FRAC-II;

FRAC-II an elute derived from slower moving dark purple fluorescent band observed on CC after 40% HOAc fractionation of n-BuOH soluble an cellulose column chromatogram and a two catechol grouping flavonoids bearing fraction, was repeatedly chromatographed on whatman NO.3PC using BAW (4:1:5) as a developing solvent. Three dark purple fluorescent bands were observed on paper chromatogram and each was cut and eluted separately in order to their decreasing mobilities. An elute derived from faster and slower moving bands gave spot -5 and spot-8, respectively. An elute derived from middle band was evaporated to dryness under reduced pressure and it was chromatographed on sephadex LH-20 CC using 55% MeOH as an eluent. Two dark purple fluorescent bands were observed on CC and each was eluted and collected separately. An elute derived from faster and slower moving bands gave compounds, 1 and 2 respectively.

RESULTS AND DISCUSSION

The compounds (1-5), an antioxidative positive, have been isolated from catecheol grouping flavonoids bearing fractions of n-BuOH soluble, were identified as follows:

Compounds (1), a dark purple fluorescent on paper chromatogram under UV (360 nm) light, gave positive colour reactions to $FeCl_3$, Mg + HCl and α - naphthol, indicating a flavonoid glycosidic compound. Complete acid hydrolysis of 1 afforded an aglycone with dull yellow fluorescent spot on paper chromatogram under UV light and a sugar moiety, rhamnose (CoPC). The dull yellow fluorescent spot of an aglycone on PC under UV light with and without the presence of NH_3 vapours turned to orange after spraying with NA (Naturstoffreagenz-A) reagent indicating a flavonol with free hydroxyl groups at C-5, C-3 and C-4 (Mabry et al; 1970).

EIMS of the aglycone gave a molecular ion at m/e 302 (M)+ for $C_{15}H_{10}0_7$. ^{1}HNMR of the aglycone is DMSO-d_6 (400 MHz) gave five aromatic proton signals at δ 6.20(1H,d,J=2.0Hz), δ 6.42 (1H, d, J = 2.0 Hz), δ 6.90 (1 H, d, J=8.5 Hz) , δ 7.55 (1H, dd, J=8.5 and 2.0 Hz) and δ 7.69 (1 H, d, J=2.0 Hz) were assigned to H-6, H-8, H-5 , H-6 , and H-2 respectively. The chromatographic behavior, MS and ^{1}HNMR spectral data of the aglycone were found similar to those reported in the literature to quercetin (Mabry et al. 1970).

FABMS (-) of the compound (1) gave a molecular ion at m/e 448 and other prominent ion was observed at m/e 302 (m/e 463-rhamnose), representing the release of rhamnose sugar from quercetin. The glycoside appeared as dark purple fluorescent on PC while its acid released aglycone gave dull yellow colour indicating the release of rhamnose sugar moiety from 3-position of quercetin. Finally, the compound (1) was identified on the basis of its ^{1}HNMR in DMSO-d_6 (400 MHz).

^{1}HNMR of 1 showed five aromatic proton signals at δ 6.18 (1H,d,2.0 Hz), δ 6.40(1H,d,2.0Hz), δ 6.88(1H,d,8.5 Hz), δ 7.20 (1 H, dd, 2.0 and 8.5 Hz) and δ 7.32 (1H, d, 2.0 Hz) were asisgnable to H-6, H-8, H-5 , H-6 , and H-2 respectively of aglycone, quercetin. An aliphalic proton signal appeared at δ 5.25 (1H, d, J=1.5 Hz) was attributed to an anomeric proton signals of rhamnose (α-configuration). A three proton doublet appreared at δ 1.20 (3H, d, J=6.0) represent CH_3 group of rhamnose. Remaining protons sugar, rhamnose appeared at δ 3.0 to δ 4.0 on the basis of acid hydrolysis, MS and ^{1}HNMR studies, the compound 1 was identified as quercetin - 3.-O - rhamnopyranoside.

Compound (2), a dark purple fluorescent on PC under UV light (360 nm) and a chromatographically similar to compound 1 with the reagents, UV, UV/NH_3, UV/ NA and UV/ $ZrOCl_2$+ citric acid but a slightly slower moving component than to 1 in BAW (4:1:5) was identified as a flavonoid glycoside on the basis of its colour reactions with $FeCl_3$, Mg + HCl and α-naphthol. FABMS (–) of compound (2) gave a molecular ion at m/e 463 $(M-H)^-$ and a prominent ion observed at m/e 301 (m/e 463-glucose), indicating the release of glucose moiety from quercetin. Complete acid hydroysis of 2, with 2NHCl gave an aglycone, quercetin (CoPC) and a sugar, glucose (CoPC). The compound (2) was identified as quercetin -3-O-β-glucopyranoside on the basis of ^{1}HNMR (in

DMSO-d_6-400 MHz) studies; The ^{1}HNMR of compound 2 in aglycone region was found similar to the corresponding signals of aglycone of compound (1). In aliphatic region, a doublet appeared at δ 5.60 (1 H, d, J=7.5 Hz) was correspond to the anomeric proton signal of glucose (β-configuration) and remaining signals of glucose sugar appeared between δ 3.0 to δ 4.0.

Compound (3), a dark purple fluorescent on paper chromatogram under UV light turned to yellow green with NH_3 vapours, indicating a flavonoid with the presence of 4 ,5 dihydroxyl system (Mabry et al; 1970). The dark purple fluorescent spot of compound on cellulose TLC turned to orange with NA reagent, indicating the presence of ortho-dihydroxyl group in the B-ring (Markham, 1982). Ethanolic solution of B gave positive colour reactions with $FeCl_3$, Mg + HCl and α-naphthol, supporting the flavonoid glycosidic nature. The Compound (3) was completely acid hydrolyzed with 2NHCl at 100^{O}C for two hours and the hydrolysed reaction mixture as neutralized with $BaCO_3$ followed by partition with isopropanol. Paper chromatographic examination of isopropanol soluble gave three spots at Rf 60, 31 and 21 in BAW (4:1:5, v/v, upper layer). The spot at Rf 60, a faster moving component on PC and a dull yellow fluorescent under UV light with and without fuming NH_3 vapours, was identified as quercetin (CoPC). The slower moving spots on PC at Rf 31 and 21, a non UV fluorescent, and visualised after spraying with Benzidine reagent followed by heating at 110^{O}C for 10 minutes, were identified as rhamnose (CoPC) and glucose (CoPC) respectively. FABMS (-) of the compound (3) gave a molecular ion at m/e 609 $(M-H)^-$ and other prominent ions observed at m/e 463 (m/e 609 - rhamnose)$^-$ and m/e 301 (m/e 463 - glucose)$^-$, indicating the release of rhamnose and glucose from quercetin. H_2O_2 oxidation of 3 gave quercetin (CoPC) and a rutinose (CoPC) sugar. Enzymatic hydrolysis of 3 with α-rhamnosidase gave a compound, quercetin-3-0-β-D- glucopyranoside (CoPC). Finally the structure of the compound (3) was identified as quercetin -3-O- rutinoside on the basis of ^{1}HNMR spectra (TMS ether in CCl_4);

^{1}HNMR of 3 showed five signals in aromatic region at δ 6.18, δ 6.38, δ 6.80, δ 7.40 and δ 7.50 were assignable to H-6, H-8, H-5 , H-2 and H-6 of quercetin. In aliphatic region two signals appeared at δ 4.20 (1H, d, 1.0 Hz) and δ 5.80 (1 H, d, 7.80 Hz) were attributed to the anomeric protons of rhamnose (α-configuration) and glucose (β-configuration) respectively. The rhamnosyl methyl doublet appeared at δ .90 (3 H, d, 6.0) and remaining 10 protons of glucose and rhamnose appeared δ 3.0 to δ 4.0. On the basis of ^{1}HNMR and enzymatic hydrolytic methods the compound (3) was identified as quercetin-3-rutionoside.

Compound (4), a dark purple fluorescent on paper chromatogram under UV light, was identified as a flavonoid glycoside on the basis of colour reactions with $FeCl_3$, Mg + HCl and α-naphthol. The dark purple fluorescent spot of 4 on cellulose TLC turned to yellow green with NH_3 vapours and orange with methanolic solution of Naturstoffreagonz-A reagent, indicating a flavonoid with free hydroxyl groups at positions C-5, C-3 and C-4 and 3-hydroxyl group is substituted (Mabry et al; 1970; Markham, 1982). UV spectra of 4 in MeOH gave λmax at 257 (band -II) and 358 (Band-I) and shifts obtained with diagnostic reagents, NaOAc (266, 378) and NaOAc/H_3BO_3 (261 and 373) suggesting the presence of a free hydroxyl group at position C-7 and further supporting the catechol grouping in the B-ring. FABMS (-) of 4 gave a molecular ion at m/e 625 $(M-H)^-$ and other prominent ions observed at m/e 463 (m/e 625-glucose) and m/e 301 (m/e 463-glucose), indicating the release of two molecules of glucose from an aglycone, quercetin. It has further been supported by complete acid hydrolysis of 4 with 2NHCl, which resulted formation of quercetin (CoPC) and glucose. An enzymatic hydrolysis of 4 with β-glucosidase afforded quercetin - 3-O-β-D- glucopyranoside (CoPC). Thus, it has been confirmed that one of the glucose moiety is linked to primary glucose moiety. The structure of 4 was finally identified by ^{1}HNMR and ^{13}CNMR is DMSO-d_6.

^{1}HNMR of 4 gave five signals in aromatic region δ 6.20 (1H, d, 2.0 Hz), δ 6.40 (1H, d, J=2.0 Hz), δ 6.83 (1H, d, 8.5 Hz), δ 7.55 (1H, d, J = 2.0 Hz) and δ 7.68 (1H, d, 8.5 Hz) were assignable to H-6, H-8, H-5 , H-2 and H-6 respectively.

Two doublets each with J = 7.5 Hz appeared at δ 5.30 and δ 4.00 were attributed to anomeric protons of glucose moietis, H-1" and H-1 (each with β-configuration), respectively and also suggesting 1 --> 2 linkage between two glucose (Altona and Haasnoot, 1980; Overend, 1972).

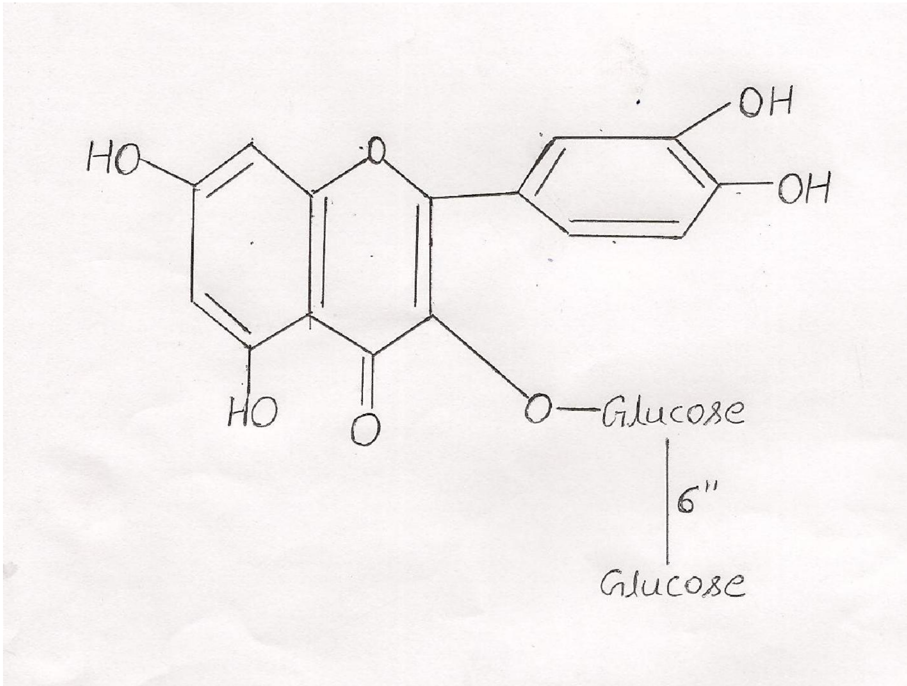
Table No. 1. ^{13}CNMR of 4 in DMSO-d_6 (ppm)

C-2	156.2
C-3	133.2
C-4	177.2
C-5	161.1
C-6	98.8
C-7	164.6
C-8	93.6
C-9	156.4
C-10	103.8
C-1	121.0
C-2	115.2
C-3	144.8
C-4	148.5
C-5	116.1
C-6	121.6

SUGAR REGION SUGAR REGION OF QUERCETIN-3-O-GLUCOSIDE

C-1″	100.8	100.9
C-2″	73.2	74.05
C-3″	76.6	76.5
C-4″	69.6	69.7
C-5″	76.3	77.4
C-6″	68.0	60.9
C-1	101.3	
C-2	74.0	
C-3	76.3	
C-4	69.7	
C-5	76.4	
C-6	60.7	

^{13}CNMR Spectra of Compound 4 (table NO.1) revealed the presence of 12 carbon signals. A downfield shift of C-6" at 68.0 of compound 4 compared to the corresponding signal C-6" of quercetin-3-O-β-glucopyranoside, at 60.9, indicating the 1 --> 6 linkage between two glucose moieties.



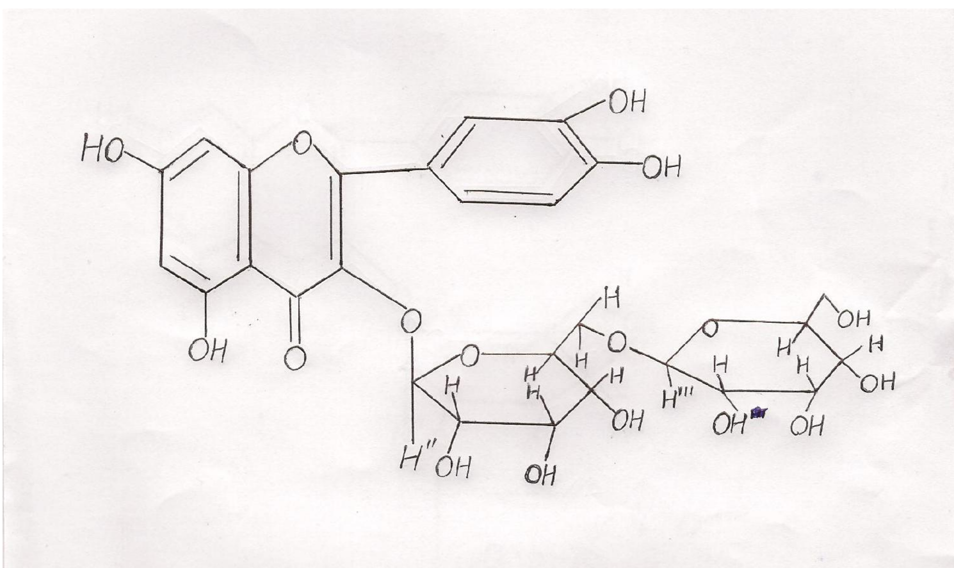


Figure 1. Chemical

COMPOUND (5), a dark purple fluorescent catechol grouping flavonoid glycoside on the basis of colour reactions with Mg + HCl, α-naphthol and NA (Naturstoffreagenz- A) reagents, gave a molecular ion at m/e 625 $(M-H)^-$ and other prominent ions were observed at m/e 463 (m/e 625 - glucose) and m/e 301 (m/e 463- glucose). Complete acid hydrolysis of 5 with 2NHCl gave quercetin (CoPC) and glucose (CoPC). Enzymatic hydroloysis of 5 with β-glucosidase gave quercetin -3-O-glucopyranoside (CoPC) and glucose (CoPC). MS spectral data, acid and enzymatic hydrolysed products of 5 were found similar to those reported to the compound 4. H_2O_2 oxidation of 5 afforded quercetin (CoPC) and sophrose (CoPC). Liberation of a disacharide sugar, sophrose from H_2O_2 clearly indicated that the glycosidic linkage between two glucose moieties has been conformed as 1 --> 2. Further, 1-->2 glycosidic linkage was confirmed by ^{1}HNMR studies of compound, 5 (in DMSO-d_6, 400 MHz).

Table No. 2. ^{1}HNMR of 5 in DMSO-d_6 (400 MHz)

Shift (δ)	**Multiplicity** J = Hz	**Attribution**
6.20	1 H, d, 2.0	H-6
6.40	1H, d, 2.0	H-8
6.83	1H, d, 8.5	H-5
7.53	1H, d, 2.0	H-2
7.66	1H, dd, 2.0, 8.5	H-6
12.60	1H, (brs)	5.OH
5.70	1H, d, 7.5	H-1"
4.65	1H, d, 1.0	H-1
3.0 - 4.0	(m)	Remaining protons of glucose.

^{1}HNMR of 5 in DMSO-d_6 showed five signals in aromatic region at δ 6.20 (1 H, d, J= 2.0 Hz), δ 6.40 (1H, d, J= 2.0 Hz) δ 6.83 (1 H, d, J= 8.5 Hz), δ 7.53 (1H, d, J = 2.0 Hz) and δ 7.66 (1 H, d, J = 2.0 Hz) were attributed to H-6, H-8,
H-5 , H-2 and H-6 , respectively.

Two doublets appeared in aliphatic region at δ 5.70 (1H, d, 7.5 Hz) and δ 4.65 (1 H, d, 1.0 Hz) were attributed to two anomeric protons, of H-1" and H-1 respectively of two glucose moietis linked with each other by 1-->2 linkage (Overend 1972; Altona and Haasnoot, 1980). On Comparing the ^{13}CNMR signals in the sugar regions of quercetin -3-O- glucoside (Table No.3). A β-glucidase enzymatic hydrolysed product of 5, with the corresponding 13 carbon signals of 5, (Table No.2) the down field shift of C-2" (74.3 --> 82.0) and C-1 signals, further, supporting the 1--> 2 linkage between two glucose moieties.

On the basis of chromatographic, hydrolytic (enzymatic and acid), ^{1}HNMR, MS and ^{13}CNMR studies,the compound 5 was identified as quercetin -3-O-β-glucopyranosyl (1-->2)- β-glucopyranoside.

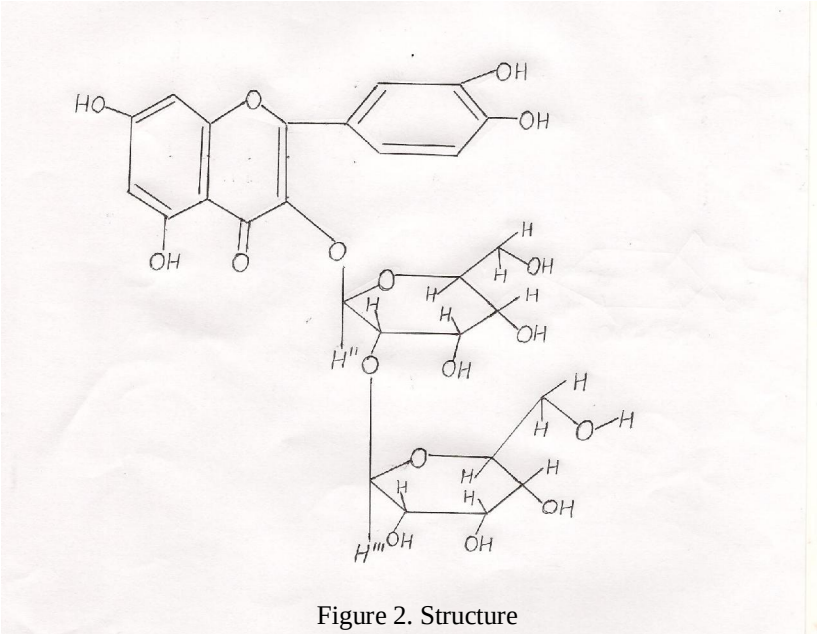


Figure 2. Structure

TABLE No. 3. ^{13}CNMR of 5 in DMSO-d_6 (sugar region)

C-1"	98.6
C-2"	82.0
C-3"	76.6
C-4"	70.0
C-5"	76.6
C-6"	61.0
C-1"'	103.6
C-2"'	74.3
C-3"'	76.7
C-4"'	70.5
C-5"'	76.7
C-6"'	61.4

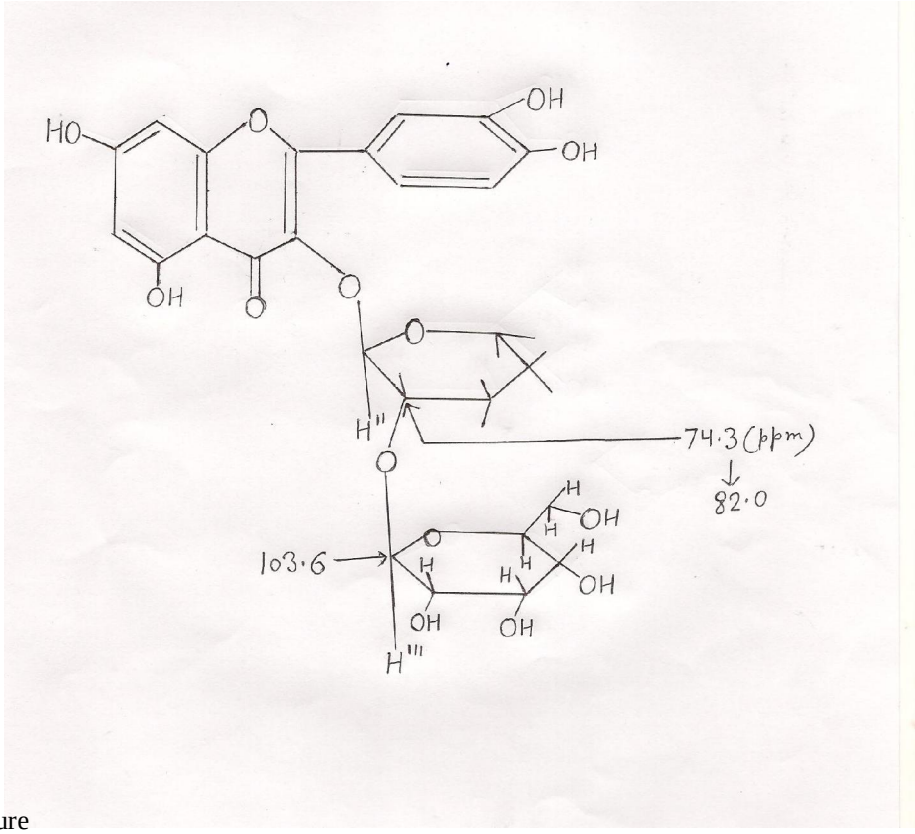


Figure 3. Structure

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1/12/2011