A NEW FLAVONOID ISOLATED FROM *THEVETIA PERUVIANA* AND EVALUATION OF ANTI-BACTERIAL ACTIVITY OF QUERCETIN-3-O-RUTINOSIDE

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Abstract : The isolation of Quercetin-3-O-rutinoside flavonoid was obtained from *Thevetia peruviana* and the structure was established based on analysis of UV, ^{1}H NMR and ^{13}C NMR spectroscopy methods. Moreover, the absence of toxicity of plant extracts and the isolation of active compounds are important to propose these plants as alternative approaches to resistance management and the results indicate the potential utility of isolated compounds, in the treatment of various bacterial infections.

***IndexTerms* - *Thevetia peruviana*, Apocynaceae, Flavonoid, Quercetin-3-O-rutinoside.**

I. INTRODUCTION

Phytochemicals derived from plants have delivered the basis for numerous commercial prescriptions used today for the treatment of a wide range of diseases. Phytochemical technique mainly applies to the quality control of herbal medicine of different chemical constituents such as tannins, saponins, alkaloids, volatile oils, flavonoids and anthraquinones. It is essential to use the phytochemical methods to screen and analyze bioactive components, not only for the quality control of crude drugs, but also for the interpretation of their therapeutic mechanisms.[1] The study of the distribution of flavonoids in plants is a continuing exercise and known flavonoids are being regularly exposed from new sources.[2]

Thevetia peruviana belongs to the family Apocynaceae and it often known as yellow oleander.[3] *Thevetia peruviana* has been deliberated as a hypothetically important plant for industrial and pharmacological application. Research admired that phytochemicals, working together with nutrients found in fruits, vegetables and nuts, may help slow the aging process and reduce the risk of numerous diseases such as high blood pressure, pain, asthma, cancers, heart diseases and urinary tract infections.[4]

The ethno-medical uses of *Thevetia peruviana* is seeming in treating the external wounds, infected area, ring worms, tumours etc., the use of grinded leaves of *Thevetia peruviana* in ethno-veterinary medicine is the evidence for its plenteous use for healing of wounds.[5] In the present study, yellow flowers of *Thevetia peruviana* were selected and the flavonol glycoside has been isolated and its therapeutic applications have been investigated.

II. MATERIALS AND METHODS

2.1. Extraction and fractionation

The fresh flowers (2 kg) of *Thevetia peruviana* (Apocynaceae) collected from Kumbakonam were extracted with 85 % MeOH (5 X 500 mL) under reflux. The alcoholic extract was concentrated *in vacuo* and the aqueous concentrate successively fractionated with peroxide - free ether (4 X 250 mL) and ethyl acetate (8 X 250 mL). The EtOAc fraction alone was taken up for the study.

2.2. Ether fraction: flavonol - quercetin

The Et_2O fraction was concentrated *in vacuo* and left in an ice chest for about a week. A yellow solid that separated was filtered and studied. It came out as pale yellow needles m.p. 315-317˚C on crystallization from MeOH. It was soluble in organic solvents and sparingly soluble in hot water. It gave a red colour with Mg-HCl, olive green with alc.Fe^{3+}, golden yellow colour with NH_3 and NaOH and appeared yellow under UV and UV/NH_3. It answered the Horhammer-Hansal, Wilson's boric acid and Gibb's tests. It had λ_{max}^{MeOH} nm 256, 268 sh, 370; +NaOMe 261 sh, 321, 422; +$AlCl_3$ 266, 302, 457; + ($AlCl_3$ - HCl) 268, 303, 352, 427; +NaOAc 274, 327, 390 and + (NaOAc - H_3BO_3) 261, 303 sh, 385 nm. The aglycone part of the compound was identified as quercetin and the same was confirmed by CO, mixed PC and m.m.p. with an authentic sample of quercetin from *Wrightia tinctoria*.[6]

2.3. Ethyl acetate fraction: Quercetin-3-O-rutinoside

The ethyl acetate fraction was concentrated *in vacuo* and left in an ice-chest for few days. A yellow solid that separated was filtered and studied. It developed a green colour with alc. Fe^{3+} and a pink colour with Mg-HCl. It appeared deep purple under UV that turned yellow on exposure to NH_3. It responded to Wilson's boric acid, Molisch and Gibb's tests, but did not answer the

Horhammer-Hansal tests. It had λ_{max}^{MeOH} nm 252, 268sh, 298sh, 359; +NaOMe 271, 330, 412; +$AlCl_3$ 276, 302 sh, 431; + ($AlCl_3$ / HCl) 266, 303 sh, 362 sh, 400; +NaOAc 278, 334, 391; and + (NaOAc/ H_3BO_3) 266, 299 sh, 388. Its R_f are represented in Table 1 and 2. The ^1H and ^{13}C spectral values of the glycoside are given in Table 3. It was identified as quercetin-3-O-rutinoside and the same was confirmed by CO and mixed PC with an authentic sample from *Wrightia tinctoria*.[6]

Table 1. R_f (X100) values of the Quercetin -3-O- rutinoside from *Thevetia peruviana*
(Whatman No.1, Ascending, 30 ± 2˚C)

Compound	*Developing Solvents								
	a	b	c	d	e	f	g	h	i
Quercetin -3-O- rutinoside	31	43	54	62	66	52	51	75	55
Quercetin from Glycoside (Complete hydrolysis)	-	-	4	17	38	84	45	47	71
Quercetin (authentic)	-	1	4	17	39	85	45	48	72
Glycoside from partial hydrolysis	8	06	24	34	60	59	54	65	67
Quercetin -3-O-glucoside (authentic)	8	06	24	35	60	59	55	66	66

***Solvent key**

a → H_2O f → BAW (n-BuOH: HOAc: H_2O=4:1:5, upper phase)
b → 5% aq.HOAc g → phenol saturated with water
c → 15% aq.HOAc h → forestol (HOAc: Conc. HCl)
d → 30% aq.HOAc i → TBA (t-butanol-acetic acid-water, 3:1:1)
e → 60% aq.HOAc

2.4. Hydrolysis of the glycoside

The glycoside (50 mg) was dissolved in hot aqueous methanol (5 mL; 50 %) and an equal volume of H_2SO_4 (7 %) was added to it. The reaction mixture was then refluxed at 100˚C for about 2 hrs. The excess of alcohol was distilled off *in vacuo* and the resulting aqueous solution was extracted with ether. The residue from ether fraction was studied as described below.

2.5. Identification of aglycone (flavonol- quercetin)

The residue from the Et_2O fraction of the hydrosylate when taken up in Me_2CO and left under chilled condition for a few days gave a yellow solid was obtained. Colour reactions, UV spectral values were all similar to those for free aglycone described earlier.

2.6. Identification of sugar (Glucose and rhamnose)

The filtrate after the removal of the aglycone was neutralized with $BaCO_3$. The concentrate 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. R_f (X100) values of the sugar from the Quercetin -3-O- rutinoside of *Thevetia peruviana*
(Whatman No.1, Ascending, 30 ± 2˚C)

Compound	*Developing Solvents			
	f	g	h	i
Sugar from Quercetin -3-O- rutinoside	17	37	37	-
Glucose (authentic)	17	38	37	-
Sugar from Quercetin -3-O- rutinoside	35	57	59	54
Rhamnose (authentic)	34	58	59	55

***Solvent key**

f → BAW (n-BuOH: HOAc: H_2O=4:1:5, upper phase); g → phenol saturated with water
h → forestol (HOAc: Conc. HCl); i → TBA (t-butanol-acetic acid-water, 3:1:1)
Spray reagent: Aniline hydrogen phthalate

2.7. Anti-bacterial activity by disc diffusion method

The bacterial strains of *Strptococcus pyogenes, Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were obtained from Microbial Type Culture Collection Centre (MTCC), Chandigarh. The bacterial cultures were swabbed on to Muller Hinton agar media was prepared and autoclaved at 15 lbs pressure for 20 minutes and cooled to 45 ºC. The cooled media was added 10 mL / L tartaric acid (10 %) act as anti-bacterial agents and poured on to sterile petriplates and allowed for solidification. A total of 6 mm diameter wells were punched into agar and filled with isolated compound from plant extracts and also placed control and standard (Ciprofloxacin purchased from Sigma-Aldrich, India) discs. The plates were then incubated at 37 °C for 48 hours. The anti-bacterial activity was evaluated by measuring the zone of inhibition and expressed in mm.

III. RESULTS AND DISCUSSION

Quercetin-3-O-rutinoside has been isolated from the fresh flowers of *Thevetia peruviana*. The free aglycone from ether fraction has been characterized as isorhamnetin on the basis of m.p., R_f values, UV data, ^{1}H-NMR and ^{13}C-NMR. The aglycone had λ_{max}^{MeOH} nm 256, 268sh, 370 nm suggesting the presence of a flavonol skeleton. A bathochromic shift of 52 nm perceived in NaOMe spectrum (band I) indicated the presence of free -OH group at C-3′. The presence of free OH group at C-5 was evident from the bathochromic shift of +57 nm in $AlCl_3$-HCl spectrum (band I). A shift of 6 nm towards the longer wavelength (band II) in the NaOAc spectrum indicated the presence of a free OH group at C-7.

The glycoside answered to Wilson's boric acid test representing the presence of free OH group at C-5. This was also apparent at from a bathochromic shift of 41 nm in its $AlCl_3$-HCl spectrum. A shift of 9 nm to the longer wavelength (band II) noticed in the NaOAc spectrum specified the presence of a free OH at C-7. A bathochromic shift of 29 nm (band I) in its NaOAc-H_3BO_3 spectrum exposed the presence of catechol type of substitution in the B-ring. A bathochromic shift detected in MeOH spectrum (band I) of the aglycone obtained on hydrolysis of the glycoside as compared to the glycoside suggested the site of glycosylation to be at C-3. It was also sustained by the fact that the glycoside did not respond to Horhammer-Hansel test.

^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the glycoside quercetin-3-O-rutinoside, the signal appearing at δ 12.0 ppm corresponds to -OH at C-5. The signal resonates at 10.2 ppm is due to the hydroxyl proton at C-7. The doublet appearing in the region of δ 7.538 ppm and δ 7.534 ppm correspond to the protons at C-2′ and C-6′. The signal appearing at δ 7.483 ppm corresponds to C-5′ proton. C-8 proton due to meta coupling with C-6 proton seems as a doublet at δ 6.701 ppm. C-6 proton due to meta coupling with C-8 proton looks as a doublet at δ 6.697 ppm. H-1″ of glucose resonates at δ 5.520 ppm while the H-1‴ of rhamnose at δ 4.608 ppm. The methyl protons of the rhamnose appear at δ1.42 ppm, the remaining sugar protons appear in the region of δ 3.36 to 4.59 ppm.

Table 3. ^{1}H and ^{13}C NMR data of Quercetin -3-O- rutinoside from *Thevetia peruviana*

Position	Quercetin -3-O- rutinoside		Position	Quercetin -3-O-rutinoside	
	δ_C	δ_H		δ_C	δ_H
2	156.48		1‴	100.42	4.608
3	133.25		2‴	73.18	
4	177.62		3‴	69.98	
5	161.20		4‴	74.48	
6	98.72	6.697	5‴	68.08	
7	164.28		6‴	16.35	
8	93.72	6.701	Rham-Me		1.42
9	155.82		5-OH		12.0
10	104.12		7-OH		10.2
1′	123.1		Rest of sugar protons		3.36-4.59
2′	115.15	7.538			
3′	145.61				
4′	148.53				
5′	116.37	7.483			
6′	120.93	7.534			
1″	101.52	5.520			
2″	71.10				
3″	76.78				
4″	68.08				
5″	76.51				
6″	67.39				

Supporting evidence for the structure of the flavonol glycoside is provided by the ^{13}C-NMR (400 MHz, DMSO-d_6, TMS) spectral data. A complete assignment of different signals is provided in Table 3. The ^{13}C-NMR spectral data for the corresponding aglycone taken out from the literature are also verified for easy comparison. Due to the glycosylation, the signal of

C-3 shifted up field by δ 1.51 ppm. The downfield shift of ortho-related C-2 signal by δ 10.6 ppm also confirms the same. The large shift in C-2 resonance also replicates the semi-olefinic characters of the flavonol C-2, C-3 double bond. The signal at δ 104.12 ppm of C-10 is less intense due to longer relaxation time of the quaternary carbon. The signal of C-6‴ of rhamnose at δ 16.35 ppm and that of C-6″ signal at δ 67.39 ppm clearly show that the glycoside is a quercetin-3-O-rutinoside.[7] Based on these observations, the glycoside obtained from EtOAc fraction of the flower from *Thevetia peruviana* could be confirmed as quercetin-3-O-rutinoside (Fig. 1).

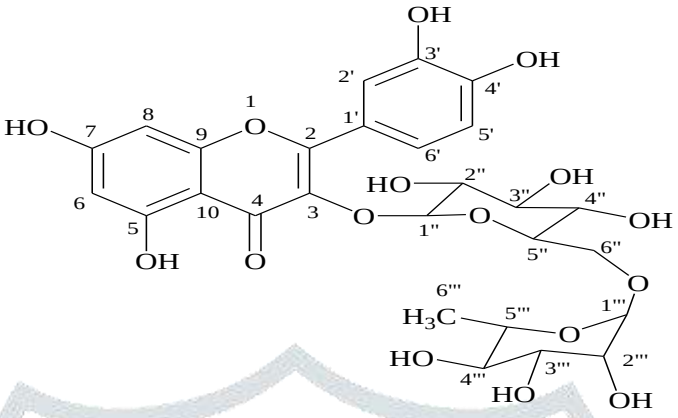

Fig. 1. Quercetin-3-O-rutinoside

The anti-microbial activity of quercetin-3-O-rutinoside was studied in the concentrations of 50, 100,150 and 200 mg / ml against five pathogenic bacterial strains (Fig. 2). All the bacterial strains were selected for the basis of its application purpose of further formulation study. Anti-microbial potential of test samples were assessed in terms of zone of inhibition of bacterial growth and the results were compared with standard (Ciprofloxacin). The results of the anti-microbial activities are presented in Table 4.

Table 4. Anti-bacterial activity of Quercetin-3-O-rutinoside isolated compounds by disc diffusion method

S. No.	Micro organisms	Zone of inhibition mm in diameter (M ± SD)				
		Quercetin-3-O-rutinoside				Standard Ciprofloxacin (30 mg)
		50 mg	100 mg	150 mg	200 mg	
1	*Streptococcus pyogenes*	04	07	06	04	18
2	*Bacillus subtilis*	10	-	11	01	16
3	*Staphylococcus aureus*	08	06	03	02	21
4	*Escherichia coli*	-	02	07	03	12
5	*Pseudomonas aeruginosa*	09	10	-	-	22

The test samples dose at 100 mg revealed moderate anti-microbial activity with zone of inhibition ranging from 2.0 to 11.0 mm (Fig. 2) and had shown to all pathogens. These observations may be due to the nature of biological active component and the stronger extraction capacity of methanol could have been produced active constituent that are responsible for anti-bacterial activities. Standard Ciprofloxacin (30 mg) shown moderate anti-microbial activity with zone of inhibition ranging from 12.0 to 22.0 mm and had shown to all pathogens (Fig. 2). It may be due to the presence of broad spectrum of anti-microbial compounds in the flowers of selected four medicinal plants. On the basis of the results reached in the present investigation, isolated compound quercetin-3-O-rutinoside showed considerable inhibitory activity against *Streptococcus pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Our results indicate the potential utility of isolated compounds, in the treatment of various bacterial infections.

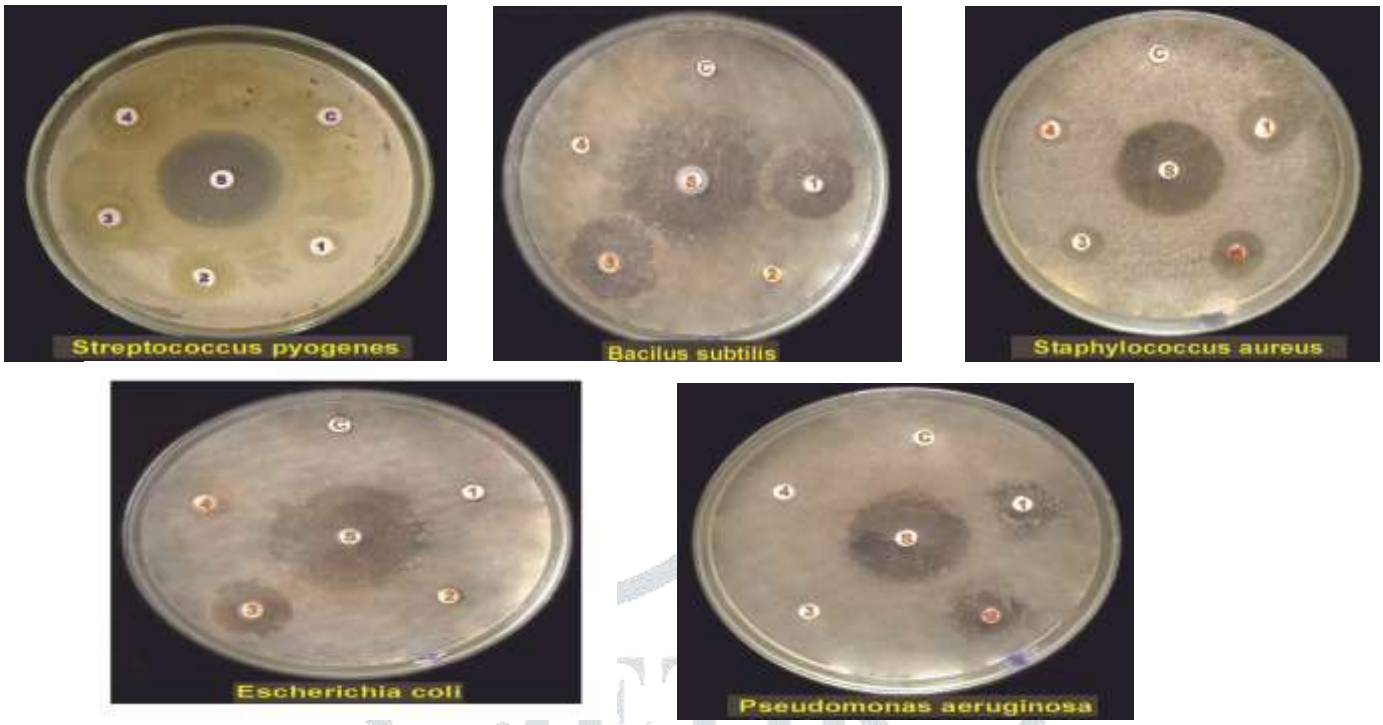

Fig. 2. Anti-bacterial activity of Quercetin-3-O-rutinoside by disc diffusion method

IV. CONCLUSION

The absence of toxicity of plant extracts and the isolation of active compound are important to propose medicinal plants *Thevetia peruviana* as alternative approaches to indicate the potential utility of isolated compound, in the treatment of various bacterial infections. By ways of solvent extraction, hydrolysis, precipitation and crystallization, high purity of flavonoid with quercetin-3-O-rutinoside structure were acquired in our research. The method is suitable for isolation of flavonoid from the *Thevetia peruviana* in industry.

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