Pharmacognostical, Phytochemical And In Vitro Antioxidant Evaluation Of The Flowers Of Nymphaea Pubescens Willd. And Nymphaea Nouchali Burm. F., With Isolation Of Rutin, 1,3,6,8-Tetrahydroxyanthraquinone And An Apigenin C-Glycoside

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ABSTRACT

Background: Flowers of Nymphaea pubescens Willd. and Nymphaea nouchali Burm. f. (Nymphaeaceae) are used in traditional medicine, yet comparative pharmacognostic and phytochemical standards for them are limited. Objective: To establish pharmacognostical, physicochemical and phytochemical profiles of both flowers and to evaluate the in vitro antioxidant activity of their ethyl acetate extracts. Methods: Fresh flowers were examined macroscopically, in transverse section and as powders. Loss on drying, ash values and extractive values were determined by standard procedures. Successive petroleum ether, chloroform, ethyl acetate and hydroalcoholic extracts were screened for secondary metabolites and profiled by thin-layer chromatography (TLC) and high-performance TLC (HPTLC). Total phenolics (Folin–Ciocalteu) and flavonoids (aluminium chloride) were quantified in the ethyl acetate extracts, which were tested for DPPH and hydrogen peroxide scavenging and fractionated on silica gel; isolates were characterised by infrared, nuclear magnetic resonance and mass spectrometry. Results: Physicochemical values were comparable between species (total ash 10.6% and 11.4% w/w; loss on drying 7.16% and 6.99% w/w). The ethyl acetate extracts showed the richest phytochemical profile. Total phenolics were 38.88 and 30.44 mg gallic acid equivalent/g, and total flavonoids 31 and 35 mg quercetin equivalent/g, for N. pubescens and N. nouchali respectively. At 100 µg/mL, DPPH scavenging was 78.86% and 80.65%. HPTLC resolved six to eight bands per extract. Rutin and 1,3,6,8-tetrahydroxyanthraquinone were isolated from N. pubescens, and rutin and an apigenin C-glycoside from N. nouchali. Conclusion: Both flowers are rich sources of phenolic antioxidants, and the data provide useful diagnostic standards for their identification and quality control.

Key words: Nymphaea pubescens; Nymphaea nouchali; pharmacognosy; HPTLC; antioxidant activity; rutin

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INTRODUCTION

Medicinal plants remain a principal source of therapeutic leads and the foundation of several traditional systems of medicine, and the World Health Organization has encouraged the scientific validation of locally used plant materials [1,2]. A persistent barrier to the wider acceptance of herbal drugs is the lack of standardised quality-control parameters, such as macroscopic and microscopic identity, physicochemical constants and chromatographic fingerprints, against which a crude drug can be authenticated [3,4]. At the same time, reactive oxygen species are implicated in the pathology of many chronic diseases, and plant phenolics and flavonoids are valued for their ability to scavenge them [5,6].

The genus Nymphaea (Nymphaeaceae) comprises aquatic perennial herbs that are cultivated as ornamentals and used in folk medicine. Flavonoids, phenolic compounds, tannins and anthocyanins have been reported from several species, including N. caerulea, N. odorata and four Mexican species [7–10], and anthocyanins have been characterised from N. marliacea cultivars [11]. The flowers of N. caerulea were found to contain antioxidant constituents [8], and the traditional uses of the related N. stellata have been reviewed [12].

Nymphaea pubescens Willd. (pink water lily) and Nymphaea nouchali Burm. f. (white water lily) grow in ponds, tanks and ditches across India. Their flowers are used as an astringent and cardiotonic and in the management of diabetes and digestive complaints [12,13]. Ethanolic extracts of N. pubescens produced antidiabetic, hypolipidaemic and antioxidant effects in alloxan-induced diabetic rats [13,14], while flower extracts of N. stellata Willd., which is treated as a synonym of N. nouchali [15], showed hepatoprotective and antihyperglycaemic activity [16,17]. A hydroalcoholic seed extract of N. nouchali also showed concentration-dependent radical scavenging [15].

Despite this interest, we are not aware of a side-by-side study that combines pharmacognostic standards, chromatographic fingerprints, antioxidant activity and the isolation of principal constituents from the flowers of both species. The present work therefore aimed to establish these parameters for N. pubescens and N. nouchali flowers, so that the crude drugs can be identified and their antioxidant potential compared.

METHODOLOGY

Collection of Plant material

Flowers of N. pubescens and N. nouchali were collected and authenticated at the Durava Herbal Centre, Chennai. Fresh flowers were used for macroscopic and microscopic study. The remaining material was washed, shade-dried, powdered and passed through a No. 40 sieve.

Pharmacognostical and physicochemical studies

Organoleptic characters were recorded from fresh flowers. For anatomy, flowers were fixed in formalin–acetic acid–alcohol for 24 h, dehydrated through a tertiary butyl alcohol series, embedded in paraffin wax (58–60 °C), sectioned at 10–12 µm on a rotary microtome and stained with toluidine blue [18,19]. Powders were cleared with chloral hydrate and stained with safranin, phloroglucinol–HCl, Sudan red III or iodine [20]. Loss on drying (105 °C), total, water-soluble and acid-insoluble ash, alcohol- and water-soluble extractive values and foreign organic matter were determined by standard procedures [4,21]. Values are mean ± error of replicate determinations (n = [to be confirmed]).

Extraction and phytochemical screening

Coarsely powdered flowers (500g) were macerated for 72 h with petroleum ether, chloroform, ethyl acetate and hydroalcohol, and each extract was concentrated under reduced pressure. Extracts were screened for alkaloids, phenolics and tannins, carbohydrates, proteins, steroids, triterpenoids, glycosides, flavonoids, saponins, gums, fixed oils and volatile oil by standard tests [21,22].

Total phenolic and flavonoid content determination

Total phenolics in the ethyl acetate extracts of N. pubescens (EAENPF) and N. nouchali (EAENNF) were measured by the Folin–Ciocalteu method at 760 nm against gallic acid [23]. Total flavonoids were measured by the aluminium chloride method at 510 nm against quercetin [24]. Standards and extracts were read at 10–50 µg/mL.

TLC and HPTLC

Extracts were chromatographed on silica gel 60 F254 plates in solvent systems selected for alkaloids, flavonoids, glycosides and anthocyanins, and viewed under 254 and 366 nm light after derivatisation. HPTLC was performed on a CAMAG system controlled by winCATS software (v1.4.7), and Rf values, peak heights and areas were recorded [25,26].

Antioxidant assays

DPPH radical scavenging was measured at 517 nm after 30 min (extract 10–100 µg/mL; ascorbic acid 10 µg/mL) [27]. Hydrogen peroxide scavenging was measured at 230 nm after 10 min (20–100 µg/mL) [28]. Percentage inhibition was calculated as (control − test)/control × 100, and IC50 values were obtained by linear regression of inhibition against concentration.

Isolation and structural characterisation

The ethyl acetate extract (20 g) was adsorbed on silica gel (60/120 mesh) and applied to a column of 200g silica gel, eluted with petroleum ether, chloroform, ethyl acetate and methanol in order of increasing polarity. Isolates were characterised by FT-IR (Shimadzu, KBr discs), 1H NMR (400 MHz, Bruker, CD3OD, TMS reference), 13C NMR (125.8 MHz, 1024 scans, proton-decoupled) and mass spectrometry (electron impact for rutin; electrospray, negative mode for EANP-2 and positive mode for EANN-2) [29].

RESULTS

Macroscopy and microscopy

The macroscopic characters of the two flowers are summarised in Table 1 and shown in Figure 1.

Table 1. Macroscopic characters of the flowers of N. pubescens and N. nouchali.

Character	***N. pubescens***	***N. nouchali***
Colour	White to pink	White
Odour	Pungent	Characteristic
Taste	Tasteless	Characteristic
Flower	Smooth, oblong; 5–9 cm	Slightly emergent; 3–15 cm in diameter
Perianth segments	Oblong	Oblong-lanceolate; 2.5–8 cm
Stigma	Rays 12–15(–30); appendages linear	Rays 10–30; appendages triangular-tapered



Figure 1. (a) N. pubescens in its habitat; (b) N. nouchali plant with white flowers.

In transverse section, the sepal of N. pubescens was dorsiventral, 550 µm thick at the midrib and 350 µm laterally, with spindle-shaped outer epidermal cells (20 µm), papillate inner epidermis, about ten layers of thin-walled mesophyll and a small collateral vascular strand. The petal midrib was about 400 µm thick, with papillate epidermis on both faces, 8–10 layers of parenchyma and xylem elements up to 50–70 µm wide. The anther was winged, with four pollen chambers and an endothecium of about 40 µm within a 70 µm wall. In N. nouchali, the sepal midrib was also 550 µm thick, tapering to 70 µm at the margin, with a conical mass of thick-walled supporting cells; the petal midrib was 500 µm thick with two vascular segments and five layers of thick-walled

adaxial cells; and the anther had two pairs of flat longitudinal pollen chambers with an endothecium showing annular thickenings.

In powder microscopy (Figure 2), N. pubescens showed petal fragments with rectangular vein-islets, sepal epidermis with trichome bases, stomata, astrosclereids, filiform sclereids, and fertile (40 µm) and sterile (20 µm) pollen grains. N. nouchali showed oblong to hexagonal epidermal cells (70–140 × 200–340 µm), glandular cells (100–140 µm wide), astrosclereids and acicular sclereids (1.2 mm × 30 µm) in vertical rows.

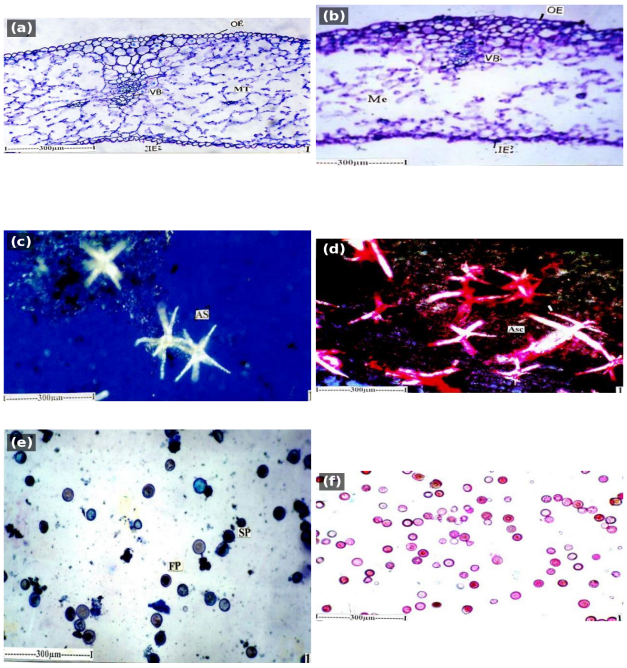


Figure 2. Microscopy of the flowers. (a) T.S. of sepal through the midrib, N. pubescens; (b) T.S. of petal through the midrib, N. nouchali; (c, d) astrosclereids in the powder of N. pubescens and N. nouchali; (e, f) pollen grains in the powder of N. pubescens and N. nouchali.

Physicochemical parameters

Table 2. Physicochemical parameters of the flowers (% w/w).

Parameter	***N. pubescens***	***N. nouchali***
Loss on drying at 105 °C	7.16 ± 0.741	6.99 ± 0.441
Total ash	10.6 ± 0.091	11.4 ± 0.112
Acid-insoluble ash	0.62 ± 0.471	0.43 ± 0.391
Water-soluble ash	4.6 ± 0.740	5.1 ± 0.770
Alcohol-soluble extractive	5.6 ± 0.511	5.9 ± 0.575
Water-soluble extractive	12.7 ± 0.271	11.9 ± 0.190
Foreign organic matter	0.02 ± 0.0011	0.02 ± 0.0013

Both flowers gave comparable values (Table 2). Total ash was slightly higher in N. nouchali (11.4%) than in N. pubescens (10.6%), whereas water-soluble extractive value was higher in N. pubescens (12.7% vs 11.9%). Acid-insoluble ash was below 1% and foreign organic matter was 0.02% in both.

Phytochemical screening

Table 3. Preliminary phytochemical screening of successive extracts (N.p / N.n).

Constituent (test)	**Pet. ether**	**Chloroform**	**Ethyl acetate**	**Hydroalcoholic**
Alkaloids (Dragendorff)	– / –	+ / +	++ / ++	++ / ++
Phenolics, tannins ($FeCl_3$)	+ / +	++ / +	++ / ++	+ / +
Flavonoids (Mg/HCl)	+ / ++	++ / ++	+++ / +++	++ / ++
Carbohydrates (Molisch)	+ / +	++ / ++	++ / ++	++ / ++
Proteins, amino acids (ninhydrin)	– / –	+ / +	+ / +	+ / +
Steroids (Liebermann–Burchard)	– / –	++ / ++	++ / ++	+ / +
Triterpenoids (Noller)	– / –	++ / ++	++ / ++	+ / +
Glycosides (Borntrager)	– / –	+ / +	+ / +	– / –
Saponins, gums, fixed oils, lignins	– / –	– / –	– / –	– / –

N.p: N. pubescens; N.n: N. nouchali. +++, intense; ++, moderate; +, weak; –, absent. Volatile oil was moderately present (++) in both species.

The ethyl acetate extract of both species had the richest profile, with strong reactions for flavonoids, phenolics, alkaloids, steroids and triterpenoids (Table 3). The petroleum ether extract reacted mainly for carbohydrates and phenolics and, in N. nouchali, flavonoids, while saponins, gums, fixed oils and lignins were absent from all extracts.

Total phenolic and flavonoid content

Table 4. Absorbance (mean ± SEM) of standards and ethyl acetate extracts, and total phenolic and flavonoid content.

Conc. (µg/mL)	Gallic acid	EAENPF	EAENNF	Quercetin	EAENPF	EAENNF
	Folin–Ciocalteu (760 nm)			$AlCl_3$ method (510 nm)		
10	0.115 ± 0.0021	0.117 ± 0.0007	0.107 ± 0.0021	0.077 ± 0.0074	0.069 ± 0.0017	0.071 ± 0.0041
20	0.183 ± 0.0018	0.154 ± 0.0005	0.121 ± 0.0031	0.082 ± 0.0067	0.071 ± 0.0044	0.075 ± 0.0077
30	0.277 ± 0.0048	0.193 ± 0.0009	0.175 ± 0.0040	0.094 ± 0.0055	0.079 ± 0.0057	0.084 ± 0.0087
40	0.359 ± 0.0024	0.259 ± 0.0004	0.214 ± 0.0141	0.114 ± 0.0041	0.087 ± 0.0047	0.096 ± 0.0049
50	0.491 ± 0.0115	0.353 ± 0.0003	0.277 ± 0.0410	0.121 ± 0.0044	0.091 ± 0.0077	0.099 ± 0.0056
Content		**38.88 mg GAE/g**	**30.44 mg GAE/g**		**31 mg QE/g**	**35 mg QE/g**

EAENPF, ethyl acetate extract of N. pubescens flowers; EAENNF, ethyl acetate extract of N. nouchali flowers; GAE, gallic acid equivalent; QE, quercetin equivalent.

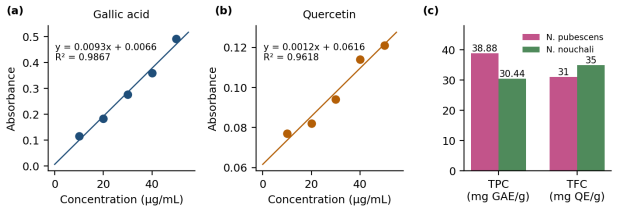


Figure 3. Calibration curves of (a) gallic acid and (b) quercetin, fitted to the tabulated absorbances, and (c) total phenolic content (TPC) and total flavonoid content (TFC) of the ethyl acetate extracts.

Total phenolic content was higher in EAENPF (38.88 mg GAE/g) than in EAENNF (30.44 mg GAE/g), whereas total flavonoid content was higher in EAENNF (35 mg QE/g) than in EAENPF (31 mg QE/g) (Table 4, Figure 3).

TLC and HPTLC profiles

Table 5. Number of TLC spots resolved in each extract (N.p / N.n).

Class (detection)	Pet. ether	Chloroform	Ethyl acetate	Hydroalcoholic
Alkaloids (Dragendorff)	3 / 3	5 / 6	4 / 3	4 / 5
Flavonoids (UV 254/366 nm)	4 / 4	6 / 5	6 / 6	6 / 6
Glycosides (UV 254/366 nm)	2 / 3	3 / 3	3 / 3	4 / 4
Anthocyanins (n-butanol–acetic acid, 18:2)	3 / 3	4 / 3	5 / 5	4 / 4

Best resolution of flavonoids: ethyl acetate–formic acid–acetic acid–water (100:11:11:27); of flavonoids and anthocyanins: n-butanol–acetic acid–water (40:10:20). Ethyl acetate extracts gave six flavonoid spots in both species (Rf 0.16–0.82 in N. pubescens; 0.22–0.81 in N. nouchali).

Table 6. HPTLC data of the extracts of N. pubescens and N. nouchali.

Extract	Spots	R_f values	Total height	Total area
N. pubescens				
Pet. ether	7	0.10, 0.29, 0.44, 0.53, 0.64, 0.70, 0.75	204.9	6702.2
Chloroform	8	0.11, 0.29, 0.38, 0.45, 0.55, 0.62, 0.72, 0.80	111.2	3030.2
Ethyl acetate	8	0.11, 0.25, 0.29, 0.45, 0.56, 0.62, 0.72, 0.80	141.3	4886.8
Hydroalcoholic	7	0.11, 0.28, 0.46, 0.56, 0.62, 0.72, 0.83	59.7	1775.1
N. nouchali				
Pet. ether	6	0.11, 0.29, 0.44, 0.54, 0.64, 0.71	172.8	5545.6
Chloroform	7	0.10, 0.29, 0.38, 0.45, 0.55, 0.60, 0.72	105.5	2674.9
Ethyl acetate	7	0.11, 0.29, 0.38, 0.45, 0.52, 0.62, 0.72	109.8	2650.9
Hydroalcoholic	7	0.11, 0.28, 0.46, 0.56, 0.62, 0.72, 0.83	59.7	1775.1

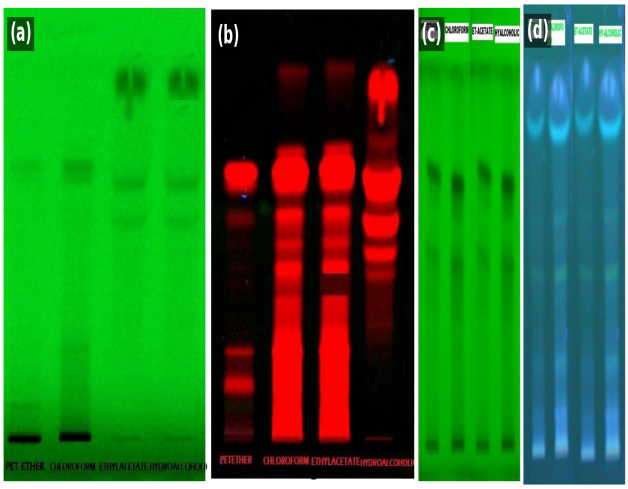


Figure 4. HPTLC plates of the four successive extracts (lanes: petroleum ether, chloroform, ethyl acetate, hydroalcoholic). N. pubescens: (a) 254 nm, (b) 366 nm. N. nouchali: (c) 254 nm, (d) 366 nm.

HPTLC resolved 7–8 bands in the N. pubescens extracts and 6–7 in the N. nouchali extracts (Table 6, Figure 4). Bands at Rf 0.11, 0.29, 0.45, 0.62 and 0.72 were common to both species, which supports the close chemical similarity seen in the screening data.

Antioxidant activity

Table 7. DPPH radical scavenging activity of the ethyl acetate extracts (mean ± SEM).

Concentration (µg/mL)	Absorbance, EAENPF	Scavenging (%), EAENPF	Scavenging (%), EAENNF
Control	1.282 ± 0.003	–	–
Ascorbic acid, 10	0.108 ± 0.002	91.57	91.57
10	0.932 ± 0.003	27.30	20.28
30	0.705 ± 0.002	45.00	31.82
50	0.584 ± 0.002	54.44	51.71
70	0.409 ± 0.002	68.09	69.18
100	0.271 ± 0.003	78.86	80.65

Absorbances of EAENNF were 1.022 ± 0.003, 0.874 ± 0.002, 0.619 ± 0.003, 0.395 ± 0.003 and 0.248 ± 0.002 at 10, 30, 50, 70 and 100 µg/mL, respectively.

Table 8. Hydrogen peroxide scavenging activity (% inhibition, mean ± SEM) and IC50 values.

Concentration (µg/mL)	Ascorbic acid	EAENPF	EAENNF
20	50.50 ± 0.0110	30.18 ± 0.0141	28.81 ± 0.0311
40	63.11 ± 0.0211	47.37 ± 0.0211	41.73 ± 0.0244
60	77.44 ± 0.0114	54.78 ± 0.0217	51.87 ± 0.0259
80	85.04 ± 0.0741	69.35 ± 0.0219	65.53 ± 0.0271
100	91.74 ± 0.7440	75.68 ± 0.0301	71.86 ± 0.0321
IC_{50}, H_2O_2 (µg/mL)†	**14.9**	**50.3**	**56.4**
IC_{50}, DPPH (µg/mL)†	**–**	**43.6**	**51.0**

Calculated by linear regression of % inhibition on concentration using the tabulated values ($R^2 \geq 0.97$ for all fits).

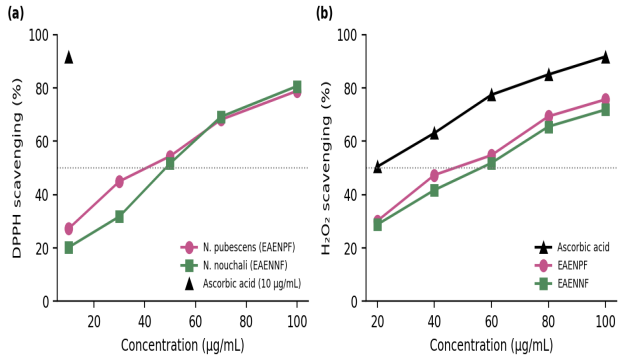


Figure 5. In vitro antioxidant activity of the ethyl acetate extracts: (a) DPPH radical scavenging and (b) hydrogen peroxide scavenging. The dotted line marks 50% inhibition.

Both extracts scavenged DPPH and hydrogen peroxide in a concentration-dependent manner (Tables 7 and 8, Figure 5). At 100 µg/mL the DPPH scavenging was 78.86% for EAENPF and 80.65% for EAENNF; the calculated IC50 values were 43.6 and 51.0 µg/mL, respectively. In the hydrogen peroxide assay the IC50 values were 50.3 µg/mL (EAENPF) and 56.4 µg/mL (EAENNF), compared with 14.9 µg/mL for ascorbic acid.

Isolated compounds determination

Table 9. Physical and spectral data of the compounds isolated from the ethyl acetate extracts.

Compound	Physical data	Spectral data	Identity
EANP-1 (and EANN-1)	Cream amorphous powder; m.p. 241–242 °C; $C_{27}H_{30}O_{16}$	IR 3132.8 (phenolic OH), 2924.5 (aromatic C–H), 1732.7 (C=O), 1400.1 (C=C) cm^{-1}. ^1H NMR δ 7.6, 7.5 (d), 6.7 (d, J = 8.4 Hz), 6.3, 6.1 (d, J = 2 Hz), 3.15–3.8 (sugar H). EI-MS m/z 301.0 (aglycone region).	Rutin (flavonol glycoside)
EANP-2	Yellowish-green needles; m.p. 284–286 °C; $C_{14}H_8O_6$	IR 3426.6 (phenolic OH), 2925 (aromatic C–H), 1721 (C=O) cm^{-1}. ^1H NMR δ 7.4 and 7.9 (anthraquinone α and β protons). MS m/z 271.2. Borntrager test positive.	1,3,6,8-Tetrahydroxy-anthraquinone
EANN-2	Fluorescent yellow powder; m.p. 235–236 °C; $C_{21}H_{20}O_{10}$	IR 3127 (OH), 1642 (C=O) cm^{-1}. ^1H NMR δ 6.609 (H-3), 6.410 (H-6), 8.014 and 6.819 (2H each, 1,4-disubstituted ring), 5.300 (anomeric H, d, J = 4.1 Hz). ESI-MS $(M+H)^+$ m/z 433.1. Shinoda test positive.	Apigenin-8-C-glucoside (vitexin-type)

EANN-1 matched EANP-1 in physical behaviour and Rf and was assigned as rutin by comparison.

Column chromatography of the ethyl acetate extracts gave two compounds from each species (Table 9). On the basis of melting point, chemical tests and IR, NMR and mass spectral data, EANP-1 and EANN-1 were assigned as rutin, EANP-2 as 1,3,6,8-tetrahydroxyanthraquinone, and EANN-2 as an apigenin 8-C-glucoside.

Spectral characterisation of the isolates

The isolates were characterised by IR, 1H NMR, 13C NMR and mass spectrometry (Figures 6–8; Tables 10–13). Peak positions below are those labelled on the recorded spectra.

Rutin (EANP-1/EANN-1). The IR spectrum (Figure 6a) showed a broad O–H stretch at 3132.8 cm−1, C–H stretches at 2924.5 and 2853.2 cm−1, a carbonyl band at 1732.7 cm−1, a band at 1400.1 cm−1 and an out-of-plane C–H bend at 743.4 cm−1 (Table 10). The 1H NMR spectrum (400 MHz, CD3OD; Figure 6b) showed a B-ring ABX system at δ 7.57 (H-2′), 7.54 (dd, J ≈ 8.4 and 2 Hz, H-6′) and 6.78 (d, J ≈ 8.4 Hz, H-5′), and two meta-coupled A-ring doublets at δ 6.31 (H-8) and 6.12 (H-6) (J ≈ 2 Hz), each integrating for one proton (Table 11). A doublet at δ 5.01 (J ≈ 7.6 Hz) is typical of the anomeric proton of a β-glucosyl unit, a signal at δ 4.49 fits the rhamnosyl anomeric proton, sugar protons appeared between δ 3.15 and 3.8, and a doublet at δ 1.02 (J ≈ 6 Hz, 3H) is characteristic of a rhamnosyl methyl group. Together these signals form the pattern expected for quercetin 3-O-rutinoside [29,30]. The 13C spectrum (Figure 6c) showed anomeric-region signals at δ 100.8 and 98.5, a cluster of oxygenated sugar carbons between δ 63 and 79, and a methyl carbon at δ 18.9, in keeping with a rhamnosyl unit (Table 12); signals of the aromatic aglycone carbons were not resolved in this spectrum. The electron-impact mass spectrum (Figure 6d) had its base peak at m/z 57.5 with other fragments at m/z 71.6, 85.6, 111.7, 129.7 and 145.7, and ions at m/z 252.9 and 301.0, the latter in the region of the quercetin aglycone ion (Table 13). The displayed scan range ends near m/z 460, so the molecular ion of C27H30O16 (610.5) was outside it.

1,3,6,8-Tetrahydroxyanthraquinone (EANP-2). The IR spectrum (Figure 7a) had a strong C–H stretch at 2930 cm−1 and bands at 1630, 1500, 1390, 1240, 1090 and 760 cm−1 (Table 10); the 1630 cm−1 band is in the range expected for a hydrogen-bonded (chelated) quinone carbonyl, and 1500 cm−1 for aromatic C=C. The 1H NMR spectrum (Figure 7b) showed broad low-intensity downfield signals near δ 7.9–8.3, assigned to the aromatic protons (δ 7.4 and 7.9 reported for the α- and β-type protons), together with intense signals between δ 1.6 and 4.3 that were not assignable to the proposed structure (Table 11). The 13C spectrum (Figure 7c) had signals at δ 173.1, 169.0, 145.2, 145.0, 138.2, 127.8, 120.6, 109.5 and 109.0 in the carbonyl, oxygenated-aromatic and aromatic regions (Table 12). Negative-ion ESI-MS (Figure 7d) gave a base peak at m/z 271.2, which corresponds to [M−H]− of C14H8O6 (M = 272.2), with further ions at m/z 131.3, 169.1, 213.2, 257.2 and 307.2 (Table 13).

Apigenin 8-C-glucoside (EANN-2). The IR spectrum (Figure 8a) showed O–H at 3128.0 cm−1, C–H at 2924.5 cm−1, a carbonyl band at 1727.9 cm−1 (a band at 1642 cm−1 was also reported for the conjugated γ-pyrone carbonyl) and a band at 1400.1 cm−1. The 1H NMR spectrum (400 MHz, CD3OD; Figure 8b) showed two 2H doublets at δ 8.014 and 6.819 for a 1,4-disubstituted B-ring (H-2′/6′ and H-3′/5′), singlets at δ 6.609 (H-3) and 6.410 (H-6) typical of a 5,7-dihydroxyflavone, a doublet at δ 5.300 (J = 4.1 Hz) for the anomeric proton, and sugar protons at δ 3.52–3.90 (Table 11). The positive-ion ESI-MS (Figure 8c) showed [M+H]+ as the base peak at m/z 433.1, successive losses of water at m/z 415.0, 397.0 and 379.1, a fragment at m/z 312.9 ([M+H−120]+), its dehydration product at m/z 295.0, and the protonated aglycone (apigenin) at m/z 271.0 (Table 13). The 120 Da loss and the aglycone ion are the hallmark fragmentation of flavone C-hexosides [31].

Table 10. Infrared absorption bands (cm−1, KBr) of the isolated compounds.

Assignment	**Rutin (EANP-1)**	**1,3,6,8-THAQ (EANP-2)**	**Apigenin C-glucoside (EANN-2)**
O–H stretch	3132.8	3426.6	3128.0
C–H stretch	2924.5, 2853.2	2930 (2925)	2924.5
C=O stretch	1732.7	1721; 1630 (chelated)	1727.9; 1642
Aromatic C=C	1400.1	1500	1400.1
C–O / O–H bend	–	1390, 1240, 1090	–
Aromatic C–H out-of-plane bend	743.4	760	–

THAQ, tetrahydroxyanthraquinone. For EANP-2, 3426.6 and 1721 cm−1 are the values reported in the compound data (Table 9); the other bands are the labelled maxima of the spectrum in Figure 7a.

Table 11. 1H NMR data of the isolated compounds (CD3OD).

Compound	δ (ppm)	Multiplicity (J, Hz)	Assignment
Rutin	7.57	1H, d (≈2)	H-2′ (B ring)
	7.54	1H, dd (≈8.4, 2)	H-6′
	6.78	1H, d (≈8.4)	H-5′
	6.31	1H, d (≈2)	H-8 (A ring)
	6.12	1H, d (≈2)	H-6 (A ring)
	5.01	1H, d (≈7.6)	H-1″ (β-glucosyl, anomeric)
	4.49	1H, br	H-1‴ (rhamnosyl, anomeric)
	3.15–3.8	m	Sugar protons
	1.02	3H, d (≈6)	Rhamnosyl CH_3
EANP-2	7.9, 7.4	As reported	β- and α-type anthraquinone aromatic protons
	≈7.9–8.3	Broad, weak	Aromatic region of the recorded spectrum
	4.95, 4.34, 4.19, 3.70, 3.38, 2.42, 2.09, 1.84, 1.82, 1.59	Intense, aliphatic	Not assigned to the proposed structure
EANN-2	8.014	2H, d	H-2′, H-6′
	6.819	2H, d	H-3′, H-5′
	6.609	1H, s	H-3
	6.410	1H, s	H-6 (5,7-dihydroxy A ring)
	5.300	1H, d (4.1)	Anomeric H-1″
	3.898; 3.524–3.797	m	Sugar protons (H-6″; H-3″, H-4″, H-5″)

Rutin values are derived from the labelled peak positions in Figure 6b (coupling constants calculated at 400 MHz).

Table 12. 13C NMR signals (δ, ppm) labelled in the spectra of rutin and EANP-2

Compound	Labelled signals
Rutin (EANP-1)	100.76, 98.54 (anomeric region); 78.60, 78.40, 76.98, 76.83, 75.10, 74.11, 73.76, 72.32, 71.85, 70.27, 66.61, 66.12, 63.47, 63.34, 53.24 (oxygenated sugar carbons and others); 26.52; 18.93 (rhamnosyl CH_3)
EANP-2	173.13, 169.04, 145.20, 144.98, 138.21 (carbonyl / oxygenated aromatic); 127.83, 120.58, 109.47, 109.00 (aromatic); 79.35, 77.25, 74.19, 66.29, 61.24, 55.63, 51.60; 48–47 (solvent); 43.68–10.05 (aliphatic)

Spectra were recorded at 125.8 MHz (1024 scans); the rutin spectrum header gives D2O and the EANP-2 spectrum CD3OD as solvent.

Table 13. Mass spectral data of the isolated compounds.

Compound	**Ionisation**	**Principal ions, m/z (approx. relative intensity %)**	**Interpretation**
Rutin (EANP-1)	EI, positive	57.5 (100), 71.6 (62), 85.6 (42), 111.7 (30), 129.7 (27), 145.7 (46), 183.9, 219.9, 252.9 (28), 301.0, 316.3, 340.7	Sugar and aglycone-region fragments; scan range to ≈460
EANP-2	ESI, negative	271.2 (100), 131.3 (60), 62.3 (33), 109.2, 169.1, 213.2, 257.2, 307.2	$[M-H]^-$ of $C_{14}H_8O_6$ (M = 272.2)
EANN-2	ESI, positive	433.1 (100), 415.0 (≈20), 397.0 (≈10), 379.1 (≈12), 337.1, 312.9 (≈30), 295.0, 271.0 (≈17)	$[M+H]^+$ of $C_{21}H_{20}O_{10}$ (M = 432.4); $[M+H-18n]^+$; $[M+H-120]^+$; $[aglycone+H]^+$

Relative intensities are approximate values read from the printed spectra.

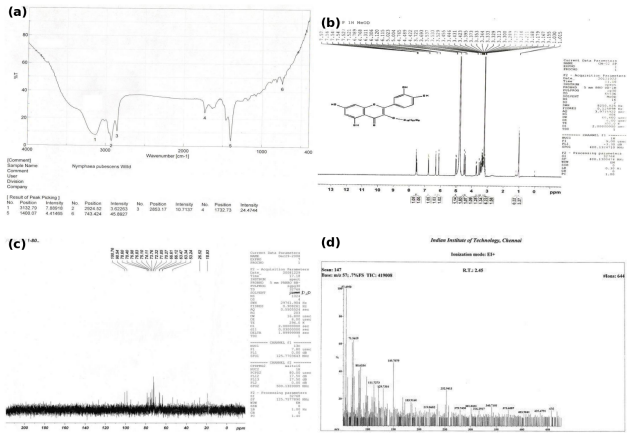


Figure 6. Spectra of rutin (EANP-1): (a) IR (KBr); (b) 1H NMR (400 MHz, CD3OD); (c) 13C NMR (125.8 MHz); (d) EI mass spectrum.

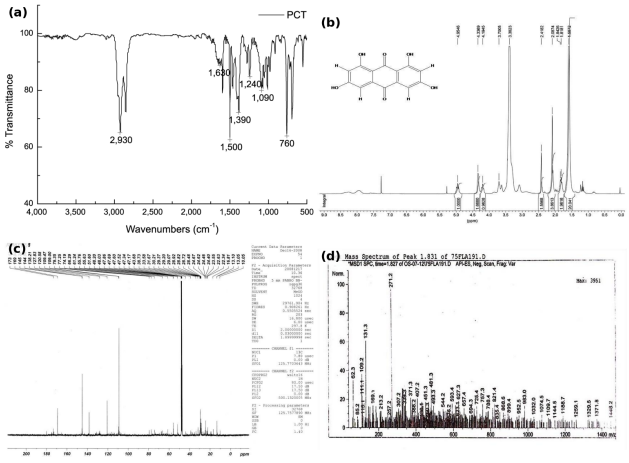


Figure 7. Spectra of 1,3,6,8-tetrahydroxyanthraquinone (EANP-2): (a) IR; (b) 1H NMR; (c) 13C NMR (125.8 MHz, CD3OD); (d) negative-ion ESI mass spectrum.

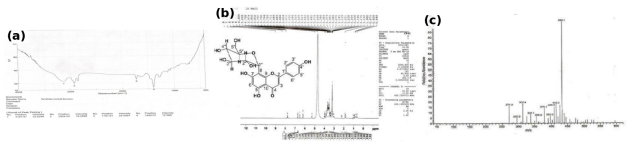


Figure 8. Spectra of the apigenin C-glucoside (EANN-2): (a) IR; (b) 1H NMR (400 MHz, CD3OD); (c) positive-ion ESI mass spectrum.

DISCUSSION

The anatomical and powder features recorded here, notably the winged anther with a well-developed endothecium, astrosclereids in the perianth and the two pollen size classes in N. pubescens, provide diagnostic characters for distinguishing the two flowers in whole and powdered form, which is the basis of pharmacognostic identification [19,20]. The low acid-insoluble ash (below 1%) and foreign organic matter (0.02%) indicate little siliceous or extraneous contamination of the samples, and the similar ash and extractive values suggest that a single set of quality limits could be proposed for both species once more batches have been analysed [3,4].

The screening results are consistent with earlier chemical work on the genus. Flavonoids and phenolic compounds were reported as the major metabolites of the flowers of N. caerulea and of N. odorata [8,10], and flavonoid glycosides and anthocyanins have been characterised from other Nymphaea species [7,9,11]. The strong response of the ethyl acetate extract fits the moderate polarity of these compounds. The TLC data also suggest the presence of anthocyanins in N. nouchali flowers, which we believe has not been shown previously for this species; confirmation by HPLC–MS is needed before this is firmly claimed.

Direct comparison of phenolic content with earlier reports is limited by differences in plant part, solvent and standard. A hydroalcoholic seed extract of N. nouchali contained 179.56 mg gallic acid equivalents/g phenolics and 23.55 mg quercetin equivalents/g flavonoids [15]. The flavonoid content of our flower ethyl acetate extracts (31 and 35 mg QE/g) was higher than that seed value, while the phenolic content (30.44 and 38.88 mg GAE/g) was lower, which may reflect fractionation by ethyl acetate rather than a true organ difference. The DPPH IC50 values calculated here (43.6 and 51.0 µg/mL) are of the same order as the 42.82 µg/mL reported for the N. nouchali seed extract [15] and fit the antioxidant effects described for N. pubescens extracts in vivo [14]. Higher phenolic content in N. pubescens accompanied a slightly lower IC50 in both assays, in line with the general relationship between phenolic content and radical scavenging [6,32,33]. However, the species differences are small, no statistical test was applied, and the extracts were less active than ascorbic acid in the hydrogen peroxide assay, as expected for crude extracts compared with a pure standard.

The isolated compounds are plausible contributors to this activity. Rutin is a well-known antioxidant flavonol glycoside with a wide range of reported pharmacological effects [34], and vitexin-type apigenin C-glycosides are also reported to have antioxidant and other bioactivities [35]. Their presence is in keeping with flavonoid-rich chemistry of Nymphaea flowers [8,9]. The isolation of a hydroxyanthraquinone from N. pubescens is unexpected, as we are not aware of earlier reports from the genus, and

should be confirmed with an authentic standard. The antioxidant constituents may also help to explain the antidiabetic and hepatoprotective activities reported for N. pubescens and N. stellata flowers [13,14,16,17], although this was not tested here.

The spectral data support these assignments to different degrees. For rutin, the 1H NMR coupling pattern (ABX B ring, meta-coupled A-ring doublets, a β-glucosyl anomeric doublet near 7.6 Hz and a rhamnosyl methyl doublet) is diagnostic of a quercetin rutinoside [29,30], whereas the 13C spectrum resolved only the sugar carbons and the displayed mass spectrum did not reach the molecular ion; confirmation of the molecular mass by soft-ionisation MS is therefore advisable. For the apigenin glycoside, the ESI-MS fragmentation (loss of 120 Da and an ion at m/z 271) indicates a C-linked hexose [31], and the 5,7-dihydroxy A-ring singlets and AA′BB′ B-ring doublets fit apigenin, but the reported anomeric coupling (4.1 Hz) is smaller than the roughly 10 Hz normally seen for β-C-glucosides, and distinguishing the 8-C (vitexin) from the 6-C (isovitexin) isomer needs HMBC data or comparison with an authentic standard. The EANP-2 data are the least conclusive: the negative-ion ESI-MS base peak at m/z 271.2 agrees with C14H8O6, but the 1H NMR spectrum was dominated by aliphatic signals and the 13C spectrum lacked labelled signals in the usual anthraquinone carbonyl range (about 180–190 ppm), so the sample may have contained impurities and this identification should be treated as tentative.

This study has limitations. Material came from a single collection, assays were chemical rather than cell-based or in vivo, the quercetin calibration was only moderately linear (Figure 3b), and compound identification relied on spectral interpretation (with incomplete 13C data for rutin and impure-looking NMR spectra for EANP-2) without co-chromatography with authentic standards. Quantitative HPLC of the marker compounds and multi-site sampling would strengthen the quality-control proposals.

CONCLUSION

The flowers of N. pubescens and N. nouchali have been characterised by macroscopic, microscopic, physicochemical and chromatographic parameters that can serve as identification standards. Their ethyl acetate extracts are rich in phenolics and flavonoids and show concentration-dependent DPPH and hydrogen peroxide scavenging, with rutin, 1,3,6,8-tetrahydroxyanthraquinone and an apigenin C-glycoside isolated as constituents. These findings support the traditional use of the flowers and justify further quantitative marker analysis and in vivo studies.

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