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Isolation, Characterization, and Membrane Stabilizing Activity of Isorhamnetin 3-O-sophoroside from *Thespesia populnea* Flowers

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Abstract

A phytochemical investigation of the fresh flowers of *Thespesia populnea* was conducted. The ethyl acetate fraction yielded a flavonoid glycoside, identified as isorhamnetin 3-O-sophoroside, alongside its aglycone, isorhamnetin. The structure of the glycoside was elucidated through extensive spectroscopic analysis, including UV, ^{1}H-NMR, and ^{13}C-NMR, and confirmed by chemical hydrolysis and chromatographic (R_f) studies. The isolated glycoside was evaluated for its anti-inflammatory potential via a human red blood cell (HRBC) membrane stabilization assay against hypotonicity-induced hemolysis. It demonstrated significant, dose-dependent activity, exhibiting maximal protection at a concentration of 50 µg/mL. This is the first report on the isolation of isorhamnetin 3-O-sophoroside from *Thespesia populnea* flowers and its notable membrane-stabilizing properties.

***Key words*:** Hemolysis, HRBC, Isorhamnetin, Malvaceae, Sophoroside

Thespesia populnea (L.) Sol. ex Corrêa, a member of the Malvaceae family [10], [12], is a tree of significant pharmacological interest. Commonly known as the Portia tree or 'poovarasu' in Tamil, it is highly adaptable to tropical climates, exhibiting considerable drought and wind resistance, which makes it a common feature in coastal areas. In traditional medicinal systems, including Ayurveda, various parts of *Thespesia populnea* (referred to as 'Parisha,' 'Kapitana,' 'Parshvipala,' and 'Kartabanda') have been employed extensively for the treatment of skin diseases and other ailments [13], [17].

This traditional use is supported by scientific studies demonstrating that extracts from its roots, fruits, and leaves possess membrane-stabilizing properties on the red blood cell (RBC) lysosomal membrane system [4]. Furthermore, different parts of the tree, including the leaves, bark, and seeds, have been investigated and shown to exhibit anti-inflammatory, anti-diabetic, and antioxidant activities [6], [22]. Phytochemical investigations have revealed a rich profile of bioactive compounds, such as gossypol, flavonoids (e.g., quercetin, kaempferol, and their glycosides), terpenoids (e.g., lupenone, lupeol), sterols (e.g., β-sitosterol), and specific unique compounds like thespesin and mansonones [18].

However, despite the extensive research on other plant parts, the floral components have received scant attention. Given that flowers are often a rich source of polyphenols and flavonoids, this study was designed to investigate the biodynamic compounds present in the petals of *Thespesia populnea*. The specific objectives were to isolate and characterize flavonoids from the fresh flowers and to evaluate the anti-inflammatory potential of the isolated compound(s) through a hypotonicity-induced human red blood cell (HRBC) membrane stabilization assay. To the best of our knowledge, this constitutes the first report on the isolation of isorhamnetin and its glycoside from the flowers of *Thespesia populnea* and the first evaluation of their hypotonicity-induced hemolysis activity.

MATERIALS AND METHODS

Plant material collection and extraction

Fresh flowers (1 kg) of *Thespesia populnea* (L.) Sol. ex Corrêa were collected from the banks of the Arasalaru River in Karaikal, India, during November 2020. The plant was identified by Dr. S. John Brito, Director at the Rapinat Herbarium. The yellow petals (650 g) were carefully separated and extracted with boiling 85% ethanol (5 × 500 mL) under reflux. The combined ethanolic extract was concentrated under reduced pressure using a rotary evaporator to obtain a crude extract. The concentrate was subsequently fractionated sequentially between water and organic solvents of increasing polarity (n-hexane, chloroform, ethyl acetate, and n-butanol).

Isolation and purification

The ethyl acetate (EtOAc) fraction was subjected to column chromatography on a silica gel (60-120 mesh) column. Elution was performed with a stepwise gradient of petroleum ether (60-80°C), benzene, chloroform, and ethyl acetate. Fractions were collected and monitored by thin-layer chromatography (TLC). Fractions with similar TLC profiles were pooled. A compound, designated G6, was eluted with a chloroform:ethyl acetate (1:3, v/v) mixture. This compound

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was found to be homogeneous on TLC in multiple solvent systems.

The pooled fractions containing G6 were concentrated, dissolved in a minimal volume of acetone, and stored at 4°C for 24 h to facilitate crystallization. The resulting yellow solid was filtered and recrystallized from aqueous methanol to yield yellow needles of pure compound G6 (yield 0.08%, 181-182°C).

Characterization of compound G6

The isolated compound was characterized by its melting point, UV-Visible spectroscopy with shift reagents, and specific colour reactions. UV-Vis spectral data (λmax, nm) were recorded in methanol: 255, 267, 356; + NaOMe: 271, 323, 410 (dec.); + $AlCl_3$: 270, 313, 359, 410; + $AlCl_3$/HCl: 270, 359, 410; + NaOAc: 271, 315, 395; + NaOAc/H_3BO_3: 257, 293, 360. The compound was soluble in ethanol and ethyl acetate but insoluble in cold water. It gave a pink colouration with Mg-HCl, a green colour with alcoholic $FeCl_3$, appeared deep purple under UV light, and turned yellow with a pale-green fluorescence when fumed with NH_3 vapours. It gave positive results for Wilson's boric acid, Gibb's, and Molisch's tests but was negative for the Horhammer–Hansel test [3]. R_f values in various solvent systems are presented in (Table 1).

Table 1 R_f (X 100) values of the constituents of the flowers of *Thespesia populnea* (Whatman No: 1, Ascending, 30 ± 2 °C)

Compound	Developing solvents							
	H_2O	Amount of Hoac (aq), 5%	Amount of Hoac (aq), 15 %	Amount of Hoac (aq), 30%	Amount of Hoac (aq), 60%	BuOH: HOAc: H_2O = 4:1:5 (upper phase)	Water saturated phenol	HOAc:ConC. HCl:H_2O = 30:3:10
Glycoside (G6)	52	55	60	68	77	70	50	89
Aglycone from G6	-	-	05	20	45	88	81	61
Isorhamnetin (authentic)	-	-	04	19	45	89	80	61

Acid hydrolysis of G6 and identification of aglycone and sugar

Compound G6 (0.05 g, ~0.1 mmol) was hydrolyzed by refluxing with 5% H_2SO_4 (20 mL in 50% aqueous methanol) at 100°C for 2 hours. The reaction mixture was cooled and diluted with water. The liberated aglycone was extracted with diethyl ether. The ether layer was evaporated, and the residue was recrystallized from acetone to yield a yellow solid (aglycone), with a melting point of 305-307°C. The identity of the aglycone as isorhamnetin was confirmed by co-TLC and mixed melting point determination with an authentic standard. The aqueous layer was neutralized with $BaCO_3$, filtered, and concentrated. The sugar residue was identified as glucose by paper chromatography (PC) using multiple solvent systems and co-chromatography with an authentic standard. The aglycone-to-sugar ratio was determined to be 1:2 using the Folin-Wu method [21]. R_f values for the sugar are listed in (Table 2).

Table 2 R_f (X 100) values of the sugar from the glycoside G6 from *Thespesia populnea* (Whatman No: 1, Ascending, 30 ± 2 °C)

Compound	Developing solvents					
	BuOH: HOAc: H_2O = 4:1:5 (upper phase)	Water saturated phenol	HOAc: Conc. HCl:H_2O = 30:3:10	EtOAc: Pyridine: H_2O = 10:4:3	BuOH: Benze: Pyridine: H_2O	BuOH: amount of HOAc, 27 % = 1:1
Sugar from the hydrolysate of G6	17	36	37	86	25	25
Glucose (authentic)	17	38	37	86	24	25

Hypotonicity-Induced hemolysis assay

The membrane-stabilizing activity was assessed using a hypotonicity-induced hemolysis assay. Fresh blood was collected from healthy adult male and mixed with an equal volume of Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, 0.42% NaCl). The blood was used within 5 hours of collection. A 1% (v/v) human red blood cell (HRBC) suspension was prepared in isotonic phosphate-buffered saline (PBS, 0.15 M, pH 7.4).

The reaction mixture consisted of 2 mL of hypotonic saline (0.36% NaCl), 1 mL of phosphate buffer (0.15 M, pH 7.4), 0.5 mL of HRBC suspension, and 1 mL of the test compound (G6) at various concentrations *[e.g., 10, 25, 50, 75, 100 µg/mL in isotonic saline]*. A control tube representing 100% hemolysis contained 1 mL of isotonic saline (0.85% NaCl) instead of the drug. A negative control contained no test compound. All test tubes were incubated at 37°C for 30 min and then centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant, representing released hemoglobin, was measured at 560 nm using a spectrophotometer. The percentage inhibition of hemolysis was calculated using the following formula:

% inhibition of Hemolysis = [1 - (Absorbance of Test / Absorbance of Control)] × 100

*All experiments were performed in triplicate (n=3), and data are presented as mean ± standard deviation

*Statistical analysis was performed using [e.g., one-way ANOVA followed by Dunnett's test].

RESULTS AND DISCUSSION

Structural elucidation of the isolated compound

Phytochemical investigation of the ethyl acetate fraction of *Thespesia populnea* flowers led to the isolation of a yellow compound (G6). Acid hydrolysis of G6 yielded isorhamnetin and glucose in a 1:2 molar ratio, confirming it to be a glycoside of isorhamnetin.

The structure was elucidated using spectroscopic data. The UV-Vis spectrum of G6 in methanol showed absorption maxima (λmax) at 255 and 356 nm, characteristic of a flavonol skeleton [15]. A bathochromic shift of 54 nm in Band I upon addition of NaOMe indicated a free hydroxyl group at the C4′

position. A positive Wilson's boric acid test and a bathochromic shift in the $AlCl_3$/HCl spectrum confirmed a free C5-OH. A bathochromic shift of 16 nm in Band II with NaOAc suggested a free C7-OH. The absence of a significant shift with NaOAc/H_3BO_3 and the minimal change between the $AlCl_3$ and $AlCl_3$/HCl spectra indicated the absence of an ortho-dihydroxy (catechol) group on the B-ring [7], [10].

The ^{1}H-NMR spectrum (270 MHz, DMSO-d_6) provided further evidence. A sharp singlet at δ 12.56 ppm was assigned to the chelated C5-OH proton [1]. A singlet at δ 10.86 ppm was attributed to the C7-OH proton. The B-ring protons appeared as a characteristic AA′XX′ system: a doublet at δ 8.00 ppm (J = 2 Hz, H-2′) and a quartet at δ 7.46 ppm (J = 2, 8 Hz, H-6′), alongside a doublet at δ 7.00 ppm (J = 8 Hz, H-5′). The methoxy group at C3′ resonated as a singlet at δ 3.96 ppm. The A-ring meta-coupled protons H-6 and H-8 were observed as doublets at δ 6.40 and 6.24 ppm (J = 2 Hz each). Two anomeric proton signals at δ 5.36 and 5.16 ppm, along with a cluster of signals between δ 3.00–3.75 ppm, confirmed the presence of two sugar units [16].

Table 3 ^{13}C NMR spectral data and their assignment for the glycoside from the flowers of *Thespesia populnea* [13]

Compound	C2	C3	C4	C5	C6	C7	C8	C9	C10
Glycoside	156.90	133.90	177.60	161.30	99.10	164.70	93.80	156.60	104.20
Isorhamnetin	147.10	136.10	176.30	161.20	98.60	164.40	93.90	156.80	103.50

Compound	C1'	C2'	C3'	C4'	C5'	C6'
Glycoside	121.40	115.13	149.50	148.50	113.37	122.40
Isorhamnetin	122.60	112.70	149.40	147.90	116.00	122.40

Compound	C1''	C2''	C3''	C4''	C5''	C6''
Glycoside	99.10	80.00	78.01	70.40	78.01	62.40

Compound	C1'''	C2'''	C3'''	C4'''	C5'''	C6'''
Glycoside	103.70	75.20	78.01	70.20	78.01	61.20

The ^{13}C-NMR data (Table 3) provided conclusive evidence for the structure. The significant up field shift of C-3 (δ 133.90 in glycoside vs. δ 136.10 in aglycone) and downfield shifts of the adjacent C-2 and C-4 confirmed glycosylation at the C-3 position [9]. The two anomeric carbon signals at δ 99.10 (C-1″) and 103.70 (C-1‴) indicated a disaccharide. The downfield shift of C-2″ (δ 80.00) compared to its typical position in a monosaccharide (δ ~74-75) is diagnostic of a (1→2) interglycosidic linkage, confirming the disaccharide as sophorose [2-3]. The methoxy carbon resonated at δ 55.96 ppm. Based on this collective evidence, compound G6 was unequivocally identified as isorhamnetin 3-O-sophoroside (Fig 1).

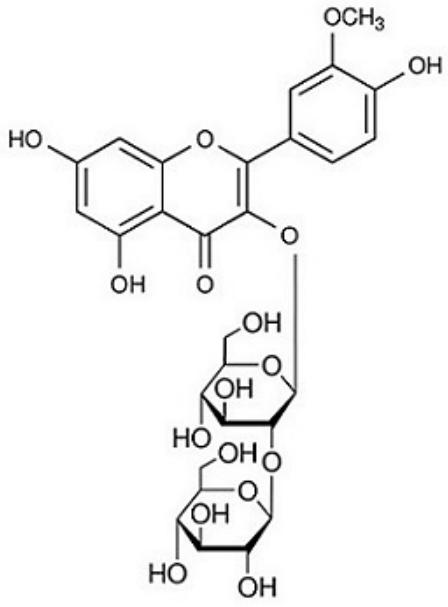

Fig 1 Isorhamnetin 3-0-sophoroside

Membrane stabilizing activity

The anti-inflammatory potential of isorhamnetin 3-O-sophoroside was evaluated by assessing its ability to stabilize human red blood cell (HRBC) membranes against hypotonicity-induced lysis, a well-established model for predicting anti-inflammatory activity [5], [19].

The results demonstrated a significant dose-dependent inhibition of hemolysis (Fig 2). The compound exhibited maximal protective activity, inhibiting hemolysis by 30% at concentrations of 50 and 75 µg/mL. This membrane-stabilizing effect is a key mechanism of action for many non-steroidal anti-inflammatory drugs (NSAIDs) [8].

The proposed mechanism may involve the flavonoid glycoside integrating into the lipid bilayer of the erythrocyte membrane, thereby increasing its surface area and reducing its fragility and permeability under osmotic stress [1, 13]. This prevents the influx of excess fluid and subsequent lysis. By stabilizing the HRBC membrane, which is analogous to the lysosomal membrane, the compound may prevent the release of inflammatory lysosomal enzymes (e.g., proteases, phospholipases) from activated leukocytes at sites of inflammation, thereby mitigating tissue damage [20].

To our knowledge, this is the first report on the isolation of isorhamnetin 3-O-sophoroside from *Thespesia populnea* flowers and the first demonstration of its significant membrane-stabilizing activity [22].

CONCLUSION

Phytochemical investigation of the fresh flowers of *Thespesia populnea* led to the isolation and characterization of a rare flavonoid glycoside, isorhamnetin 3-O-sophoroside, alongside its aglycone, isorhamnetin. The structure was unequivocally determined using comprehensive spectroscopic techniques (UV, ^{1}H-NMR, ^{13}C-NMR), chromatographic analysis, and chemical hydrolysis. Furthermore, the isolated

glycoside demonstrated significant, dose-dependent human red blood cell (HRBC) membrane stabilization activity in vitro, exhibiting potent inhibition of hypotonicity-induced hemolysis. This membrane-stabilizing effect suggests a potential mechanism for the anti-inflammatory properties traditionally attributed to this plant. To our knowledge, this is the first report of the isolation of this compound from *Thespesia populnea* flowers and the first evaluation of its bioactivity in this model, highlighting the flowers as a valuable source of bioactive flavonoids worthy of further investigation.

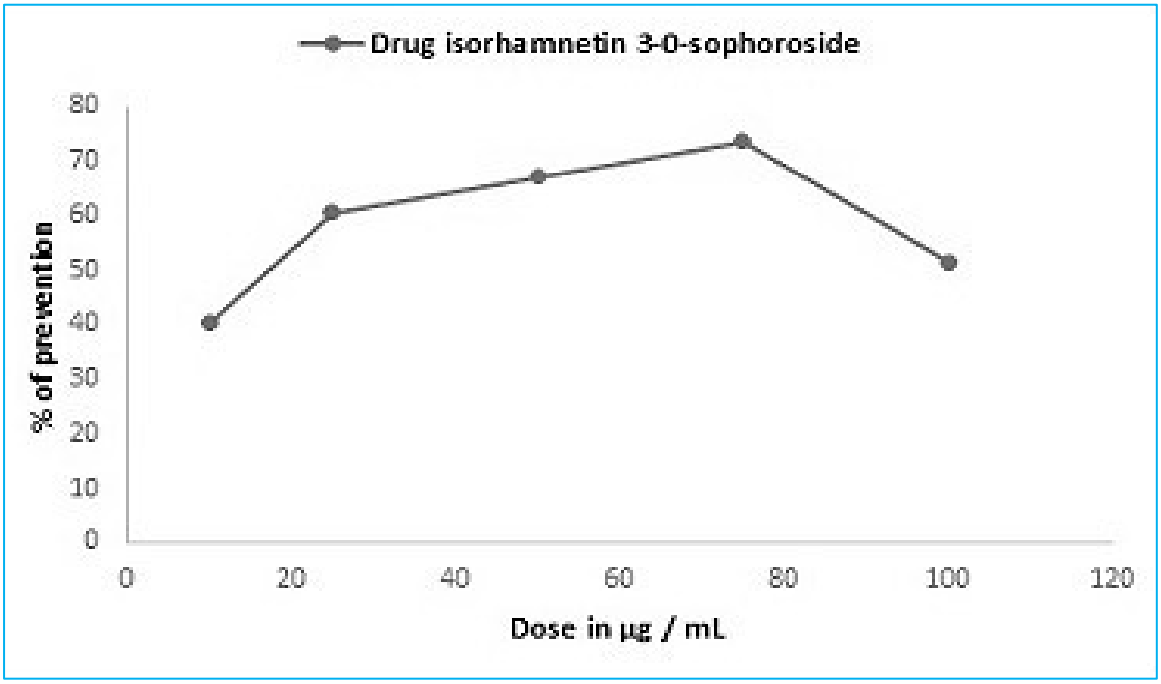

Fig 2 Effect of flavonoids isolate of *Thespesia populnea* against hypotonicity induced hemolysis

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