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Anti Bacterial Activity of Apigenin 7-O-(6''caffeoyl) neohesperidoside from chrysanthemum indicum

* M.Jerome Rozario** Dr.A.John Merina *** Dr.V.Srinivasana

* Dept. Of Chemistry, RMK Engineering College, Chennai

** PG and Research Dept., of Chemistry, Govt. College for Women (Autonomous), Kumbakonam

*** Dept of Chemistry, RMK Engineering College, Chennai

ABSTRACT

The fresh florets of chrysanthemum indicum is examined to contain the flavonoid Apigenin 7-O-(6''caffeoyl) neohesperidoside by Modern physical methods like UV, NMR and chemical reactions. PC and hydrolytic studies were used to ascertain the structure. The isolated Glycoside has been found to contain Anti-bacterial activity as it is compared with a standard.

Keywords : Antibacterial activity, Flavonoids, Apigenin 7-O-(6'' caffeoyl) neohesperidoside

INTRODUCTION

Chrysanthemum indicum Linn is a wild shrubby plant belongs to Compositae family. It is used in ayurveda and yunani. Crushed leaves contain apigenin 7-O-glucoside. The yellow florets from Chrysanthemum indicum were chosen for phyto-chemical investigation and the results are presented below.

EXPERIMENTAL

Extraction and fractionation

Fresh yellow coloured florets (2kg) of Chrysanthemum indicum collected from Bangalore during July – September were extracted with 85% methanol (5x500mL). The alcoholic extract was concentrated in vacuo. The aq. Concentrate was fractionated successively with light petrol (60-80°C x 250 ml), peroxide free Et₂O (4x250ml) and EtOAc (8x250ml). The EtOAc fraction alone was taken up for the study.

EtOAc fraction : (Flavone glycoside : Apigenin 7-O-(6'' caffeoyl) neohesperidoside)

The EtOAc fraction was concentrated in vacuo and left in an ice chest for few days. The yellow solid that separated when subjected to PC revealed the presence of a single glycoside. A yellow solid on recrystallization from methanol was obtained. It developed a green colour with alc. Fe³⁺ and orange red colour with Mg-HCl, yellow colour with NaOH and appeared as purple spot under UV turning yellow on exposure to NH₃. It responded to Wilson's boric acid and Gibb's test but did not answer the Horhammer-Hansel test. It responded to Molisch's test showing that it could be a flavones glycoside. It had λ_{max}. MeOH 265, 269sh 330; +NaOMe 245sh, 269, 301, 386; +AlCl₃, 276, 300, 348, 386; +AlCl₃-HCl 277, 299, 341, 382; +NaOAc 267, 256sh, 355 386; and +NaOAc-H₃BO₃ 267, 340mm. It had R_f values as recorded in Table - 1. It was identified as apigenin 7-O-(6'' Caffeoyl) neohesperidoside and the identity was confirmed by co and mixed PC and with an authentic samples of apigenin from Chrozophora rotteri².

Acid hydrolysis of the glycoside:

The glycoside (0.05g 0.1m mole) was dissolved in hot aq.MeOH was hydrolyzed with 7% H₂SO₄ at 100°C for about 2hr. The excess alcohol was distilled off in vacuo and the resulting aq. Solution was extracted with Et₂O. The residue from Et₂O fraction was studied as presented below.

Identification of the aglycone (Flavone : apigenin)

The aglycone recovered from the hydrolytic fraction of the

glycoside on recrystallization gave yellow needles (m.p346-480C,). It was soluble in organic solvents and sparingly soluble in hot water. It gave a red colour with Mg-HCl; brown colour with alc.Fe³⁺, golden yellow colour with NH₃ and appeared deep purple under UV changing to yellowish green on exposure to NH₃. It had λ_{max} MeOH 267, 296sh, 336; +NaOMe 275, 324, 392; +AlCl₃, 276, 301, 348, 384; +AlCl₃-HCl 276, 299, 340, 381; +NaOAc 274, 301, 376; and +NaOAc-H₃BO₃, 268, 302sh, 338mm and had R_f values as recorded in Table-1 and could be identified as apigenin and the identity was confirmed by co and mixed PC with an authentic sample of apigenin from Chrozophora rotteri.

Identification of sugar moiety :

The filtrate after the removal of the aglycone was neutralized with BaCO₃. The concentrated filtrate when examined by paper chromatography gave R_f values corresponding to those of glucose and rhamnose. The running properties of the glycoside were also in favour of a bioside. The identity of the sugars was confirmed by comparison with authentic samples of glucose and rhamnose. (Table-2).

Enzymatic hydrolysis with pectinase:

The glycoside was resistant to hydrolysis by pectinase which indicates that it is a neohesperidoside and not a rutinoside.

Partial hydrolysis of the glycoside :

The glycoside was subjected to partial hydrolysis by treatment with 10% formic acid in cyclohexane and the resulting solution extracted with ethyl acetate and subjected to PC. The R_f values of the EtOAc fraction agreed with those of quercetin 3-O-glucoside (isoquercetin). The R_f values are indicated in Table-I. On this basis it can be concluded that glucose is directly linked to the aglycone moiety.

Alkaline hydrolysis of the glycoside :

The concentrated aqueous solution of the glycoside (G7) (2mL) was mixed with 2M NaOH(5ml) in a 10ml syringe and the mixture was left in the syringe (with all air expelled) for two hours at room temperature. The mixture was ejected in to a vial containing 2M HCl (6ml) and evaporated to dryness in vacuo. The dried mass was extracted with warm ether. The residue from the other solution fluoresced green on PC under UV, had R_f (BAW) 0, 91, (water) 0.42, 0.85 and λ_{max}. MeOH 245, 325 and +NaOMe 306 sh, 334 nm. It was identified as caffeic acid². The remaining residue containing deacylated

glycoside exhibited the same λ_{max} in MeOH. Its R_f values are depicted in Table-1.

RESULTS AND DISCUSSIONS

From the fresh yellow florets of *Chrysanthemum indicum* apigenin-7-O-(6''-caffeoyl) neohesperidoside has been isolated. During a chemical investigation of *Chrysanthemum indicum*, neohesperidoside was identified based on ^1H , ^1H - ^1H NOESY, ^1H - ^{13}C HSQC and ^{13}C -NMR. The UV spectrum of the glycoside apigenin-7-O-(6''-caffeoyl) neohesperidoside showed two absorption maxima at 330 (band I) and 265nm (band II) indicating the presence of a flavone skeleton. A bathochromic shift of 56nm in band I observed in its NaOMe spectrum indicated the presence of free OH group at C-4'. The AlCl_3 -HCl spectra of the glycoside as well as the aglycone consist of 4 major absorption peaks which indicates the presence of a free -OH group at C-5 in both. It was also confirmed by a bathochromic shift of 7nm, indicating the presence of a free -OH at C-7 (as a result of hydrolysis). In both glycoside and in the aglycone no additive bathochromic shift was noticed in AlCl_3 spectrum (band I) with respect to AlCl_3 -HCl spectrum which confirms the absence of O-dihydroxyl grouping in the B ring. No change was noticed in band I of the glycoside and the aglycone on the addition of NaOAc-H $^3\text{BO}_3$ thereby revealing the absence of O-dihydroxyl grouping in the B ring.

In the ^1H -NMR spectrum (400MHz, DMSO- d_6 , TMS) of the glycoside, the signal at $\delta 13\text{ppm}$ indicates the presence of -OH at C-5. The absence of the signal $\delta 10\text{ppm}$ indicates the absence of free -OH at C-7. The two pair of ortho coupled doublets of C-2, C-6 appear at $\delta 7.99\text{ppm}$ and C-3, C-5 protons appear respectively at $\delta 7.4\text{ppm}$ (d, $J=8\text{Hz}$). The doublet due to C-3' and C-5' protons occur upfield from that of C-2' and C-6' due to the shielding effect of the oxygen substituents and to the deshielding influences of C-ring functions on C-2' and C-6' protons. The protons C-6 and C-8 resonated respectively at $\delta 6.2\text{ppm}$ (d- $J=2.5\text{Hz}$) and $\delta 6.7\text{ppm}$ (d, $J=2.5\text{Hz}$). The C-3 proton singlet overlaps with the C-6 proton doublet $\delta 6.5\text{ppm}$. H-1'' of glucose and H-1' of rhamnose resonate at $\delta 5.15\text{ppm}$ and $\delta 4.5\text{ppm}$. The remaining sugar protons appear in the region of 3.00 - 3.8ppm . The methyl protons of the rhamnosyl unit can be seen at $\delta 1.25\text{ppm}$. The α , β protons of caffeoyl group resonate at $\delta 7.53\text{ppm}$ (d, $J=15.9\text{Hz}$) and $\delta 6.29\text{ppm}$ (d, $J=15.9\text{Hz}$). The remaining protons of C-2, C-5 and C-6 resonate at $\delta 7.16$ (d, $J=2.3\text{Hz}$), $\delta 6.86$ (d, $J=8.3\text{Hz}$) and $\delta 7.03\text{ppm}$.

^1H - ^1H NOESY spectrum is very similar in appearance to the COSY spectrum. In the case of NOESY spectrum off-diagonal peaks indicate NOE interactions (exchange of magnetization) rather than spin-spin coupling interactions. 2D NMR Noesy shows a diagonal strong NOE cross peaks C-8 at $\delta 6.8\text{ppm}$. This contour peak shows the cross peak and correlation to a doublet located at $\delta 5.2\text{ppm}$ which is assigned to anomeric proton of the sugar moiety. The 2D NMR reveals steric proximity of anomeric proton of sugar moiety with C-8 proton. This clearly indicate that glycosylation has been at C-7 in Apigenin 7-O-(6''-caffeoyl) neohesperidoside compound.

Supporting evidence for the structure of the glycoside is also provided by the analysis of the ^1H and ^{13}C NMR spectral data presented in Table-3 & Table-4. As a result of glycosylation the signal of C-7 is shifted upfield and appears at $\delta 162.9\text{ppm}$. The C-1'' of glucose appears at $\delta 99.5\text{ppm}$ and C-1' of rhamnose of $\delta 99.52\text{ppm}$. The downfield shifts of the signals of the two anomeric carbons indicate their involvement in glycosylation. The appearance of C-6'' signal at $\delta 21.8\text{ppm}$ and the signal of C-6' at $\delta 60.60\text{ppm}$ (not at $\delta 66.0\text{ppm}$) show that the

glycoside is a 7-O-(6''-caffeoyl) neohesperidoside. The signal at $\delta 164.26\text{ppm}$ represents the caffeoyl carbonyl group. As a result of attachment of the carbonyl group at C-6'' of glucose the signal of carbonyl group is shifted upfield and appears at $\delta 164.26\text{ppm}$ (not at $\delta 266.3$). On the basis of the above mentioned physical and chemical evidences, the glycoside obtained from *Chrysanthemum indicum* has been characterized as apigenin 7-O-(6''-caffeoyl) neohesperidoside.

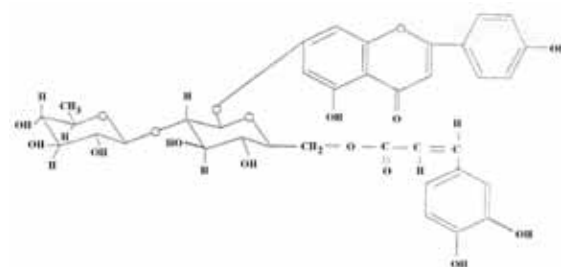


Table-1
 $R_f(\times 100)$ values of the APIGENIN 7-O-(6''-CAFFELOYL) NEOHESPERIDOSIDE from the yellow florets of *Chrysanthemum indicum*
(Whatman No.1, Ascending, $30 \pm 2^\circ$)

Compound	*Developing solvents								
	a	b	c	d	e	f	g	h	i
Glycoside Apigenin 7-O-(6''-caffeoyl) neohesperidoside	56	34	23	48	78	40	56	86	86
Deacylated Glycoside Apigenin 7-O-(6''-caffeoyl) neohesperidoside	58	37	25	49	80	42	56	88	88
Aglycone from APIGENIN 7-O-(6''-caffeoyl) neohesperidoside (complete hydrolysis)	-	-	10	32	66	90	90	73	-
Apigenin	-	-	10	32	66	90	91	73	-
Glycoside Apigenin 7-O-(6''-caffeoyl) neohesperidoside from Partial hydrolysis	11	12	47	70	84	74	70	80	-
Apigenin 7-O-glucoside	12	13	47	70	85	77	70	80	68

TABLE-2
 $R_f(\times 100)$ values of the Apigenin 7-O-(6''-caffeoyl) neohesperidoside from the florets of *Chrysanthemum indicum*
(Whatman No.1, Ascending, $30 \pm 2^\circ$)

Compound	Developing solvents				
	f	g	h	i	J
Sugar from Apigenin 7-O-(6''-caffeoyl) neohesperidoside	18	38	37	-	25
Glucose authentic	17	38	37	-	24
Sugar from Apigenin 7-O-(6''-caffeoyl) neohesperidoside	34	58	58	55	-
Rhamnose authentic	34	58	59	55	-

TABLE-3
2D NMR Values of the Compound Apigenin 7-O-(6''-caffeoyl) neohesperidoside ^1H - ^{13}C [HSQC] for confirmation of assignment

Protons chemical shift (δ)	Correlated carbon (δ)	Assignment
7.99 (H-2')	128.54	C-5'
7.99 (H-6')	128.5	C-6'
6.4 (H-5')	115.9	C-2'

6.7 (H-8)	94.7	C-8
6.2 (H-6)	99.8	C-6
5.15 (H-1" Glu)	99.8	C-1"
4.48 (H-"" rham)	100.5	C-1"
3.35 (H-2""	70.3	C-2"
3.20 (H-3""	70.1	C-3"
3.20 (H-5""	68.3	C-5"
3.20 (H-3")	76.7	C-3"
3.20 (H-4")	70.7	C-4"
3.20 (H-2")	76.7	C-2"

2.99 (H-4")	72.2	C-4"
2.99 (H-5")	77.3	C-5"
1.2 (H-6")	21.8	C-6"
7.4 (α - H)	121.4	α -C
6.2 (β - H)	144.7	β -C
7.1 (H-2)	113.5	C-2
6.8 (H-5)	114.8	C-5
7.0 (H-6)	115.7	C-6
3.6 (H-6"β)	60.60	C-6"

TABLE-4

¹³C – NMR spectral data and their assignments for the aglycone and glycoside Apigenin 7-O-(6" caffeoyl) neohesperidoside from the yellow florets of *Chrysanthemum indicum*

Compound	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
Apigenin from the literature (δ ppm)	163.5	102.85	181.8	161.23	98.91	164.28	94.02	157.34	103.68
Glycoside Apigenin 7-O-(6" caffeoyl) neohesperidoside (δ ppm)	164.46	103.15	181.88	161.12	99.8	162.9	94.71	156.93	105.35
Apigenin 7-O- neohesperidoside from literature (δ ppm)	165.2	103	182.1	161.68	99.8	163	94.9	157.3	105.7

Compound	C-1'	C-2'	C-3'	C-4'	C-5'	C-6'
Apigenin from literature (δ ppm)	121.18	128.52	116.0	161.47	116.0	128.52
Glycoside Apigenin 7-O-(6" caffeoyl) neohesperidoside (δ ppm)	121.36	128.52	115.99	161.2	115.99	128.52
Apigenin 7-O- neohesperidoside from literature (δ ppm)	121	129.1	116.0	161.6	116.0	129.1

Compound	C-1'	C-2'	C-3'	C-4'	C-5'	C-6'
Glycoside Apigenin 7-O-(6" caffeoyl) neohesperidoside (δ ppm)	99.88	76.79	76.79	70.7	77.1	60.60
Apigenin 7-O- neohesperidoside from literature (δ ppm)	99.5	77.0	77.0	70.9	77.3	61.0

Compound	C-1'	C-2'	C-3'	C-4'	C-5'	C-6'
Glycoside Apigenin 7-O-(6" caffeoyl) neohesperidoside (δ ppm)	99.52	70.3	70.1	72.2	68.31	21.8
neohesperidoside from literature (δ ppm)	100.6	70.6	70.2	72.3	68.4	20.9

Compound	CO	CH	CH	C1	C2	C3	C4	C5	C6
Caffeoyl literature	166.3	121.1	144.7	128.3	113.3	145.5	148.2	114.6	115.6
Caffeoyl	164.5	121.4	144.7	128.5	113.5	145.5	148.4	114.8	115.7

MATERIALS AND METHODS

5% w/v test solution of each extract was prepared by dissolving 250mg of each extract separately in 5ml of sterile dimethyl formamide.

Nutrient agar medium was prepared and sterilized by an autoclave. In an aseptic room, they poured into sterile petridishes to a uniform depth of 4mm and then allowed to solidify at room temperature. After solidification, the test organisms were inoculated with the help of a sterile swap soaked in a bacterial culture or suspension. Thus provides the uniform surface growth of bacterium and is used for antibacterial sensitivity studies. Then the sterile filter paper discs (6mm) containing sample (100μl) were immersed in plant extracts and was placed over the solidified agar in such a way that there is no overlapping of zone of inhibition⁴ Plates were kept at room temperature for half an hour for the diffusion of the sample into the agar media. The organisms inoculated petridishes were incubated at 37°C for 48 hours. After the incubation period is over, the zone of inhibition produced by the sample with different organisms in different plates were measured and recorded immediately by using a zone reader.⁵

Anti Bacterial Activity of Apigenin 7-O-(6" caffeoyl) neohesperidoside

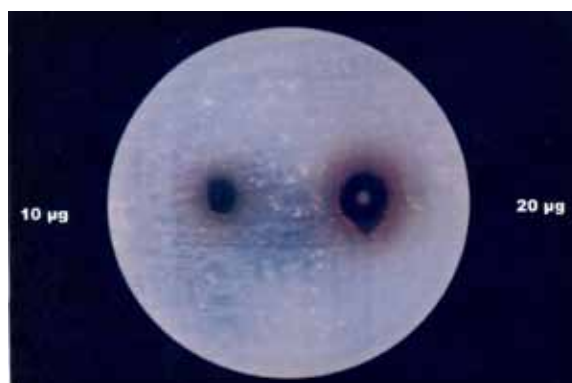


TABLE-5
Anti Bacterial activity of Apigenin 7-O-(6''caffeoyl) neohesperidoside

Drug	Concentration	Micro-Organism used			
		S.aureus	% of inhibition	E.coli	% of inhibition
S1	2mg/mL(Penicillin)	19mm	100	-	-
S2	2mg/mL(Norfloxacin)	-	-	21mm	100
Apigenin 7-O-(6''caffeoyl) neohesperidoside	100µg	17mm	89.47	-	-
	200µg	17mm	89.47	19mm	90.48

RESULTS AND DISCUSSION

The isolated compound Apigenin 7-O-(6'' caffeoyl) neohesperidoside was screened for its antibacterial activity at two different concentrations (100&200g) against the Gram positive organism *S. aureus* and Gram negative organism *E. coli*. Table-5 shows of inhibition along with the standard antibiotics namely Penicillin against the *S. aureus* and Norfloxacin against the *E. coli*.

The % of inhibition of the growth of the *S.aureus* was found to be inhibited by 89.47% at 200g concentration with a least inhibition at its lower concentration by Apigenin 7-O-(6'' caffeoyl) neohesperidoside. The *E.coli* was inhibited by 90.48% at it's a higher concentration with least inhibition at its lower concentration by the same drug.

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