Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2012, 4(7):3665-3671

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Isolation of flavonoids and biological activities of *Crotalaria Grahamiana*

P. Vanitha[1], R. Valliappan[2], A. Charles[3] and D. Sukumar[3]

[1]*Department of Chemistry, Annamalai University,* Chidambaram, *Tamilnadu, India,*
[2]*Department of Chemistry, SRM University, Ramapuram, Chennai, Tamilnadu, India,*
[3]*Department of Chemistry, Bharathiyar College of Engineering and Technology, Karaikal.*

ABSTRACT

The flavonoids of Crotalaria grahamiana (family Popilionaceae) were isolated from the alcoholic extract by partition with benzene, diethyl ether, ethyl acetate respectively. On the basis of chemical and spectral analysis their structures are elucidated as 4'hydroxy flavone-7-O-rhamnoside and its aglycone.Identification of unknown compounds were established through comparison of chromatographic and spectral data (PC, UV, and NMR) with literature values. Isolated yellow pigment for further studies was promoted to anti-microbial and SRBC membrane stabilization. It reveals that isolated compound shows significant antimicrobial and anti inflammatory activity.

Key words: *Crotalaria grahamiana,* Flavonoids, antimicrobial, membrane stabilization.

INTRODUCTION

Phytochemistry, the natural product chemistry deals with different heads namely structure, stereochemistry, dynamic aspects (reaction), synthesis, biosynthesis and biological properties. Facilitating the development of the subject in the format of organic chemistry and advancing it logically to the door-step into Biology [1].

Both primary and secondary metabolites are studied vividly and reported in literature the structures of the secondary metabolites like alkaloids, carotenoids, flavonoids, steroids, terpenoids *etc.*, are characterized by means of modern physical methods like UV, IR, MASS and NMR spectroscopic techniques. Amongst the mentioned compounds flavonoids play important roles which are structurally derived from the parent substance flavones. Among the natural phenolic compounds, of which several thousand structures are known, the flavonoids form the largest group, but simple monocyclic phenols [2].

Crotalaria grahamiana Wight & Arn. Belongs to *Popilionaceae* [3].The Hepatotoxicity studies are seen in *C. grahamiana.* It appears to be the plant species primarily responsible for the observed equine toxicosis on Easter Island which has continued over the last 15 years. *Crotalaria grahamiana* was intentionally introduced to Easter Island prior to 1982 to Control roadside soil erosion. The plant is effectively opportunistic and thoroughly spread throughout the Easter Island subsequent to introduction. Easter Island horse owners indicated that condition referred to as "Gazy horse and coco disease" was first recognized in 1984 [4].*C.grahamiana* acting as a nitrogen fixation in the soil it is acting as a green manure for agriculture [5].

In the present work we are concentrated on the isolation of flavonoids from the alcoholic flowers extract of *Crotalaria grahamiana* by using of different solvent system.The isolated compound was characterized by using of

chemical test, UV, NMR and PC comparatively with literature data the compound is depicted [7-16].Further more studies was conducted the biological activity of the isolated compound. The isolated yellow pigment was treated to anti microbial and SRBC membrane stabilization studies. Erythrocytes have been used as a model system by a number of workers for the study of interaction of drugs with membranes. Drugs like anesthetics tranquilizers and non-steroidal anti-inflamatories stabilize erythrocytes against hypotonic haemolysis at low concentration. When the RBC is subjected to hypotonic stress the release of hemoglobin (Hb) from RBC is prevented by anti-inflammatory agents because of membrane stabilization. So, the stabilization of SRBC membrane by drugs against hypotonicity induced haemolysis serves as a useful in vitro method for assessing the anti-inflammatory activity of various compounds. [6]

Fig 1 Floral diagram of *Crotalaria grahamina* Wight & Arn.

EXPERIMENTAL SECTION

Extraction and fractionation:

The flowers of *C.grahamiana* collected from the hills of Kodaikanal during March – April were extracted with 85% EtOH (5 x 600ml) under reflux. The alcoholic extract was concentrated in vacuo successively fractioned with benzene (3x 250ml) peroxide free Et_2O (4 x 300 ml) and EtOAc (4 x 500 ml). The benzene fraction didn't yield any isolable material.

Et_2O fraction: (4', 7- dihydorxy flavone)
The yellow compound isolated from Et_2O fraction was recrystallised from MeOH to get greenish yellow crystals. It appeared fluorescent light blue under UV which turned fluorescent yellowish green on fuming with NH_3.

It had λ_{Max}^{MeOH} nm 253sh, 312sh, 323; +NaOMe 251, 263sh, 329, 386; + $AlCl_3$ 231sh, 255sh, 313sh, 327, 383sh; + $AlCl_3$ / HCl 246sh, 255sh, 310sh, 328sh, 396; + NaOAc 261, 309, 320sh, 369; + NaOAc / H_3BO_3 256sh, 314sh, 329. It responded only to Shinoda test 15. But it never answered Harhammer – Hansel, Wilson boric acid and Molisch tests. It had R_f, values as depicted in table (I -1). It was identified as 4', 7 – dihydroxy flavone.

EtOAc fraction: (4'hydroxy flavone-7-O-rhamnoside)
The solid isolated from EtOAc recrystallisation came out as yellow crystals. It appeared fluorescent light blue under UV which turned yellowish green on exposure to NH_3.

It had λ_{Max}^{MeOH} nm, 255sh, 311sh, 323 ; + NaOMe 251sh, 294, 304sh, 385 ; $AlCl_3$, 255sh, 310sh, 327 ; + $AlCl_3$, / HCl 253sh, 310sh, 327 ; + NaOAc 257sh, 307, 331, 386sh ; + NaOAc / H_3BO_3 256sh, 312, 328. It responded to Shinoda and Molisch tests and it did not answer to Harhammer – Hansel and Wilson boric acid tests. It had R_f values as depicted in table (I-1).The ^1H and ^{13}C NMR of the glycosides are appended. It was identified as 4'hydroxy flavone-7-O-rhamnoside[7-21].

Hydrolysis of the glycoside
The glycoside (0.05g, 0.2m mole) dissolved in hot aq. MeOH (2ml, 50%) was hydrolysed with H_2SO_4 (5%) at 100° C for about 2h and the hydrolytic products identified as described below.

Identification of aglycone
The glycoside on recrystallation from MeOH affoarded a yellow crystalline solid which was identified as 4', 7 – dihydroxy flavone by colour reactions, behaviour under UV and R_f values (Table I-1). It had the same UV spectral values mentioned under Et_2O fraction.

Identification of the Sugar
The filtrate after removal of the aglycone was neutralized with $BaCO_3$.The concentrated filtrate when examined by PC gave R_f values (Table I-2) corresponding to those of rhamnose.The identify of the sugars were confirmed by comparison with authentic samples of rhamnose.

ANTI-MICROBIAL ACTIVITY
The flavonoids isolated from C.grahamina have been investigated for their anti-bacterial activity.A standard volume (2.2ml) of Muller-Hinton agar medium that would support the growth of the test organisms was added to several, labeled, sterile, stoppered and identical petri-dishes.Solutions of the test compound at low different concentrations viz., 25,50 µg/ml in sterile water were prepared. Standards containing concentration of streptomycin at concentrations of 50,100,200,400 µg and a control containing no drug were prepared. A standard inoculums of a suspension of turbidity equal to a Mc Farland standard 0.5 of the test organisim was added to all the petri-dishes.The small amount of the drug that is carried over this inoculums is easily removed by diffusion into agar and the effect is spreading the inoculums over a large area.All these manipulations were carried out with almost care under aseptic conditions.After inoculums the plates were immediately incubated at 37 ^{0}C and minimum inhibitory concentration (MIC)is found out after 24hr of incubation.

SRBC MEMBRANE STABILIZATION.

The SRBC membrane stabilization was used as a method to study the anti-inflammatory activity of isolated compound [22-25]. Sheep blood was purchased and mixed with equal volume of sterilized Alsever solution. Alsever solution contains dextrose, sodium citrate, sodium chloride in water.

The blood was centrifuged and the packed cells were washed with isosaline and 10% v/v suspension was made with Isosaline. The drug samples were prepared by suspending the residues in hot water. The assay mixture contained the drug, 1 ml phosphate buffer; 2 ml hypo saline, 0.5 ml SRBC suspension.Instead of hypo saline 2 ml of distilled water used in the control. All the assay mixture were incubated at 37^0C for 30 minutes and centrifuged. The hemoglobin content in the supernatant solution was estimated using photoelectric colorimeter at 560nm. The percentage haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100 %. The results obtained was showed in the Table I-4 and graph shown Fig 2

Calculation

The percentage of SRBC membrane stabilization was calculated using the formula,

$$\textbf{Percentage protection} = \frac{100 - \text{Optical density of drug treated sample}}{\text{Optical density of control}} \times 100$$

Table I – 1 R_f *(x 100) values of the constituents of the flowers of C. GRAHAMIANA*
(Whatman No.1, Ascending, 30° ± 2° C)

Compound	a	b	c	d	e	f	g	h	i
Aglycone (From Et_2O fraction)	-	06	11	22	44	86	75	74	85
Glycoside(From EtOAc fraction)	13	25	58	65	72	60	72	75	44

Table I – 2 R_f *(x 100) values of sugar from the glycoside of C. GRAHAMIANA*
(Whatman No.1, Ascending, 30° + 2° C)

Compound	**f**	**g**	**h**	**i**
Rhamnose	**34**	**58**	**58**	**54**
Rhamnose (Authentic)	**34**	**58**	**58**	**55**

** Solvent Key : **a**-H_2O, **b**-5% aq. HOAc , **c**-15% aq. HOAc, **d**-30% aq. HOAc, **e**- 60% aq. HOAc, **f**-n-BuOH : HOAc : H_2O = 4: 1 : 5 : (Upper phase) ,**g**-Phenol Saturated with water , **h**- BuOH : HOAc : H_2O = 3 : 1 : 1 , **i**-HOAc : Conc. HCl : H_2O = 30 : 3 : 10*

Table I – 3 ^{13}C – NMR spectral data and their assisgnment for the glycoside isolated from the flowers of *C. grahamiana*

COMPOUND	**C_2**	**C_3**	**C_4**	**C_5**	**C_6**	**C_7**	**C_8**	**C_9**	**C_{10}**
Literature (δ ppm)	162.5	104.5	176.5	126.4	114.7	162.4	102.4	157.4	116.1
Glycoside (δ ppm)	162.2	103.0	176.0	128.6	115.4	162.2	103.0	159.2	115.4

COMPOUND	**C_1'**	**C_2'**	**C_3'**	**C_4'**	**C_5'**	**C_6'**
Literature (δ ppm)	121.8	128.0	115.9	160.7	115.9	128.0
Glycoside (δ ppm)	121.5	128.6	115.4	159.6	115.4	128.6

COMPOUND	C_1"	C_2"	C_3"	C_4"	C_5"	C_6"
Literature (δ ppm)	97.5	71.5	70.5	73.4	69.9	18.0
Glycoside (δ ppm)	97.5	71.5	70.5	73.4	69.9	18.0

Table I-4 SRBC Membrane Stabilization of C.Grahamiana

S.No.	Conc. of drug(µg)	Transmission (%)
1	10	16
2	25	17
3	50	17
4	75	17
5	100	16
6	200	16

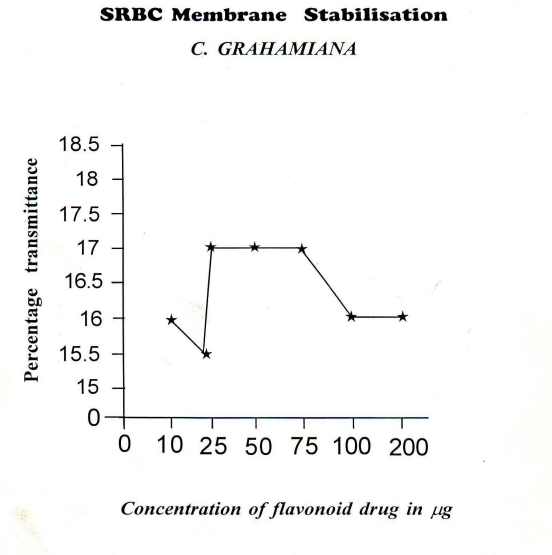


Fig 2.Graphical representation of SRBC membrane stabilization studies of C.grahamiana

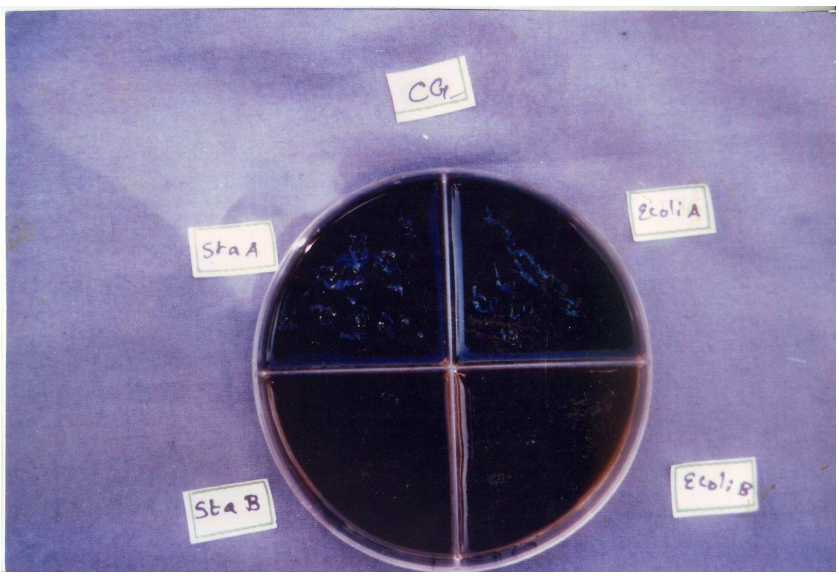


Fig 3. ***Anti microbial activity of C. grahamiana***

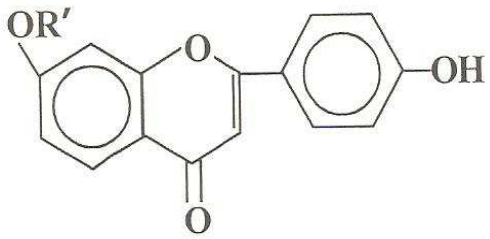
RESULTS AND DISCUSSION

The fresh flowers of *C. grahamiana* has been found to contain 4'hydroxy flavone-7-O-rhamnoside and its aglycone.

The UV spectrum of the EtOAc soluble exhibited two peaks at 325 nm (band I) and 255 nm (band II) indicated a flavone skeleton. A bathochromic shift of 60mm (band I) on the addition of NaOMe revealed the presence of a free 4' – OH group in the B – ring. The absence of a free 5 – OH group was evidenced from the absence of the required shift on the addition of $AlCl_3$ – HCl. The $AlCl_3$ spectrum was exactly same as that of $AlCl_3$ – HCl revealed the absence of catechol of the free OH group at C – 7 was observed by a shift of only +2 nm on the addition of NaOAc. The corresponding aglycone however showed a bathochromic shift of +8nm, supporting the presence of a free OH at C – 7. The absence of catechol type of B – ring in the glycoside can be inferred from the fact that the NaOAc spectrum was unaffected on the addition of H_3BO_3.

In the ^1H – NMR spectrum (400 MHz, DMSO – d6, TMS) of the glycoside. The C_4' hydroxyl group resonate at δ 9.55 ppm. The C_3' and C5' protons occur at δ 7.01 ppm and the C_2' & C_6'protons shows up at δ 7.9 ppm. The protons of C_6 & C_8 resonate respectively at δ 6.16 ppms. The ^1H" of glucose resonates at δ 5.45 ppm. The ^1H" of rhamnose resonates at δ 5.45 ppm. The remaining sugar protons appear in the region of δ 3.25 ppm to δ 3.51 ppm. The rhamnosyl CH_3 appears as a doublet at δ 0.88ppm (J=7Hz).The various signals noticed the 13C – NMR spectrum (100 MHz, DMSO – d6, TMS) of the glycoside can be assigned to different carbons as indicated in (Table 1-3).

From the above chemical & spectral evidences the glycoside has been characterized as 4'hydroxy flavone-7-O-rhamnoside.



a. R' = H = 7, 4' dihydroxy flavone
b. R' = Rha = 4'hydroxy flavone-7-O-rhamnoside.

Biological studies revealed that the antimicrobial activity of the isolated pigments was worked on the Gram positive organism *Staphylococcus aures* and gram negative organisim *Escherichia coli*. At a higher concentration of 75 µg *Crotalaria grahamiana* is observed to be having traces of the gram positive organism while the concentration is decreased to 25 µg not even traces of organisms were observed. Hence that it is found to lower concentration the *Staphyllococus aures* is fully contained by the drug. The same care is observed in the case of E.coli at lower concentration of 75 µg.The drug is able to at fully on the organisms.

The SRBC membrane stabilization studies have been carried out with the EtOAc soluble of *C. grahamina*. As the concentration is increased from 10 µg.The protective property gets stabilized at concentration of 25 µg.Beyond the concentration upto that of 75 µg.No alteration is seen in the curve.Beyond 75 µg the value declaims.Thus it has been inferred the drug is able to retained its capacity only in-between concentration 25 µg and 75 µg

CONCLUSION

It deals with the studies on the isolation and characterization of flavonoids from the flower of *Crotalaria grahamiana* respectively using UV, NMR and chromatographic techniques, *Crotalaria grahamiana* have been

found to contain 4'hydroxy flavone-7-O-rhamnoside and its aglycone respectively. These results suggest that the isolated compounds have excellent scope for further development as antimicrobial and anti inflammatory agent.

Acknowledgement
The authors wish to thank the Department of Chemistry, Government College (Autonoums), Kumbakonum for providing laborotary facilities to carry this work.

REFERENCES

[1]N.R.Krishnaaswamy, *Chemistry of Natural Products*, University Press, **1999**, x.
[2]J.B.Harborne, *Phytochemical methods*, Chapman and Hall, London, **1973**, 52.
[3]K.M.Mathew, *The Flora of Tamilnadu Carnatic*, **1983**, Vol III Rapinant Herbarium, Trichirappali, India.
[4]G.Zizka, *Flowering plants of Easter Island Frankfurt palmengarten*, **1991**, 45.
[5]Mukadasi Buyinza and Nabalegwa Wambede, *Environmental Research Journal Year*, *2008*, 2(3), 131-137.
[6]R.A.Nambi, D.Sukumar. V.Sethuraman, N.Suluchana, and J.Sadique, "Satellite *symposium on Traditional Medicine as Asian Congress of Pharmacology*". Tamil University Thanjavur, **1985**,140.
[7]K.R.Markham,*'Technique of Flavonoid Identification'*Academic Press, London, **1982**, 39.
[8]L.Horhammer and R.Hansel, *Arch.Pharm.Berl.* **1955**,288,315.
[9]C.W.Wilson, *J.Amer.Chem.Soc..*, **1939**,61,2303**.**
[10]Shinoda, J.Chem.Pharm.Soc. **1928**, 48.
[11]L.Jurd.Ed. '*The chemistry of Flavonoids compounds'*, Pergamon, London, **1963**,107.
[12]K.R.Markham and T.J Mabry, *Phytochem*. **1968**, 1, 1197.
[13]JB Harborne, TJ Mabry and H Mabry. *The flavonoids*, Chapman and Hall, London, **1975,** 78-126.
[14]K.Venkataraman and T.A.Geissman, Ed., *The Chemistry of Flavonoids Compounds*, Pergamon Press, London, **1962**, 69.
[15]J.B.Harborne, *Comparative Biochemistry of the Flavonoids*, Academic Press, London, **1967**,208.
[16]PK Agrawal. *In C-13 NMR of flavanoids*, Elsevier, Amsterdam, **1998;** 41-182
[17] R.Prabakaran, S. Arivoli., A. Hema and C. Kamatchi *J. Chem. Pharm. Res.*, **2011**, 3(3):805-813.
[18]Donatus Ebere Okwu, Fred Uchenna Nnamdi, *J. Chem. Pharm. Res.*, **2011**, 3(2):1-10
[19]Venkata Sai Prakash Chaturvedula and Indra Prakash, *J. Chem. Pharm. Res.*, **2011**, 3(6):799-804.
[20] X .Queen Rosary Sheela, Arockiasamy P, R.Kanmani, A.Charles and V Alex Ramani, *J. Chem. Pharm. Res., **2011***, 3(2):762-764.
[21]A.Charles and V.Alex Ramani, *J. Chem. Pharm. Res.*, **2011**, 3(5):205-211.
[22]S.Palanichamy and S. Nagarajan, *J.Ethnopharmacol*., **1990**, 29, 73.
[23]N.Uma, E.A.Bhaskar, L.Ambujavalli, N.Suluchana, D.Sukumar. and R.A Nambi, *Indian Drugs*, **1985**,23,18.
[24]N.Sethuraman and N.Sulochana, *Fitoterapia*, **1988**, 59,335.
[25]M.Sampath Kumar, *International Journal of Pharma and Bio science*, **2011**, 2(1) 220-226.