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ORIGINAL ARTICLE

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Assessment of HRBC membrane stabilizing activity from flavonoids of *Asystasia gangetica*

K. Shakila

[1] *Assistant Professor of Chemistry, Arignar Anna Government Arts and Science College, Karaikal, Pondicherry, India.*

*E-Mail: ***shakila.manickaraj@gmail.com***

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Flavonoids from the largest single-family of naturally occurring oxygen-containing heterocyclic compounds. Benzo pyrone of various levels of saturation, oxidation, and substitution constitutes the basic skeleton for these polyphenolics. *Asystasia gangetica* belongs to the family *Acanthaceae* and has been investigated for flavonoids The white flowers of *Asystasia gangetica* on suitable extraction and fractionation have afforded a rare flavone glycoside viz. luteolin 7 – O – rutinoside and a flavonol glycoside viz. patuletin 3 – O- glucuronide the structure of which has been established by methods like ^{1}H NMR, ^{13}C NMR, UV, PC, chemical reactions, and hydrolytic studies. A biphasic activity has been observed in the HRBC membrane stabilization, an in vitro investigation against hypotonicity induced haemolysis of the isolated flavonoids.

Key words: Patuletin, Luteolin, Asystasia gangetica, Acanthaceae family

Asystasia gangetica T. Anders. (syn. *A. coromandeliana* Nees.) of the *Acanthaceae* is a procumbent plant, sometimes climbing among bushes (Gopal *et al*., 2013). The white flowers are secud, in long lax terminal and axillary spike-like racemes. It is distributed widely in N. Circars, Decaan, Carnatic, W. Peninsula, Ceylon-Tropical and S. Africa. The juice of the plant is used as an anthelmintic. It is also given in swellings and rheumatism.

Isolation of five phytochemical constituents namely, salidroside, verbascoside, forsythiaside, 3''-O-caffeoyl-6-O-rhamnopyranosyl catalpol and 4''-O-caffeoyl6-O-rhamnopyranosyl catalpol from the leaves *of A. gangetica* has also been reported (Isna *et al*., 2020).

The structure of red blood cells (RBCs) is similar to that of lysosomal membrane components (Mounnissamy *et al*., 2008). Because the lysosomal membrane is similar to human RBC membranes, its stabilizing effects have been studied using HRBC. When RBC is subjected to hypotonic pressure, the release of hemoglobin from RBC is blocked by anti-inflammatory agents due to membrane stabilization. During the present work, it has been reported for the first time that the petals of the plant contain luteolin 7-rutinoside and patuletin 3-0-glucuronide. The results of *in vitro* stabilization action and investigation of the HRBC membrane function of flavonoid glycosides extracted from *A. gangetica* are presented here.

MATERIALS AND METHODS

The fresh flowers of *A.gangetica* collected from the banks of Arasalaru in Karaikal, Pondicherry State during December were extracted with 80 % ethanol (5x500 mL) under reflux. The alcoholic extract was concentrated in Vacuo and the aq. Concentrate successively fractionated with petrol (60-80 °C) (3x250 mL), peroxide-free Et_2O (4x300 mL) and EtOAc (4x500 mL).

The petrol and ether fractions did not yield any isolable material.

EtOAc fraction (flavanol glycosides: luteolin 7-0-rutinoside and patuletin 3-0-glucuronide)

The residue from EtOAc fraction on chromatography over silica gel afforded a pinkish-brown crystalline solid recovered from EtOAc fraction. The pinkish-brown solid that separated when subjected to PC revealed the presence of two glycosides. The mixture of glycosides was separated by preparative PC (Whatman No.3, ascending, 15 % HOAc (R_f = 0.50, 0.73). the two bands were cut, eluted with methanol and studied separately.

The eluate from the lower band was concentrated and treated with acetone and the mixture left in an ice chest for a week. A yellow solid (I) m.p. 175-78 °C (yield 0,05 %) separated on recrystallisation from methanol. It developed a green colour with alc. Fe^{3+}, and orange-red colour with Mg-HCl, yellow colour with NaOH and appeared as a purple spot under UV turning yellow on exposure to NH_3. It answered Wilson's boric acid and Gibb's tests but did not respond Horhammer – Hansel test. It responded to Molisch's test showing that it could be a flavone glycoside.

It had MeOH (λ_{max}, nm) 257, 266 sh, 351; + NaOMe 281, 297 sh, 407; + $AlCl_3$ 274, 298 sh, 327 sh, 421; + ($AlCl_3$ – HCl) 274, 294 sh, 356, 384; + NaOAc 257, 321 sh, 372, 404 and + (NaOAc – H_3BO_3) 259, 372 nm. It had R_f values are depicted in Table I. The ^1H and ^{13}C NMR spectra of the glycoside are appended (Fig.I and Fig. II). It was identified as luteolin 7-0-rutinoside and the identity was confirmed by co- and mixed-PC and m.m.p. with an authentic sample of luteolin 7-0-rutinoside from *Azima Tetracantha* (Muthu *et al*., 2019).

Hydrolysis of the glycoside

The glycoside (0.05 g ≈ 0.1 m mole) was dissolved in hot aq. MeOH (2 mL, 50 %) and an equal volume of H_2SO_4 (10 %) were added to it. The reaction mixture was refluxed at 100 °C for about 30 min and the hydrolytic products were worked up and identified as described below.

Identification of aglycone: (flavone: luteolin)

The yellow aglycone obtained above on crystallization from MeOH afforded yellow needles, m.p. 328 – 330 °C. It was soluble in MeOH, Et_2O, Me_2CO_3, CCl_4 and $CHCl_3$, but not in water. It developed orange-red colour with Mg-HCl, green colour with alc. Fe^{3+} and appeared dull ochre in UV turning very bright yellow fuming with NH_3. It answered Wilson's boric acid test but

did not respond Horhammer – Hansel and Molisch's tests. It developed a bluish green colour in Gibb's test and afforded a tetraacetate m.p. 226 – 27 °C, and a tetrabenzoate, m.p. 202 – 03 °C. It had MeOH (λ_{max}, nm) 240 sh, 253, 268, 348; + NaOMe 265 sh, 329 sh, 402; + $AlCl_3$ 275, 298 sh, 327, 425; + ($AlCl_3$ – HCl) 265 sh, 275, 295 sh, 356, 384; + NaOAc 269, 326, 384 and + (NaOAc -H_3BO_3) 259, 301 sh, 370, 430 sh. nm. It was identified as luteolin and the same was confirmed by co- and mixed-PC and m.m.p. with an authentic sample of luteolin from Tecoma stans (Thaís *et al*., 2022).

Identification of aglycone: (flavone: patuletin)

The yellow aglycone obtained from the above hydrolysate on crystallization from dilute alcohol afforded pale yellow needles, m.p. 262-64 °C (yield 0.05 %). It gave pentaacetate, m.p. 171-73 °C; fluorescent yellow under UV and UV/NH_3; gave an intense yellow colour with NaOH and red colour with Mg-HCl, answered the Wilson's boric acid, Gibb's and Horhammer – Hansel test and had MeOH (λ_{max}, nm) 256, 290 sh, 370; + NaOMe 251 sh, 296 sh, 330, 412 sh (dec.); + $AlCl_3$ 238, 275, 308 sh, 327 sh, 460; + ($AlCl_3$ – HCl) 240, 268, 302 sh, 381 sh, 428; + NaOAc 270, 340, 390 and + (NaOAc -H_3BO_3) 265, 390. nm. Its R_f values are listed in Table. IV. It was identified as patuletin and the same was confirmed by co- and mixed-PC and m.m.p. with an authentic sample of patuletin from Leucaena glauca (Campos *et al*., 2020).

Identification of sugar moiety: (glucose and rhamnose)

The aq. solution from the above was neutralized with $BaCO_3$ and filtered. An aliquot of this was cautiously neutralized with $NaHCO_3$ and the sugar was estimated quantitatively by the Folin-Wu micro method (Simoni *et al*., 2002). The sugar content was in accordance with a bioside. The concentrated filtrate on PC gave two spots and their R_f values agreed very well with those of glucose and rhamnose. The identity of the sugar was further confirmed by co-chromatography with authentic samples of glucose and rhamnose.

To 5 mg of the glycoside (from the EtOAc fraction) in distilled water (0.5 mL), about 6 flakes of the pectinase (Koch-Light, from Aspergillus niger) were added. The reaction mixture was left overnight. It was concentrated in vacuo almost to dryness. The residue was dissolved in water and chromatographed (PC) in 15 % HOAc. The completion of hydrolysis could be ascertained from the changed R_f values of the product (as compared to those of the starting material) which agreed with those of luteolin. Thus, the sugar moiety was confirmed to be rutinoside and not a neohesperidoside.

The glycoside was subjected to partial hydrolysis by treatment with formic acid in cyclohexane and the resulting solution was extracted with ethyl acetate and subjected to PC. The R_f values of the EtOAc fraction agreed with those of luteolin 7-0-glucoside and rhamnose as products thereby showing that glucose moiety is directly linked to aglycone viz. luteolin. The R_f values of luteolin 7-0-glucoside are indicated in Table I.

The eluate from the upper band was concentrated and dissolved in the minimum amount of methanol and kept under chilled conditions for a week. A pale-yellow solid (Glycoside II) that separated was filtered and examined. On recrystallisation from MeOH, it came out as yellow leaflets m.p. 160 – 62 °C (yield 0.1 %) and developed a greenish-brown colour with alc. Fe^{3+}. It appeared deep purple under UV and gave greenish-yellow fluorescence when fumed with NH_3. Its R_f values are given in Table. II. It had MeOH (λ_{max}, nm) 260, 270 sh, 335; + NaOMe 273, 338, 410; + $AlCl_3$ 278, 302 sh, 340 sh, 438; + ($AlCl_3$ – HCl) 269, 280 sh, 300 sh, 373, 407 sh; + NaOAc 273, 326 sh, 385 and + (NaOAc -H_3BO_3) 265, 381 nm.

HRBC Membrane Stabilization

The fresh blood was collected. The collected blood was mixed with an equal volume of sterilized Alsever solution (containing 2 % dextrose, 0.8 % sodium citrate, 0.05 % citric acid, 0.42% NaCl) stored at 4 °C salines at different concentrations was prepared (isosaline 0.85% and hyposaline 0.25%). The blood was centrifuged at 3000 Pm and paced cells obtained were washed with isosaline (0.85%, pH-7.2) three times and a 10 % (V/V) suspension was made with isosaline (Saleem *et al*., 2011).

Solutions of different concentrations of flavonoids are prepared. The assay mixture contained the drug (flavonoids in concentration as mentioned in Table IV), 1 mL of phosphate buffer (0.15 M: pH 7.4), 2 mL of

hyposaline (0.25 % and 0.5 mL) of 10 % RBC suspension. In another tube instead of 2 mL of hypo saline, 2 mL distilled water was taken and served as the control. All the tubes were incubated at 37° C for 30 minutes. Then they were centrifuged and the hemoglobin content in the supernatant was estimated using a photoelectric colourimeter at 560 nm (Chanbi *et al*., 2015).

RESULTS AND DISCUSSION

From the fresh flowers of *A.gangetica* luteolin, 7-0-rutinoside and patuletin 2-0-glucuronide have been isolated.

The UV spectrum of the glycoside (I) showed two absorption maxima at 351 nm (a band I) and 257 nm (band II) indicating it contains a flavone skeleton. A bathochromic shift of 56 nm in the band I observed in its NaOMe spectrum indicated the presence of a free -OH a4 C-4' (Harborne *et al*., 1975). The $AlCl_3$-HCl spectra of the glycoside, as well as the aglycone, consist of 4 major absorption peaks which indicate the presence of a free -OH at C-5 in both. It was also confirmed by a bathochromic shift of 33 nm and 36 nm respectively in the glycoside and in the aglycone on the addition of $AlCl_3$-HCl (Geissmann, 1962). No change was observed in absorption characteristics in band II of the glycoside on the addition of NaOAC. This is indicative of the absence of a free -OH as C-7. The corresponding aglycone however showed a bathochromic shift of 16 nm, supporting the presence of a free -OH at C-7 (as a result of hydrolysis). A bathochromic shift of 21 nm in the glycoside and 22 nm in the aglycone (band I) on the addition of H_3BO_3 indicated the presence of an o-dihydroxyl grouping in the B-ring (at C-3' and C-4') in both (Andersen and Markham, 2005). This is also confirmed by an additional bathochromic shift of 37 nm in the glycoside and 41 nm in the aglycone (band I) in $AlCl_3$ spectra with respect to $AlCl_3$-HCl spectra (Harborne and Williams, 2000).

In the ^{1}H NMR spectrum of the glycoside (270 MHz, DMSO-d_6, TMS the C-6 proton doublet at δ6.50 ppm (J = 2.5 Hz) occurs at a higher field than the C-8 proton doublet at δ6.73 ppm (J = 2.5 Hz), indicating 5, 7-dioxygenated system. The C-3 proton singlet overlaps with C-6 proton signal the doublet at δ6.80 ppm (J = 8.5 Hz) indicating the ortho coupled C-5' proton of the B-ring. The signals of the meta coupled C-2' proton and ortho and meta coupled C-6' proton overlap at δ7.40 ppm. H-1'' of glucose resonate at δ5.30 ppm while that of H-1''' of rhamnose appears at δ4.63 ppm (d, J = 2 Hz) (Rösler *et al*., 1965). The rhamnosyl – CH_3 protons resonate at δ0.76 ppm. This reveals the presence of rutinoside. The rest of the sugar protons appear between δ3.00 ppm and δ4.00 ppm.

The NMR data and the complete assignment are depicted in Table. III. The up-field shift of 1.50 ppm in case of C-7 and the downfield shifts of 0.60 and 0.20 ppm in the case of ortho related C-6 and C-8 resonances respectively indicate the glycosylation at C-7 which is further confirmed by a significant shift of the para related C-10 which moves down field by 3.80 ppm. The appearance of the carbonyl carbon (C-4) at δ181.82 ppm (much downfield), is due to the hydrogen bonding with C-5 -OH in the absence of C-3 -OH. The rutinosyl nature of the sugar linkage is confirmed by the appearance of the signal of C-6''' of rhamnose at δ18.00 ppm and the signal of C-6'' of glucose at δ68.40 ppm. Had it been a neohesperidoside these signals would have appeared at δ20.90 ppm and δ60.90 ppm respectively.

Based on this observation, the glycoside (I) has been characterized as luteolin 7-0-rutinoside.

The glycoside (II), on hydrolysis, yielded the flavonol patuletin and glucuronic acid. The running property of the glycoside is in favour of glucuronide. In the UV spectra of the glycoside and the aglycone, the absorption maxima were 355 nm, (band I) 260 nm (band II) and 370 nm (band I) 256 nm (band II) respectively. The hypochromic shift of +15 nm observed in band I of the glycoside with respect to the aglycone indicates possible glycosylation at C-3. This shift again indicates the presence of free C-5 hydroxyl in both glycoside and aglycone which is also evidenced by a bathochromic shift of 52 nm in the glycoside and 58 nm in the aglycone on the addition of $AlCl_3$-HCl. A shift of 55 nm in the glycoside and 42 nm in the aglycone towards the longer wavelength in the NaOMe spectra indicates the presence of free C-4' -OH. Again, the NaOMe spectrum

of the aglycone which degenerated with time is indicative of C-3-, C-3'-, C-4'-hydroxyl system which is however absent in the glycoside. The bathochromic shifts observed in the NaOAc-H_3BO_3 and $AlCl_3$ spectra (band I) of the glycoside, as well as the aglycone, indicate the presence of C-3', C-4'-ortho-dihydroxyl system in the B-ring in both. The bathochromic shift of 13 nm in the glycoside and 14 nm in the glycoside in band II on the addition of NaOAc is suggestive of free -OH in C-7 in both. Further, the degeneration of NaOAC spectra of both the glycoside and the aglycone with time indicates the presence of an alkali-sensitive grouping viz. C-5, C-6, C-7-trioxygenation pattern.

In the ^{1}H NMR (270 MHz, DMSO-d_6, TMS the signal located at δ7.70 ppm represents the overlapping protons at C-2' and C-6'. The proton at C-5' appears at δ6.88 ppm (d, J = 8.5 Hz) due to the shielding effect of the oxygen substituent in the ortho position. The C-8 proton is represented by a singlet at δ6.56 ppm. The hydroxyl protons at C-5, C-7, C-3' and C-4' resonate at δ12.66, 10.91, 9.31 and 9.21 ppm respectively. The signal at δ4.13 ppm integrates for 3 protons indicating a methoxyl group. H-1'' of glucuronic acid appears at δ5.55 ppm and the rest of the sugar protons appear at δ3.50 – 4.00 ppm.

On the basis of the above shreds of evidence, glycoside (II) has been characterized as patuletin 3-0-glucuronide.

The HRBC membrane stabilization studies on the isolates of *A.gangetica* showed two different properties namely haemolytic property and protecting biphasic properties (Hema *et al*., 2002). In the initial stage of hypo, saline induced haemolysis seemed to be predominating up to 75 µg beyond this concentration level, and the biphasic property started. Thus, at 100 µg of administration and optimum efficacy of the drug is noted. Such kind of biphasic property is quite prevalent with flavone glycoside. In addition, the haemolytic property which is considered the irritant is also observed.

Table 1: R_f [x 100) values of the constituents of the flowers of *A. gangetica*. (Whatman No: 1, Ascending, 30 ± 2° C)

Compound	Developing solvents							
	a	**b**	**c**	**d**	**e**	**f**	**g**	**h**
Glycoside (I)	21	30	50	62	74	64	66	83
Glycoside (II)	80	54	73	80	94	89	86	92
Luteolin 7-0-rutinoside (authentic)	21	31	49	61	75	64	66	84
Glycoside resulting from partial hydrolysis of I	02	10	18	30	36	44	56	69
Luteolin 7-0-glucoside (authentic)	01	10	18	32	36	44	56	70
Aglycone obtained after complete hydrolysis of I	-	02	06	25	59	89	72	66
Luteolin (authentic)	-	02	06	25	60	89	72	66
Aglycone obtained after complete hydrolysis of II	01	-	09	16	30	55	32	35
Patuletin (authentic)	01	-	10	16	30	56	31	35

Table 2: R_f [x 100) values of the constituents of the flowers of *A. gangetica*. (Whatman No: 1, Ascending, 30 ± 2° C)

Compound	Developing solvents			
	f	**g**	**j**	**k**
Sugar from the hydrolysate of II	20	13	20	04
Glucose acid(authentic)	19	14	20	04

j – n-BuOH: EtOH: H_2O = 4:1:2:2 and k – n-BuOH: Benzene: Pyridine: H_2O = 5:1:3:3

Table 3: ^{13}C NMR spectral data and their assignment for the glycosides from the flowers of *A. gangetica*

Compound	C2	C3	C4	C5	C6	C7	C8	C9	C10
Glycoside	164.20	103.20	181.82	161.60	99.80	163.20	94.40	157.00	108.00
Luteolin	164.50	103.30	182.20	162.10	99.20	164.70	94.20	157.90	104.20

Compound	C1'	C2'	C3'	C4'	C5'	C6'
Glycoside	122.00	113.60	146.20	150.20	116.60	119.60
Luteolin	122.10	113.80	146.20	150.10	116.40	119.30

Compound	C1''	C2''	C3''	C4''	C5''	C6''
Glycoside	100.40	74.00	76.80	70.80	76.00	68.40

Compound	C1'''	C2'''	C3'''	C4'''	C5'''	C6'''
Glycoside	101.20	70.40	70.30	71.80	68.80	18.00

Table 4: HRBC membrane stabilization from the flowers of *A. gangetica.*

S.no	Concentration of drug (µg)	Transmittance (Glycoside I)	Transmittance (Glycoside II)
1	10	89	75
2	25	88	79
3	50	88	81
4	75	86	80

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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