Isolation, Characterization and Anti-Inflammatory Property of *Thevetia Peruviana*

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Abstract: *Thevetia peruviana* seeds contain glucosides of neriifolin, acetylneriifolin and thevetin. Seed oil distillates of *Thevetia peruviana* have been found to contain anti-bacterial activity. In the present work, the fresh flowers of *Thevetia peruviana* was subjected to phytochemical studies. The results of the study showed that the flowers contain quercetin, kaempferol and quercetin-7-*o*-galactoside. The structure of the isolated compound was characterized by UV, ¹H NMR and ¹³C NMR spectra. The anti-inflammatory character of the isolated compound was tested by *in vitro* method and the results of the study revealed that the isolated compound showed a biphasic property.

Keywords: *Thevetia Peruviana*, Quercetin, Kaempferol, Anti-inflammatory.

Introduction

Phytochemistry is the branch of chemistry which deals with the isolation and characterization of the freely available organic and inorganic compounds in plants using the modern techniques like UV, NMR, IR and Mass spectral analysis. Amongst the organic compounds, flavonoids play an important role as pigments in plant kingdom.

The role of flavonoids in clinical conditions such as hypertension, rheumatism, arthritis and pregnancy and also their beneficial effects in frost-bite and cold injury have been well documented. Seeds of *Thevetia peruviana* were screened for their antifungal photoactivity¹.

The extracts and preparation from the plant which are hopefully safe, exhibited various additional biological effects such as antioxidant, immunomodulatory, anticancerogenic,

antimicrobial, antiparasitic and insect antifeedant or repellent activities². Each year there are thousands of yellow oleander poisoning cases in South Asia and probably hundreds of deaths, though it possess several pharmacological activities³. Several flavonoids are moderately effective against laboratory cultures of malignant cells. Anti-ulcer and anti-tumor activities of flavonoids have also been observed. In the present work, the flowers of *Thevetia peruviana* have been subjected to phytochemical studies with a view to isolate and characterize their polyphenolics and also to study their biological activity.

Thevetia peruviana an evergreen shrub, belonging to apocynaceae family, is a very poisonous shrub in nature and the kernels being the most toxic. The active principles in yellow oleander are cardiac glycosides⁴. The physical properties of the fruit and kernel are unique and different from other tree born oilseeds *i.e.* the storage, transport handling *etc.* Activities related to the fruits and kernels will require modifications in the processes and structures prevailing for other tree born oilseeds⁵⁻⁷. Though the kernel fraction in fruit of *Thevetia peruviana* is only 16.14%, the amazingly high oil content for use in different industrial purpose is an incentive for its post-harvest processing.

Thevetin is pharmacologically the most active constituent, especially on heart. Thevetoxin closely resembles Thevetin in pharmacological action but is less toxic. The seeds contain glycosides of neriifolin, acetyl neriifolin and *Thevetin*. Seed oil distillates of *Thevetia peruviana* has been found to contain anti-bacterial activity. The leaves of *Thevetia peruviana* are used to toothache due to caries. It is used in antirheumatic and decongestant. Its branches are used for febrifuge and purge.

Experimental

Fresh flowers of *Thevetia peruviana* collected were extracted with 90% methyl alcohol under reflux. The alcoholic extract was concentrated *in vacuo* and the aqueous concentrate was successively fractionated with benzene, peroxide free ether and ethyl acetate. The benzene fraction did not yield any isolable material.

Ether fraction

The ether fraction was concentrated *in vacuo* and left in an ice-chest for a few days. The yellow solid that separated when subjected to paper chromatography revealed the presence of two aglycones. The mixture of aglycones was separated by partial paper chromatography. The two solids separated were labeled as A₁ and A₂.

Quercetin (A₁)

The solid A₁ was dissolved in acetone and kept in an ice-chest for a few days. The yellow solid that separated was filtered and recrystallized from methyl alcohol. The yellow needles obtained, melted at 313-315 °C. The yield of the compound was 0.02%. It was readily soluble in organic solvents and sparingly in hot water. It gave a golden yellow when fumed with ammonia and appeared yellow in sodium hydroxide. It showed a pale green fluorescence with concentrated sulphuric acid. It reduced ammoniacal silver nitrate in the cold and Fehling's solution on heating. It answered the Horhammer-Hansel, Wilson's boric acid and Gibb's test. It did not answer Molisch's test.

It shows $\lambda_{\max}$ (nm) at 255, 269, 370; NaOMe 262, 321, 420; AlCl₃ 267, 303, 458; (AlCl₃-HCl) 267, 303, 351, 428; NaOAc 275, 328, 390; (NaOAc-H₃BO₃) 262, 303, 386 nm and had R_f values as given in Table 1. The compound was identified as quercetin by ¹H NMR and ¹³C NMR spectra and also the identity was confirmed by authentic sample of quercetin from *Wrightia tinctoria*.

Table 1. R_f (x100) values of the constituents of the flowers of *Thevetia peruviana*

Compound	Developing solvents*								
	a	b	c	d	e	f	g	h	I
Aglycone from Et ₂ O fraction	-	01	04	17	38	85	39	48	72
Al: Quercetin (Authentic)	-	01	04	17	38	85	39	48	72
Aglycone from Et ₂ O fraction	-	5	-	49	93	67	62	87	94
Kaempferol (Authentic)	-	5	-	50	93	67	62	87	94
Glycoside from EtOAc fraction	6.49	7.45	11.43	20.71	41.12	27.22	32.32	50	64.60

Table 2. R_f (x100) values of the sugar from the glycoside of *Thevetia peruviana*

Compound	Developing solvents*				
	e	f	g	h	i
Sugar from the hydrolysate of glycoside	73	09	44	89	43
Galactose (Authentic)	73	09	44	89	43

*Solvent key: a - H₂O; b - 5% aq.HOAc; c - 15% aq.HOAc; d - 30% aq.HOAc; e - 60% HOAc; f - n-BuOH: HOAc: H₂O= 4:1:5 upper phase (BAW); g - Phenol saturated with water; h - Forestal (AcOH: Conc.HCl:H₂O=30:3:10); i - TBA(t-BuOH:HOAc:H₂O=3:1:1)

Kempferol (A₂)

The isolated pigment was recrystallized from methyl alcohol (m.p. 276-278 °C, yield 0.01%). $\lambda_{\max}$ (nm)= 266, 320, 370; NaOMe 278, 316, 420; AlCl₃ 268,303, 350, 424; AlCl₃-HCl 269, 302, 352, 420; NaOAc 274, 386 and NaOAc-H₃BO₃ 267, 320, 372. It developed a red colour with Mg-HCl. It appeared yellow when viewed under ultra violet light with and without ammonia. Its R_f values are given in Table 1. The compound was identified as kaempferol by ¹H NMR and ¹³C NMR spectra and also the identity was confirmed by authentic sample of kaempferol isolated from Bauhinia acuminata.

Ethyl acetate fraction

The ethyl acetate fraction was concentrated *in vacuo* and left in an ice-chest for a week. The solid that was separated revealed the presence of a glycoside. The glycoside was recrystallized and separated as yellow needles.

Flavonol glycoside quercetin-7-O-glactoside

The glycoside recrystallized from methyl alcohol separated as yellow needles (m. p. 250-252 °C, yield 0.01%). It showed $\lambda_{\max}$ (nm) at MeOH 265, 295, 315, 340, 370; NaOMe 269, 299, 317, 337, 369 dec; NaOAc 267, 295, 312, 340, 370; NaOAc-H₃BO₃ 257, 290, 315, 367, 374; AlCl₃ 270, 300, 335, 399, 410, 435; AlCl₃-HCl 267, 293, 336, 367, 390, 405 nm. It is sparingly soluble in cold water and somewhat freely in hot water. The R_f values are tabulated in Table 1. It was identified as quercetin-7-*o*-galactoside.

Hydrolysis of the glycoside

The glycoside was dissolved (20 mg) in methyl alcohol (10 mL) and hydrolyzed with 7% sulphuric acid (10 mL) at 100 °C for 2 h. The excess of alcohol was distilled off *in vacuo* and resulting aqueous solution was extracted with ether. The residue from ether fraction was studied as described under ether fraction. The isolated aglycone was identified as quercetin.

Identification of the sugar

The filtrate after the removal of the aglycone was neutralized with barium carbonate. The concentrated filtrate when examined by paper chromatography gave R_f values (*c.f.* Table 2) corresponding to those of galactose.

The fresh flowers of *Thevetia peruviana* have been found to contain quercetin, kaempferol and quercetin 7-*o*-galactoside. The general structure for flavone is given in Figure 1. The structure of the isolated compounds are given in Figures 2-4.

Figure 1. The structure formula of flavone

Figure 2. The structure formula of quercetin 7-*o*-galactoside

Figure 3. The structure formula of kaempferol

Figure 4. The structure formula of quercetin

Results and Discussion

The fresh flowers of *Thevetia peruviana* have been found to contain quercetin, kaempferol and quercetin 7-*o*-galactoside.

UV spectrum of quercetin exhibited two major peaks at 370 nm and 255 nm which showed the presence of a flavonol skeleton. Flavonols which have free –OH groups at both the 3 and 4' positions are unstable in sodium acetate and the absorption peaks in the sodium acetate spectrum degenerate in a few minutes⁸ which is actually observed in our compound.

In the ¹H NMR spectrum (400 MHz, DMSO-d₆, TMS) of the aglycone, the hydroxyl proton at C-5 absorbs at δ12.494 ppm as a distinct singlet. The sharp singlets at δ10.796 and δ9.605 ppm correspond to –OH protons at C-7 and C-3. The doublet at δ9.378 ppm and a singlet observed at δ9.321 ppm accounts for the hydroxyl protons at C-3' and C-4'. The C-5' protons appears as a doublet at δ6.934 ppm (*J*=8.5Hz). The signals due to the protons at C-2' and C-6' overlap at δ7.677 ppm. A ring protons at C-6 and C-8 could be located respectively at δ6.185 (d, *J*=2.5Hz) and δ6.439ppm (d, *J*=2.5Hz)

The 100 MHz ¹H NMR (DMSO-d₆) spectrum of the flavonol shows a signal at the offset region around δ12.543 ppm, revealing the presence of a free 5-OH proton. The signal at δ10.120 ppm indicates the presence of free 7-OH proton. The C-6 proton occurs as a doublet at δ6.233 ppm, higher field than that of C-8 proton which also occurs as a doublet at δ6.439 ppm. Protons at C-2', 3', 5' and 6' (due to free rotation of the B-ring) appear as a two pairs of *ortho* coupled doublets. The H-3', 5' doublets occurs at δ6.872 ppm upfield from the H-2',6' doublet which in turn occurs at δ8.032 ppm due to the shielding effect of the oxygen substituent.

Table 3. ^{13}C NMR data and signal assignments for the aglycone from the flowers of *Thevetia peruviana* (* Values from literature)

Compounds	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-1'	C-2'	C-3'	C-4'	C-5'	C-6'
Aglycone from Et ₂ O fraction:A ₁	146.8	135.7	175.8	160.7	98.2	163.8	93.3	156.1	103	122	115.1	145	147.6	115.6	120
Quercetin*	146.9	135.5	175.8	160.7	98.2	163.9	93.3	156.2	103.1	122.1	115.3	145	147.6	115.6	120
Aglycone from Et ₂ O fraction:A ₂	146.8	135.7	175.8	160.7	98.2	163.8	93.4	156.1	103	121.7	129.4	115.4	159.1	115.6	129.4
Kaempferol*	146.8	135.6	175.9	160.7	98.2	163.9	93.5	156.2	103.1	121.7	129.5	115.4	159.2	115.4	129.5

Anti-inflammatory drugs may be defined as components that inhibit the whole or any portion of an acute or chronic inflammation reaction irrespective of whether the drug is clinically useful or not. Clinically anti-inflammatory drugs are judged by their action on the pain, stiffness or swelling of the affected part.

Red blood cells (RBC) have been used to study the action of prymnesin, as algae toxin membrane. Red blood cells has been used as a model system by a number of workers for the study of interaction of drugs with membranes. Drugs like anesthetics stabilize red blood cells against hypotonic haemolysis at low concentration but cause haemolysis at high concentration.

The stabilization of red blood cells against hypotonic haemolysis has attracted the attention of a number of workers. Haemolytic effect of membrane active substances has been the interest of few others. The action of phospholipases membrane degrading enzymes using red blood cells has also been investigated. In these entire cases hemoglobin leak was observed.

Procedure

Collection of blood

Blood was collected from healthy human volunteers using sterile 22 gauge hypodermic needle. The collected blood was mixed with equal volume of sterile alsever solution (containing 2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride) and stored at 4 °C.

Saline

Saline at different concentrations were prepared. (Isosaline 0.85% and hyposaline 0.25%).

Preparation of human red blood cell suspension

The blood was centrifuged and the packed cells obtained were washed thrice with isosaline (0.85%, pH 7.2) and a 2% suspension was made with isosaline.

Determination of human red blood cell membrane stabilization

Solutions of different concentrations of flavonoids were prepared. Assay mixture contained the drug flavonoid in concentrations as mentioned in (Table 4), 1 mL of phosphate buffer (0.15 M, pH 7.4), 2 mL of hypo saline (0.25%) and 0.5 mL of 2% human red blood cell suspension. All the tubes were incubated at 37 °C for 30 min. Then they were centrifuged and hemoglobin content in the supernatant was estimated using a photoelectric colorimeter at 560 nm. Percentage haemolysis was calculated assuming haemolysis produced by the cells in the presence of distilled water to be 100%. A control has been done without the drug. The values are tabulated in Table 4.

Table 4. Effect of ethyl acetate fraction of *Thevetia peruviana* on human red blood cell

S. No.	Concentration of drug, microgram	Transmittance
1.	10	97
2.	25	100
3.	50	100
4.	75	95
5.	100	91
6.	200	91
7.	400	91

The human red blood cell membrane stabilization studies may be considered as a screening investigation for anti-inflammatory properties. In our present work, the yellow pigments isolated from *Thevetia peruviana* showed a biphasic property.

The glycoside isolated from *Thevetia peruviana* showed an initial stabilization to reach a maximum at 50 µg and the value decreases and remains constant with increasing concentration. Such kind of biphasic property is prevalent in polyphenols.

Conclusion

In the present work, the fresh flowers of *Thevetia peruviana* was subjected to phytochemical studies. The results of the study showed that the flowers contain quercetin, kaempferol and quercetin-7-*o*-galactoside. The structure of the isolated compound was characterized by UV, ¹H NMR and ¹³C NMR. The anti-inflammatory character of the isolated compound was tested by in-vitro method and the results of the study revealed that the isolated compound showed a biphasic property.

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