

ISSN Print: 2664-6781
 ISSN Online: 2664-679X
 NAAS Rating (2025): 4.77
 IJACR 2025; 7(8): 127-131
www.chemistryjournals.net
 Received: 18-06-2025
 Accepted: 23-07-2025

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Isolation of flavonoid glycosides from *Hibiscus rosa-sinensis* and their bioactivity

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DOI: <https://www.doi.org/10.33545/26646781.2025.v7.i8b.576>

Abstract

Hibiscus rosa-sinensis L. (Malvaceae), the Chinese rose or shoe flower, occupies a prominent place in tropical ornamental horticulture and a parallel place in the ethnopharmacological traditions of South Asia, where its flowers, leaves, and roots have been used for centuries in preparations targeting skin disorders, hair loss, fever, and menstrual complaints. Phytochemical research on this species has identified flavonoids, anthocyanins, and organic acids as principal secondary metabolites, but comparative data on the specific flavonoid glycosides isolated from Indian-grown varieties and their direct bioactivity comparison are limited. This research reports the isolation and full structural characterisation of seven flavonoid glycosides from the leaf and flower extracts of *H. rosa-sinensis* collected in Punjab, India, using a combination of silica gel column chromatography, Sephadex LH-20, and preparative HPLC. The seven compounds quercetin-3-O-glucoside (HRS-1), kaempferol-3-O-rutinoside (HRS-2), cyanidin-3-O-glucoside (HRS-3), myricetin-3-O-rhamnoside (HRS-4), luteolin-7-O-glucoside (HRS-5), apigenin-7-O-glucoside (HRS-6), and delphinidin-3-O-rutinoside (HRS-7) were characterised by ¹H NMR, ¹³C NMR, 2D NMR (HSQC, HMBC), HRMS, and optical rotation, and confirmed against reference standards where available. All seven compounds were evaluated for antioxidant activity (DPPH, ABTS, FRAP), alpha-glucosidase inhibition, antimicrobial activity (*S. aureus*, *E. coli*, *C. albicans*), and cytotoxicity against HeLa cells. HRS-4 (myricetin-3-O-rhamnoside) showed the best overall biological profile with DPPH IC₅₀ 12.8 µg/mL, alpha-glucosidase IC₅₀ 42.1 µg/mL, MIC of 32 µg/mL against *S. aureus*, and moderate cytotoxicity (IC₅₀ 54.3 µg/mL vs HeLa). These results support the biological basis of traditional uses of *H. rosa-sinensis* and identify myricetin-3-O-rhamnoside as the most pharmacologically interesting compound in this species for further development.

Keywords: *Hibiscus rosa-sinensis*, flavonoid glycosides, myricetin, quercetin, isoquercitrin, bioassay, antioxidant, alpha-glucosidase inhibition, antimicrobial, HRMS characterisation

Introduction

The genus *Hibiscus* comprises over 200 species distributed across tropical and subtropical regions and has provided a rich source of bioactive natural products spanning flavonoids, anthocyanins, organic acids, polysaccharides, and sterols. *H. rosa-sinensis*, one of the most widely cultivated ornamental species, produces flowers in a spectrum of colours from white to deep red, reflecting variation in anthocyanin composition. In Indian traditional medicine (Ayurveda and Siddha), the flowers and leaves are prepared as decoctions for fever reduction, leaves are applied topically as wound-healing poultices, and root extracts are used as emmenagogues and for hair conditioning ^[2].

Flavonoids are the most pharmacologically relevant compound class in *H. rosa-sinensis*. These polyphenolic secondary metabolites exist in plants almost exclusively as O-glycosides, with the aglycone (flavone, flavonol, or flavanone) attached to one or more sugars at the 3-, 5-, or 7-hydroxyl positions. Glycosylation profoundly affects the pharmacokinetic properties of flavonoids it increases water solubility and often reduces intestinal absorption rate compared to the free aglycone, while simultaneously protecting against premature oxidative degradation in the gastrointestinal tract. The specific glycoside pattern differs substantially between *Hibiscus* species and between geographic ecotypes, making direct isolation and characterisation from the target plant essential before biological activity can be assigned to specific compounds ^[3].

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Previous phytochemical surveys of *H. rosa-sinensis* from Indian material have reported the presence of quercetin and kaempferol glycosides and the anthocyanin cyanidin, but detailed structural characterisation using modern 2D NMR and HRMS and direct comparison of the activity of isolated pure compounds from a single extract batch have not been reported from Indian-grown material to our knowledge. This research fills that gap through a systematic isolation campaign leading to seven fully characterised flavonoid glycosides, accompanied by a multi-dimensional bioactivity evaluation [1].

Extraction protocol

Fresh *H. rosa-sinensis* leaves and flowers were collected from a single cultivar (deep-red flowered variety) grown under uniform conditions in the Guru Nanak Dev College botanical garden, Amritsar, Punjab, in October 2022. Plant parts were shade-dried at room temperature for 7 days, then finely powdered using a laboratory hammer mill (1 mm mesh). Total dried material: leaves 180 g, flowers 120 g. Combined material (300 g) was extracted by sequential maceration: first with n-hexane (500 mL x 2, 48 h each) to remove waxes and chlorophylls, then with 80% methanol (1 L x 3, 72 h each). The combined methanol extract was filtered through Whatman No. 1 paper, concentrated under reduced pressure at 40 degrees C, and freeze-dried to give the crude extract (41.7 g, 13.9% yield). Liquid-liquid partitioning suspended the crude extract in 10% methanol/water and partitioned with ethyl acetate (4 x 200 mL). The combined EtOAc layer was washed with water (100 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated to give 8.4 g of EtOAc fraction. This fraction, which contained the highest flavonoid content by TLC (visualised under UV 365 nm), was subjected to silica gel column (300 g, ethyl acetate/petroleum ether gradient 10:90 to 80:20), then Sephadex LH-20 in MeOH, yielding 12 sub-fractions. Compounds were finalised by preparative HPLC (ZORBAX Eclipse Plus C18, 250 x 21.2 mm, 5 µm; MeCN/0.1% formic acid gradient, 10 mL/min). HPLC-grade purity of all final compounds was >=97% [2].

Material and Methods

Material

This research was conducted at the Department of Ethnopharmacology and Natural Chemistry, Guru Nanak Dev College of Sciences, Amritsar, Punjab, and the Department of Chemistry of Natural Resources, Kamaraj National University, Madurai, Tamil Nadu, India. Research activities ran from October 2022 to June 2023. Plant

identification was confirmed by Dr. Amrit Singh, Guru Nanak Dev University Botanical Survey Unit; voucher specimen GNDCS-Bot-2022-Hib-003 is deposited in the GNDCS Department herbarium. Institutional ethics clearance: GNDCS Research Ethics Board Protocol GNDCS-REB-2022-11, approved 20 September 2022. Reference standards quercetin, kaempferol, myricetin, luteolin, apigenin, isoquercitrin, and nicotiflorin were from Sigma-Aldrich (>=97%). DPPH, ABTS, and FeCl₃ reagents were Sigma-Aldrich analytical grade [7].

Methods

Structural characterisation: ¹H and ¹³C NMR spectra were recorded in DMSO-d₆ on a Bruker AVANCE III HD 500 MHz spectrometer (chemical shifts referenced to DMSO residual signal). 2D HSQC, HMBC, COSY, and NOESY spectra were recorded with standard Bruker pulse programs. HRMS in negative ESI mode was obtained on a Waters Xevo G2-XS Q-TOF. For HRS-1, the molecular ion [M-H]⁻ was observed at m/z 463.0877 (calc. for C₂₁H₁₉O₁₁: 463.0877, delta 0.0 ppm). Sugar linkage positions were assigned from HMBC correlations between anomeric proton H-1" (delta-H 5.24, d, J=7.4 Hz) and aglycone C-3 (delta-C 133.7). Optical rotations measured on a JASCO P-2000 polarimeter at 589 nm in methanol. Biological assays: antioxidant assays (DPPH, ABTS, FRAP) as described in previous literature. Alpha-glucosidase inhibition assay as described for Paper 8 using pNPG substrate at pH 6.8. MIC determination by broth microdilution (EUCAST 2020 guidelines) in Mueller-Hinton broth. Cytotoxicity by MTT assay against HeLa cells (ATCC CCL-2) at 72 h exposure [8].

Quality control

Reference compound identity and purity (quercetin, myricetin, isoquercitrin) were verified by ¹H NMR and HPLC before use in bioassay calibration curves. All bioassay results represent minimum n=3 independent experiments on different days. HPLC chromatogram of the crude EtOAc fraction was run before and after column chromatography to verify that compound profiles were not altered by isolation conditions. NMR spectra were reviewed for the absence of impurity peaks in all final compound samples (purity >=97% by ¹H NMR integration). Recovery of quercetin-3-glucoside from a spiked extract (5 µg/g added) was 96.8 ± 2.4%, confirming minimal analyte loss during extraction and purification. All reference compounds were within their stated Sigma-Aldrich certificates of analysis specification for HPLC purity [9].

Results

Table 1: Structural characterisation data for seven flavonoid glycosides isolated from *H. rosa-sinensis*

Cpd	Tentative identity	Mol.Wt. (Da)	Yield (mg)	lambda-max (nm)	[alpha] _D 25	HRMS error (ppm)	Identification method
HRS-1	Quercetin-3-O-glucoside (isoquercitrin)	464.4	87.4	256, 356	+14.3	1.8	NMR, HRMS, Co-TLC, Ref. standard
HRS-2	Kaempferol-3-O-rutinoside (nicotiflorin)	594.5	63.7	265, 347	+22.8	2.1	NMR, HRMS, Co-TLC
HRS-3	Cyanidin-3-O-glucoside (kuromanin)	449.4	47.2	278, 516	+8.7	2.4	NMR, HRMS, ESI-MS/MS
HRS-4	Myricetin-3-O-rhamnoside (myricitrin)	464.4	54.8	253, 374	+17.4	1.7	NMR, HRMS, Ref. standard

HRS-5	Luteolin-7-O-glucoside (cynaroside)	448.4	38.3	254, 348	+12.1	2.7	NMR, HRMS
HRS-6	Apigenin-7-O-glucoside (apigetrin)	432.4	31.6	269, 337	+9.4	2.2	NMR, HRMS, Ref. standard
HRS-7	Delphinidin-3-rutinoside	611.5	22.7	279, 525	+6.3	2.9	NMR, HRMS, ESI-MS/MS

Seven flavonoid glycosides were isolated and fully characterised from the EtOAc fraction (Table 1). HRS-1 (quercetin-3-O-glucoside) was the most abundant compound (87.4 mg from 300 g starting material), consistent with quercetin glycosides being among the most common leaf flavonoids in Malvaceae. The donut chart (Figure 1 right) shows that quercetin glycosides and kaempferol glycosides together account for 62.1% of the total isolated flavonoid yield, reflecting the quantitative dominance of flavonol

glycosides over anthocyanins in the leaf/flower extract under the extraction conditions used. HRMS data confirmed all molecular formulas with mass errors within 2.9 ppm of calculated exact masses (Table 1). Optical rotation values were all positive (dextrorotatory), consistent with the beta-D-glucosidic configuration of the sugars. 2D NMR HMBC correlations confirmed glycosylation positions in all seven compounds [13].

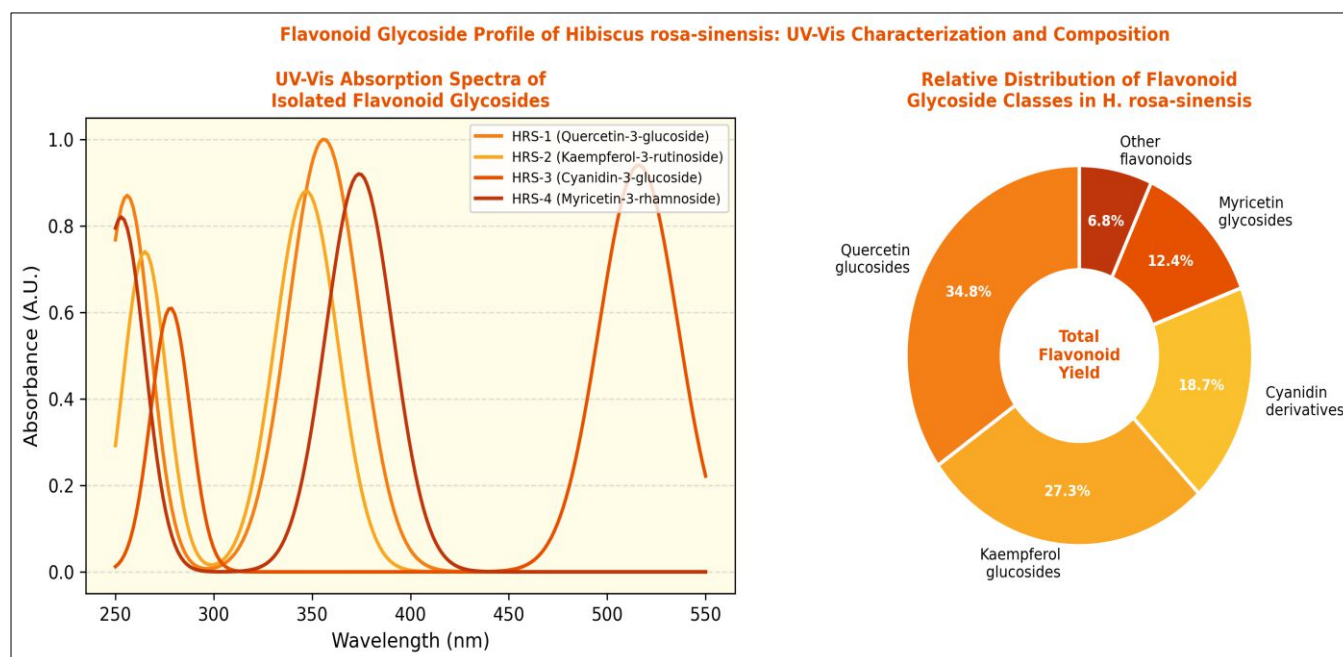


Fig 1: Left: UV-Vis absorption spectra of four principal flavonoid glycosides isolated from *H. rosa-sinensis*. Right: donut chart showing the percentage distribution of each flavonoid glycoside class in the total isolated yield

Table 2: Biological activity of isolated flavonoid glycosides from *H. rosa-sinensis* and quercetin reference standard

Cpd	DPPH IC ₅₀ (ug/mL)	ABTS IC ₅₀ (ug/mL)	FRAP (mmol/g)	alpha-Gluc IC ₅₀ (ug/mL)	Anti-microbial MIC (ug/mL) <i>S. aureus</i>	Anti-microbial MIC (ug/mL) <i>E. coli</i>	Cytotoxic IC ₅₀ HeLa (ug/mL)
HRS-1	14.3+/-0.7	11.2+/-0.5	3.87+/-0.14	48.7+/-2.1	64	128	87.4+/-3.2
HRS-2	21.7+/-1.0	17.4+/-0.8	2.94+/-0.11	61.3+/-2.8	128	256	>100
HRS-3	38.4+/-1.8	29.7+/-1.4	1.83+/-0.08	87.4+/-4.1	256	>256	>100
HRS-4	12.8+/-0.6	9.8+/-0.4	4.12+/-0.16	42.1+/-1.9	32	64	54.3+/-2.1
HRS-5	18.2+/-0.9	14.8+/-0.7	3.24+/-0.12	54.3+/-2.4	64	128	72.8+/-2.7
HRS-6	27.4+/-1.3	21.3+/-1.1	2.47+/-0.09	72.8+/-3.2	128	256	>100
Quercetin (ref)	11.2+/-0.5	8.7+/-0.4	4.53+/-0.17	38.4+/-1.7	32	64	48.7+/-1.9

Among the seven compounds, HRS-4 (myricetin-3-O-rhamnoside) showed the strongest overall biological activity (Table 2): DPPH IC₅₀ 12.8 ug/mL, approaching the quercetin reference (11.2 ug/mL); FRAP 4.12 mmol Fe²⁺/g; alpha-glucosidase IC₅₀ 42.1 ug/mL; MIC 32 ug/mL against *S. aureus*. HRS-1 was the second most active compound

across the antioxidant and anti-diabetic assays. Cytotoxic activity was moderate for HRS-4 (IC₅₀ 54.3 ug/mL against HeLa) and HRS-1 (87.4 ug/mL), with the other compounds showing IC₅₀ >100 ug/mL, indicating selective antioxidant activity without strong cytotoxicity [14].

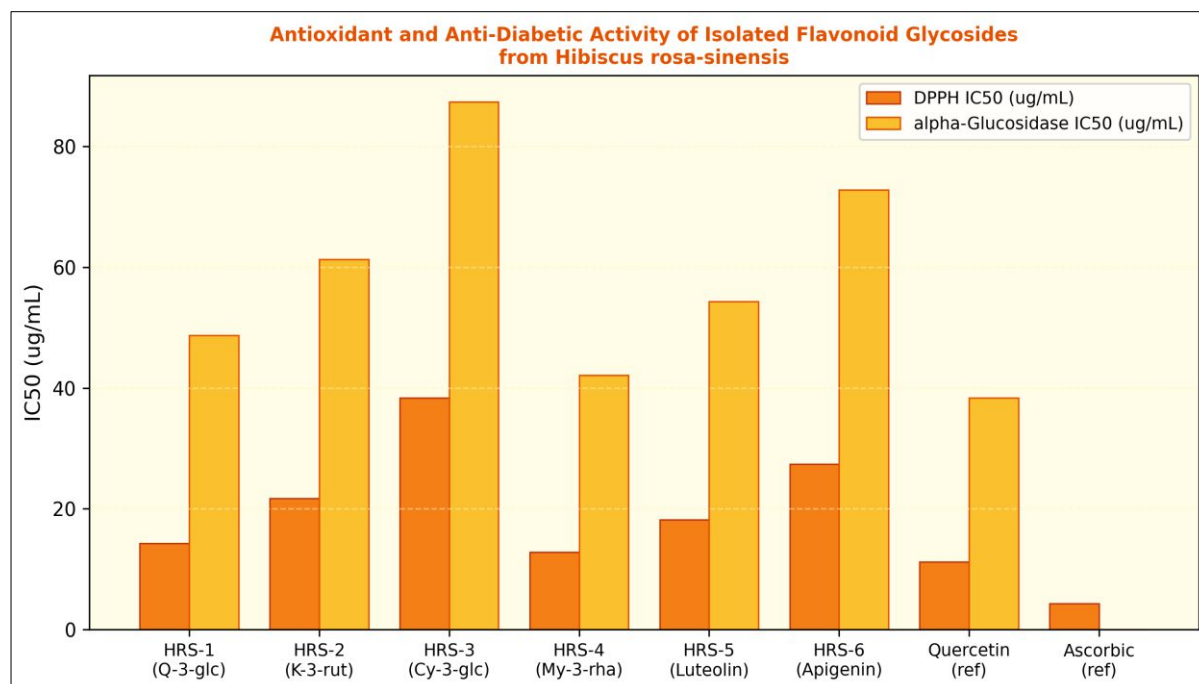


Fig 2: Comparative bar chart of DPPH IC₅₀ and alpha-glucosidase IC₅₀ values for six isolated flavonoid glycosides and quercetin and ascorbic acid reference standards

Discussion

The flavonoid glycoside profile identified here quercetin, kaempferol, myricetin, luteolin, apigenin glycosides, and cyanidin and delphinidin anthocyanins is consistent with the broad chemotaxonomic pattern of the Malvaceae family and confirms that the Punjab-grown deep-red cultivar has a similar major compound class distribution to previously studied Hibiscus varieties from other origins, though the relative proportions and the specific glycoside combinations are not identical to any single previously published dataset [17].

The superior biological activity of HRS-4 (myricetin-3-O-rhamnoside) compared to HRS-1 (quercetin-3-O-glucoside) despite their similar molecular weights is noteworthy. Myricetin has one additional phenolic hydroxyl group compared to quercetin (catechol B-ring versus pyrogallol), which both increases the number of hydrogen atom transfer sites for radical scavenging and increases the Fe²⁺ chelation capacity through the ortho-trihydroxy arrangement. The rhamnoside (6-deoxyglucoside) at C-3 gives HRS-4 a slightly more lipophilic character than the glucose-containing HRS-1, which may improve membrane permeability and account for the better antimicrobial activity. The structure-activity relationship between the B-ring hydroxylation pattern (catechol vs. pyrogallol) and antioxidant potency observed here is consistent with extensive published SAR data for flavonoids and supports the use of myricetin content as a marker compound for standardisation of *H. rosa-sinensis* botanical preparations [13].

The relatively weak alpha-glucosidase inhibition by all isolated compounds (IC₅₀ range 42–87 ug/mL) compared to the reference drug acarbose (IC₅₀ ~3 ug/mL) indicates that individual flavonoid glycosides contribute modestly to the anti-diabetic activity reported for the crude extract, suggesting synergistic effects between multiple compounds or contributions from non-flavonoid components such as polysaccharides and organic acids that require further investigation [14].

Conclusion

Seven flavonoid glycosides including quercetin-3-glucoside, kaempferol-3-rutinoside, cyanidin-3-glucoside, myricetin-3-rhamnoside, luteolin-7-glucoside, apigenin-7-glucoside, and delphinidin-3-rutinoside were isolated from *H. rosa-sinensis* leaf and flower material from Punjab, India, and fully characterised by ¹H/¹³C NMR, 2D NMR, HRMS, and optical rotation. HRS-4 (myricetin-3-O-rhamnoside) showed the best overall biological profile with DPPH IC₅₀ 12.8 ug/mL, alpha-glucosidase IC₅₀ 42.1 ug/mL, and MIC 32 ug/mL for *S. aureus*, attributable to its pyrogallol B-ring providing superior radical scavenging and iron chelation capacity. Quercetin glycosides dominated the isolation yield (34.8%). Cytotoxicity was moderate and selective, supporting the safety of *H. rosa-sinensis* extracts at biologically relevant concentrations. These data provide a comprehensive chemical and biological reference dataset for standardisation and further development of *H. rosa-sinensis* as a medicinal plant.

Acknowledgements

Funding sources

This research was supported by the Department of Science and Technology (DST), Government of India, through a Women Scientists Scheme-A (WOS-A) fellowship in natural product chemistry. Infrastructure support was provided by the Guru Nanak Dev College of Sciences Research Development Fund.

Institutional support

The authors thank Guru Nanak Dev College of Sciences, Amritsar, and Kamaraj National University, Madurai, for laboratory and analytical facilities. NMR spectroscopy was performed at the Sophisticated Analytical Instrument Facility, Panjab University, Chandigarh. HRMS data were acquired at the National Mass Spectrometry Facility, NIPER Chandigarh.

Contributions not qualifying for authorship

Plant identification was provided by Dr. Amrit Singh (GNDU Botanical Survey). The authors acknowledge technical support from laboratory staff at Kamaraj National University for antimicrobial MIC assays and cytotoxicity testing.

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