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Isolation of a free radical-scavenging antioxidant from water spinach (*Ipomoea aquatica* Forsk)

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Abstract: ***Ipomoea aquatica*** **Forsk, a green leafy vegetable that is a rich source of vitamins and amino acids with many health benefits, has been explored for the isolation and identification of its bioactive compounds. Activity-guided repeated fractionation of a methanol extract on a silica gel column followed by an XAD column yielded a compound that exhibited antioxidant activity with an EC_{50} value of $83 \pm 1.02\,\mu g\,ml^{-1}$ reaction mixture. It also showed very strong lipid peroxidation-inhibitory activity in a liposome model system with an EC_{50} value of $72.2 \pm 0.9\,\mu g\,ml^{-1}$. However, it showed negligible metal-chelating activity. Based on UV, 2D nuclear magnetic resonance and gas chromatography/mass spectrometry studies, the compound was tentatively identified to be 7-*O*-*β*-D-glucopyranosyl-dihydroquercetin-3-*O*-*α*-D-glucopyranoside. This is the first report on the antioxidant properties of *I aquatica* leaf extracts.**

Keywords: green leafy vegetable; *Ipomoea aquatica* Forsk; antioxidant activity; flavonol glycoside; 7-*O*-*β*-D-glucopyranosyl-dihydroquercetin-3-*O*-*α*-D-glucopyranoside

INTRODUCTION

Antioxidants are compounds that when added to food products, especially to lipids and lipid-containing foods, can increase the shelf-life by retarding the process of lipid peroxidation, which is one of the major reasons for the deterioration of food products during processing and storage. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have restricted use in foods, as they are suspected to be carcinogenic.[1] Therefore the importance of searching for and exploiting natural antioxidants, especially of plant origin, has increased greatly in recent years.[2] Several isolated plant constituents as well as crude extracts of vegetables and fruits have been recognised to possess beneficial effects against free radicals in biological systems as antioxidants.[3] This beneficial effect of fruits and vegetables can be attributed to the antioxidant capacity of polyphenols present in them.[4,5]

Green leafy vegetables form a rich source of dietary fibres, nutraceuticals and vitamins, eg carotene (a precursor of vitamin A), vitamin C, riboflavin and folic acid, and minerals, eg calcium, iron and phosphorus.[6] Water-soluble antioxidant components such as flavonoids and *p*-coumaric acid derivatives from spinach leaves have been isolated and identified.[7,8]

Ipomoea aquatica Forsk (IA), commonly called water spinach, belongs to the family Convolvulaceae. It is cultivated commercially as an edible green leafy vegetable in Hong Kong, Taiwan and China.[9] It is a tender, trailing or floating perennial aquatic plant found on moist soil along the margins of fresh water and in ditches, marshes and wet rice fields. It is also commonly used as a green leafy vegetable in rural India.[10]

I aquatica is one of the richest sources of carotenoids and chlorophylls.[11] An extensive study on the identification of these pigments using high-performance liquid chromatography (HPLC) showed that it contains 12 components.[12] The leaves contain adequate quantities of most of the essential amino acids, eg aspartic acid, glycine, alanine and leucine, in accordance with the WHO-recommended pattern for ideal dietary protein.[13] This is comparable to conventional foodstuffs such as soybean or whole egg, indicating the potential of *I aquatica* for utilisation as a food supplement.[14]

In the ancient science of Indian medicine (Ayurveda) and homeopathy, extracts of *I aquatica*

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leaves are administered orally to alleviate antioxidant-related disorders.[15] The plant is also used effectively against nosebleed and high blood pressure.[16,17] Further, its leaf extract can be used to reduce blood sugar levels[18,19] and as an antibiotic against *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis*.[20] The floral buds are used as an anthelmintic.[21]

Thus *I aquatica* ingredients, in terms of their health benefits and applications, have placed the plant within the nutraceutical landscape, though its bioactive compounds are yet to be identified. The purpose of the current study was to determine whether *I aquatica* leaf extracts exhibited antioxidant activity. We also wanted to determine whether the isolated fractions and pure compound were responsible, at least in part, for any antioxidant activity. Therefore fractions containing the pure compound from the most active extract were also tested to determine their effectiveness as antioxidants. Since the antioxidant activity and yield of a methanol extract from *I aquatica* leaves were high, the present work was aimed at isolating and identifying the major antioxidant component of this extract. The results are presented below.

MATERIALS AND METHODS

Plant material

Fresh and healthy leaves of *I aquatica* (IA) were obtained from a local market. The leaves were washed thoroughly in potable water and the surface water was removed by air drying under shade. The leaves were subsequently dried in a hot air oven at 48 °C for 36 h and powdered to 60 mesh in an Apex comminuting mill (Apex Constructions, London).

Chemicals

Folin–Ciocalteu reagent and all other organic solvents used for extraction and chromatography were of AR grade from E Merck (Mumbai, India). Methanol and acetonitrile used for HPLC were of HPLC grade from Ranbaxy Fine Chemicals Limited (New Delhi, India). L-Ascorbic acid, ferrozine and Tris-HCl were from Sisco Research Laboratories (Mumbai, India). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and butylated hydroxyanisole (BHA) were from Sigma Chemical (Chicago, IL, USA). 2-Thiobarbituric acid (TBA) was from ICN Biomedicals Inc (Aurora, OH, USA). Trichloroacetic acid (TCA) and acetic anhydride were from SD Fine Chemicals (Mumbai, India). Pyridine and caffeic acid were from Qualigens Fine Chemicals (Mumbai, India).

Isolation of bioactive compound from IA leaves

IA leaf powder (20 g) was serially extracted with solvents of increasing polarity, namely hexane, chloroform and methanol, in a Soxhlet apparatus for 8 h each. To obtain an aqueous extract, IA leaf powder (10 g) was constantly stirred with water (100 ml) using a magnetic stirrer for 8 h at 30 °C to prevent the degradation of thermolabile compounds. Each extract was filtered through Whatman No 41 filter paper to remove suspended particles. The hexane, chloroform and methanol extracts were concentrated individually under vacuum at 40 °C, while the water extract was concentrated at 65 °C.

Fractionation of methanol extract of IA

IA leaf powder (628 g) was extracted with methanol in a Soxhlet apparatus and the solvent was removed by distillation, yielding 146 g of crude methanol extract. The crude extract (40 g at a time) was subjected to column (450 mm × 40 mm) chromatography using silica gel (60–120 mesh) (Qualigens Fine Chemicals, Mumbai, India) and eluted stepwise with hexane, chloroform and a linear gradient of chloroform/ethyl acetate/methanol (from 100:0:0 to 0:0:100 v/v/v). Eighty fractions of 250 ml each were collected, concentrated by distillation and, after analysis by thin layer chromatography (TLC), divided into 12 groups (fraction nos 1–12, containing fractions 1–10, 11–17,

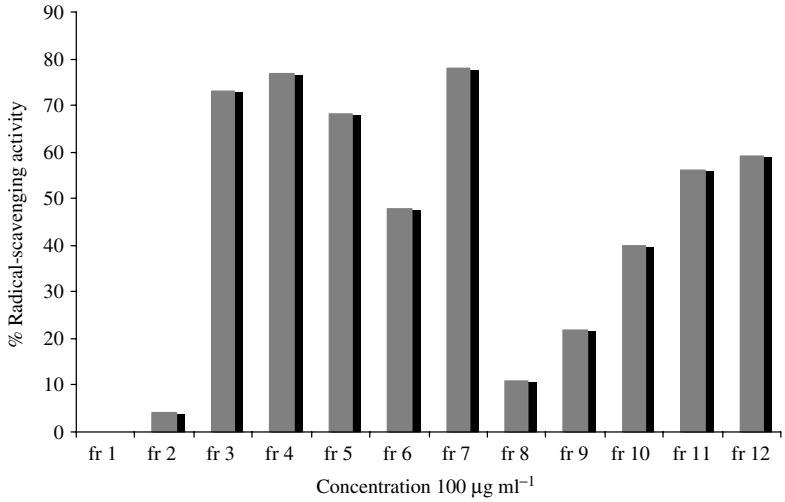

Figure 1. DPPH radical-scavenging activity of silica gel column fractions (nos 1–12) of methanol extract of *Ipomoea aquatica*.

18–20, 21–25, 26–32, 33/34, 35–41, 42–56, 57–59, 60, 61–63 and 64–80 respectively) and assayed for their DPPH free radical-scavenging potential (Fig 1).

Thin layer chromatography (TLC)

An aliquot of each column fraction was spotted on a silica gel TLC plate (20 cm × 20 cm) (Qualigens Fine Chemicals, Mumbai, India). The plates were developed in ascending direction to 18–19 cm height with different proportions of chloroform and methanol as mobile phase. After air drying, the spots on the plates were located by exposure to iodine.

Purification of major fraction

The major fraction (no 12), which showed high DPPH radical-scavenging activity, was subjected to further XAD-4 resin column (450 mm × 40 mm) (Qualigens Fine Chemicals, Mumbai, India) chromatography. Fractions were eluted with a linear gradient of methanol/water (from 100:0 to 50:50 v/v). Twenty-six fractions of 250 ml each were collected, concentrated by distillation and, after analysis by TLC, divided into seven groups (fraction nos 1′–7′, containing fractions 1/2, 3, 4–9, 10, 11–17, 18–20 and 21–26 respectively) and assayed for their DPPH free radical-scavenging potential (Fig 2).

High-performance liquid chromatography (HPLC)

The purity of the most active fraction (fraction no 6′ from the above procedure) was checked by HPLC using a Shimadzu LC-8A liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Rheodyne 7725i injection valve, a 100 µl sample loop and a 250 mm × 20 mm id, 5 µm, amino column (Supelcosil $LCNH_2$, Supelco, Bellefonte, PA, USA). The fraction was eluted with an isocratic solvent mixture of acetonitrile/water (90:10 v/v) at a flow rate of 1 ml min^{-1}. UV detection was carried out at 320 nm using a Shimadzu RID–10A detector. The fraction (2 mg ml^{-1}) was dissolved in triply distilled water, 10 µl

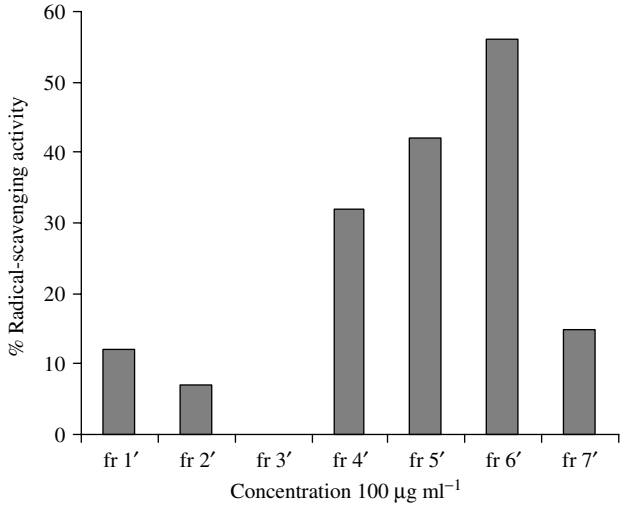

Figure 2. DPPH radical-scavenging activity of XAD gel column fractions (nos 1′–7′) of major fraction (no 12).

of this solution was injected into the chromatograph, and the chromatogram was recorded at 320 nm.

Acetylation of purified compound

Acetylation was carried out according to the method of Markham.[22] The purified compound (20 mg) was dissolved in 0.5 ml of pyridine, then 0.5 ml of acetic anhydride was added and the mixture was stirred in a stoppered conical flask overnight at room temperature. The reaction mixture was subsequently poured into ice-cold water (50 ml) with constant stirring and left for 1 h. The insoluble acetylated compound was separated by filtration using Whatman No 1 filter paper.

Identification of bioactive compound

UV spectrophotometry

The UV-vis spectrum from 200 to 800 nm was recorded on a Shimadzu UV-160A instrument at room temperature, using 2 mg of purified compound dissolved in 200 ml of distilled water.

Gas chromatography/mass spectrometry (GC/MS)

GC/MS analysis of the acetylated compound was carried out using an Agilent (Palo Alto, CA, USA) 6890 GC instrument equipped with a 5973N mass-selective detector and a PE-1 capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm). Helium was used as carrier gas at 2 ml min^{-1} constant flow rate. The oven temperature was maintained at 120 °C for 1 min, ramped to 280 °C at 10 °C min^{-1} and then held at 280 °C. Other conditions were as follows: total run time 40 min, injection temperature 250 °C, detection temperature 260 °C, inlet temperature 300 °C, interface temperature 280 °C, ion source temperature 230 °C and quadrupole temperature 150 °C.

Two-dimensional heteronuclear multiple quantum coherence transfer nuclear magnetic resonance (2D-HMQCT-NMR) spectroscopy

Spectra were recorded on a Bruker (Rheinstetten, Germany) DRX500 NMR instrument operating at 500 MHz for ^1H at room temperature. Regions of 0–12 ppm for ^1H and 0–200 ppm for ^{13}C were employed for scanning, Signals were referenced to tetramethylsilane to within ± 0.01 ppm. The purified compound (30 mg) was dissolved in 0.5 ml of D_2O for recording the spectra. Proton and carbon 90° pulse widths were 12.25 and 10.5 µs respectively. 2D-HMQCT spectra were recorded in magnetic mode with sinusoidal-shaped z gradients of strengths 25.7, 15.2 and 20.56 G cm^{-1} in the ratio 5:3:4 applied for a duration of 1 µs, each with a gradient recovery delay of 100 µs to defocus unwanted coherences. The t_1 was incremented in 256 steps. The size of the computer memory used to accumulate the 2D data was 4K. The spectra were processed using unshifted and $\pi/4$ pulse-shifted sine bell window functions in dimensions F_1 and F_2 respectively.

Determination of total phenolics

The concentration of total phenolic compounds in the extracts was determined according to the method of Taga *et al*[23] and expressed as caffeic acid equivalents. Dried samples and standards were prepared in 60:40 (v/v) acidified (3 g l^{-1} HCl) methanol/water. Test samples/standards of 100 µl were added to 2 ml of 20 g l^{-1} Na_2CO_3. After 2 min, 100 µl of 500 ml l^{-1} Folin–Ciocalteu reagent was added and the mixture was allowed to stand at room temperature for 30 min. The absorbance was measured at 750 nm. The blank consisted of all reagents and solvents without test samples or standards. The standard was caffeic acid prepared at concentrations of 0.001–1 mg ml^{-1}. The phenolic concentration was determined by comparison with the standard calibration curve.

Determination of antioxidant activity

The antioxidant activity of the methanol extract and purified compound of IA was determined by three methods.

DPPH radical-scavenging activity

We determined the antioxidant activity of *I aquatica* leaf extracts with respect to free radical-scavenging activity using the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH).[24–26] The DPPH-scavenging assay employed in the present study was a modification of the procedure of Moon and Terao.[27] First, 0.2 ml of test sample (dissolved in DMSO; for the purified compound, dissolved in Tris-HCl buffer) at different concentrations was mixed with 0.8 ml of Tris-HCl buffer (pH 7.4), then 1 ml of DPPH (500 µM in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. The absorbance of the resulting solution was measured at 517 nm in a Shimadzu UV-160A spectrophotometer. All samples were tested in triplicate, with BHA as positive control. EC_{50} represents 50% of the radicals scavenged by the test sample.

Lipid peroxidation-inhibitory activity

The lipid peroxidation-inhibitory activity of the methanol extract and purified compound was determined according to the method of Duh and Yen.[28] Egg lecithin (3 mg ml^{-1} phosphate buffer, pH 7.4) was sonicated using a UP50H Ultraschallprozessor (Dr Hielscher GmbH, Teltow, near Berlin, Germany). Test samples (100 µl) of different concentrations were added to 1 ml of liposome mixture; the control was without test samples. Lipid peroxidation was induced by adding 10 µl of $FeCl_3$ (400 mM) and 10 µl of L-ascorbic acid (200 mM). After incubation for 1 h at 37 °C the reaction was stopped by adding 2 ml of 0.25 N HCl containing 150 mg ml^{-1} TCA and 3.75 mg ml^{-1} TBA. The reaction mixture was subsequently boiled for 15 min, cooled and centrifuged at 1000 × *g* for 15 min, and the absorbance of the supernatant was measured at 532 nm.

Metal-chelating activity

The ferrous ion-chelating activity was determined by the method of Dinis *et al*.[29] Test samples at different concentrations were mixed with 2 mM $FeCl_2 \cdot 4H_2O$ and 5 mM ferrozine at a ratio of 10:1:2 and the mixture was shaken. After 10 min, Fe^{2+} was monitored by measuring the formation of ferrous ion–ferrozine complex at 562 nm.

RESULTS AND DISCUSSION

Isolation of antioxidant compound

The dry powder obtained from *I aquatica* leaves was first extracted with hexane in order to separate non-polar lipid components, the yield of which was negligible (12.7 mg g^{-1}). Then it was extracted with chloroform, known extracting low-molecular-weight phenolics; the extraction yield was 173.5 mg g^{-1}. The yield of the methanol extract was highest (232.4 mg g^{-1}), probably owing to the extraction of both low- and high-molecular-weight polar compounds,[30] while aqueous extraction yielded 48 mg g^{-1}. The phenolic content was maximum in the methanol extract, followed by water, chloroform and hexane. This indicated that the methanol and aqueous extracts consist largely of polar compounds. Table 1 summarises the extraction yields of the different solvents and the corresponding phenolic contents and DPPH-scavenging activities.

The yield as well as the antioxidant activity of the methanol extract was high. The antioxidant activity may be directly correlated with the phenolic content[31] of various extracts; thus the methanol extract showed higher activity as compared with the other extracts. Similar results were obtained in the case of pomegranate fruit, where the methanol extract had higher yield and antioxidant activity compared with other solvent extracts.[32,33] Hence methanol was selected for large-scale extraction and isolation of the antioxidant compound. The methanol extract was subjected to activity-guided repeated fractionation on a silica gel column and eluted with a series of solvents, namely hexane, chloroform, ethyl acetate and methanol, in different proportions of increasing polarity. The major fraction (no 12) obtained from repeated silica gel column chromatography was finally purified using an XAD-4 resin column.

Table 1. Yield, phenolic content and DPPH radical-scavenging activity of different solvent extracts of *Ipomoea aquatica*

Solvent	Yield (mg g^{-1})	Phenolics (mg g^{-1})	% DPPH radical-scavenging activity[a]
Hexane	12.7	26	6 ± 1.2
Chloroform	173.5	170	55 ± 1.5
Methanol	232.4	260	85 ± 1.1
Water	48	240	67 ± 0.5
BHA	—	—	95 ± 0.02

[a] Concentration of test sample was 100 µg ml^{-1}. Values are mean ± standard deviation of three replicate analyses.

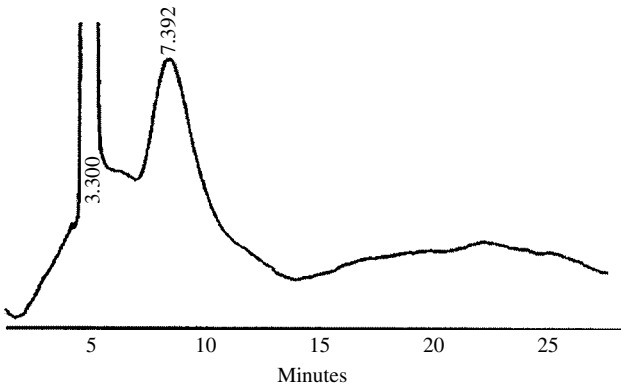

Figure 3. HPLC chromatogram (see text for conditions) of purified compound isolated from *Ipomoea aquatica*.

HPLC analysis was carried out on the purified compound. It showed a single peak with an R_t value of approximately 7.4 min (Fig 3). The weight of the purified antioxidant compound obtained from the methanol extract was 1.7 g from 628 g of dry powder. Fig 4 schematically represents the extraction procedure for the antioxidant compound.

Identification of bioactive compound

The structure of the bioactive compound was determined by a detailed spectroscopic investigation. The purified compound exhibited UV-vis absorption maxima at 218, 282 and 326 nm. The peaks at 282 and 326 nm are due to the $\pi-\pi^*$ transition of the phenyl ring and the $n-\pi^*$ transition of the non-bonded electron of the phenolic hydroxyl respectively.

The specific rotation of the compound was found to be $[\alpha]_{25}^{D} + 3.0$ (10 mg ml^{-1} H_2O). On TLC the acid-hydrolysed compound with butanol/pyridine/water (6:4:3 v/v/v) showed an R_f of 0.49, consistent with glucose. When viewed under UV (254–360 nm) light, the compound fluoresced with a yellow colour.

^{1}H and ^{13}C NMR data were obtained from a detailed 2D-HMQCT spectrum (Table 2). The compound showed ^{13}C signals for carbohydrates mainly in the region of 55–110 ppm, corresponding to glucose molecules. The ^{13}C NMR spectrum showed signals at 110.5 and 94.5 ppm, indicating an α-linked glucose and a β-linked glucose at the C-1 position. The other –CH–O– signals indicated the presence of two glucose molecules: 60 ppm (C-6 G1—glucose 1), 57.5 ppm (C-6 G2—glucose 2), 71.8 ppm (C-4 G2), 72.0 ppm (C-4 G1), 78.4 ppm (H-2 G2), 76.5 ppm (H-2 G1), 79.6 ppm (C-3 G2), 78.4 ppm (C-3 G1), 84.4 ppm (C-5 G2), 80.8 ppm (C-5 G1). The aromatic carbon signals observed were in the region of 85.3–199.0 ppm. The ^{1}H and ^{13}C signals for the aglycone flavonol moiety are given in Table 2. The phenyl ring attached to the 2 position of the flavonol showed proton signals of the ABX system which can only come from three phenyl protons with three substituents attached probably at positions 1′, 3′ and 4′. However, the position of the glucose linkage is not certain.

Based on the NMR data, it can be concluded that the flavonol moiety is a quercetin. However, quercetin shows UV maxima at 256, 268sh and 355 nm,[22] whereas the isolated compound showed UV maxima at 282 and 326 nm. This clearly showed that the

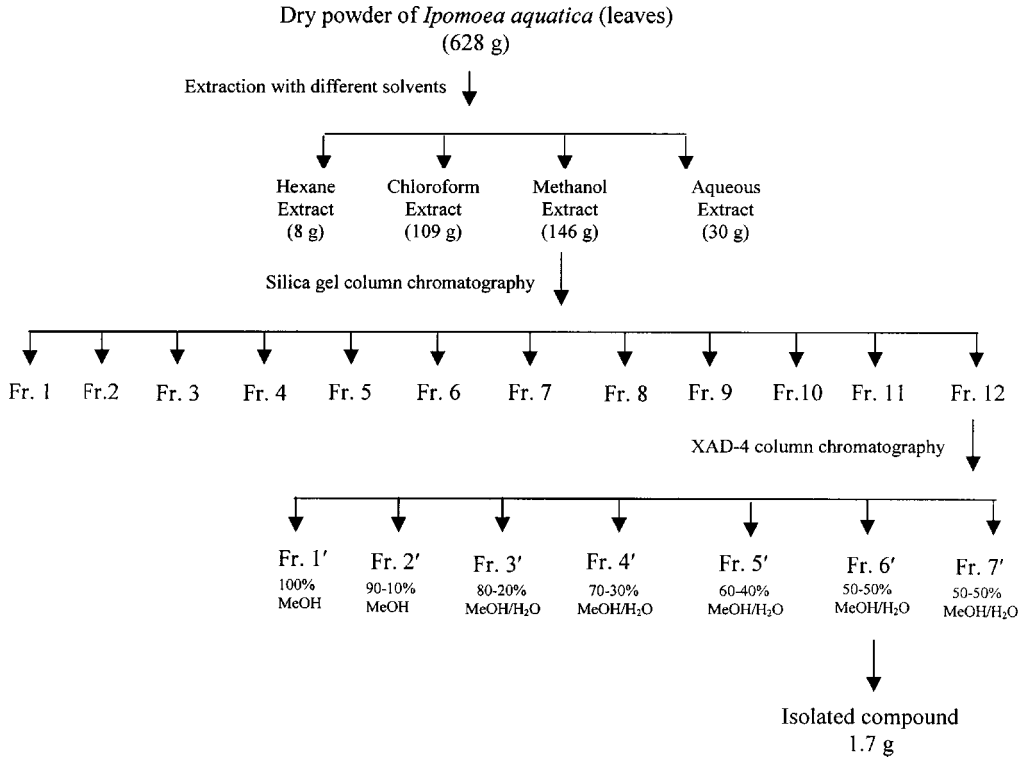

Figure 4. Extraction scheme for isolation of antioxidant compound from *Ipomoea aquatica*.

Table 2. ^{1}H and ^{13}C NMR data (ppm) of purified antioxidant compound from *Ipomoea aquatica*

^{13}C NMR	^{1}H NMR	Assignment
110.5	5.45	H-1$_\alpha$ G2
94.5	4.27	H-1$_\beta$
84.4	3.94	H-5 G2
80.8	3.87	H-5 G1
79.6	3.71	H-3 G2
78.4	4.03	H-3 G1
78.4	3.86	H-2 G2
76.5	4.21	H-2 G1
71.8	3.20	H-4 G2
72.0	3.29	H-4 G1
60.0	2.75, 2.77	H-6$_{a\&b}$ G1
57.5	3.06, 2.79	H-6$_{a\&b}$ G2
85.3	3.91	C-2
72.0	3.87	C-3
199.0	—	C-4
161.2	—	C-5
95.8	6.97 (3.7)	C-6
—[a]	—	C-7
95.2	7.42 (3.7)	C-8
—[a]	—	C-9
99.9	—	C-10
134.5	—	C-1′
111.1	7.1	C-2′
149.2	—	C-3′
148.0	—	C-4′
117.2	7.23 (8.4)	C-5′
122.6	7.33 (8.4)	C-6′

[a] Could not be detected.

isolated compound contains dihydroquercetin as the aglycone portion. The ^{1}H and ^{13}C NMR signals also correspond to the dihydroquercetin moiety.

The compound was acetylated and subjected to GC/MS, but the mass spectrogram did not show any parent *m/z* peak corresponding to 1090, probably owing to fragmentation of the larger parent ion to smaller flavonol and glucose acetates. Correspondingly, the ion at *m/z* 347 was detected for glucose tetraacetate. Other *m/z* peaks at 157, 115, 98 and 43 corresponding to various fragments of acetylated glucose and flavonol acetate were detected. A less intense peak corresponding to *m/z* 394 for flavonol acetate was detected. The most intense peak at *m/z* 43 corresponding to $-CH_3CO-$ was detected distinctly. The compound was tentatively identified to be 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside (Fig 5), as the positions of the linkages of glucose moieties are not certain.

Antioxidant activity of IA extracts and isolated compound

Antioxidants may act in various ways, eg by scavenging radicals, decomposing peroxides or chelating metal ions. Therefore antioxidant activity can and must be evaluated with different tests for different mechanisms. In our study we followed the most frequently used methods for measuring antioxidant activity. Table 3 summarises the antioxidant potential

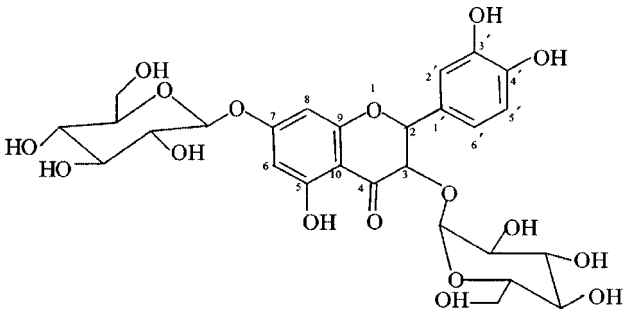

Figure 5. Structure of radical-scavenging antioxidant compound, 7-*O*-β-D-glucopyranosyl-dihydroquercitin-3-*O*-α-D-glucopyranoside, from *Ipomoea aquatica*.

Table 3. Antioxidant potential of methanol extract and 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside from *Ipomoea aquatica*

	EC_{50} value ($\mu g\,ml^{-1}$)[a]		
Bioactive compound	DPPH radical-scavenging activity	Lipid peroxidation-inhibitory activity	Metal-chelating activity
Methanol extract	58 ± 2.6	64.4 ± 1.2	—
7-*O*-β-D-Glucopyranosyl-dihydroquercetin-3-*O*-α-D glucopyranoside	83 ± 1.02	72.2 ± 0.9	—
BHA	5.3 ± 0.6	12.3 ± 1.9	—

[a] Values are mean ± standard deviation of three replicate analyses.

of the methanol extract and 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside in different antioxidant assay systems.

DPPH radical-scavenging activity

Antioxidants react with DPPH, which is a stable free radical, and convert it to α,α-diphenyl-β-picryl hydrazine. The degree of discolouration indicates the radical-scavenging potential of the antioxidant.[33] Among the different solvent extracts of *I aquatica* (Table 1), the methanol extract at a concentration of 100 $\mu g\,ml^{-1}$ showed excellent DPPH free radical-scavenging activity (85%), followed by the aqueous extract (67%). The methanol extract was very much closer to the radical-scavenging ability of the synthetic antioxidant BHA (95%). The chloroform extract showed moderate DPPH-scavenging activity (55%), while the hexane extract showed very low activity (6%). The activity of the extracts is attributed to their hydrogen-donating ability.[33] The EC_{50} value of the methanol extract was found to be 58 $\mu g\,ml^{-1}$, whereas that of the synthetic antioxidant BHA was 5.3 $\mu g\,ml^{-1}$. 7-*O*-β-D-Glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside, a major chemical constituent of *I aquatica*, showed DPPH free radical-scavenging activity with an EC_{50} value of 83 $\mu g\,ml^{-1}$ (Table 3).

Lipid peroxidation-inhibitory activity

Lipid peroxidation is a free radical-mediated propagation of oxidative damage to polyunsaturated fatty acids involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants.[34] To evaluate the antioxidant activity of the methanol extract and 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside (to inhibit lipid peroxidation in biological systems), a liposome model system was used. TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm. MDA is the major product of lipid peroxidation and is used to study the lipid peroxidation process.[33] The lipid peroxidation-inhibitory activities of the methanol extract and 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside from *I aquatica* are shown in Table 3. The EC_{50} value for 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside was found to be 72.2 $\mu g\,ml^{-1}$, while the methanol extract was more active, showing an EC_{50} value of 64.4 $\mu g\,ml^{-1}$. BHA showed very strong antioxidant activity against lipid peroxidation with an EC_{50} value of 12.3 $\mu g\,ml^{-1}$.

Metal-chelating activity

The methanol extract and 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside were also tested with respect to metal-binding capacity by assessing their ability to compete with ferrozine for ferrous ion, thus avoiding the formation of the coloured complex measured at 562 nm. Both the methanol extract and 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside showed negligible metal-chelating activity (4.6 and 1.3% respectively) at a concentration of 100 $\mu g\,ml^{-1}$ test sample.

Although 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside contains many phenolic OH groups capable of chelating ferrous ion, it showed very low metal-chelating activity. With so many OH groups the ion-chelating activity should be very high, so the low activity observed could not be explained at present. The above result suggests that the lipid peroxidation-inhibitory activity of 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside and the methanol extract is not due to iron chelation but may be due to chain termination by the scavenging of peroxyl radicals.[35] Thus both the methanol extract and 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside exert their antioxidant activity mainly through radical-scavenging activity by donating electrons.

The protective effects of antioxidants in biological systems are ascribed mainly to their capacity to scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes and inhibit oxidases.[32] 7-*O*-β-D-Glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside has free hydroxyl groups at the 5, 3′ and 4′ positions. The antioxidant activity of this compound may be related to the phenolic hydroxyl group. The presence of more than one phenolic hydroxyl group increases the antioxidant activity owing to the additional resonance stability of *o*-quinone or *p*-quinone formation.[36,37] The higher the number of hydroxyl groups on the flavonoid nucleus, the higher is the antioxidant activity.[38–40] Compared with 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside, the methanol extract showed greater ability to scavenge free radicals. This may be due to the synergistic effect of other phenolics present in the crude methanol extract, which could supplement the radical-scavenging ability. The presence of the sugar moiety in 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside may be responsible for its low antioxidant activity, and the addition of a second sugar moiety decreased the activity further, probably as a result of steric hindrance due to the addition of sugar moieties.[40] However, glycosylation of quercetin is reported to increase its bioavailability and intestinal absorption to a greater extent than the pure aglycone.[41] Both 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside and the methanol extract showed potential radical-scavenging activity and negligible metal-chelating activity. The above experimental results suggest that the mechanism of antioxidant action of the isolated compound and methanol extract is by donating electrons to free radicals.

CONCLUSIONS

In the current study we performed a serial extraction from *I aquatica* leaves and isolated a flavonol glycoside, 7-*O*-β-D-glucopyranosyl-dihydroquercetin-3-*O*-α-D-glucopyranoside, possessing antioxidant activity. This is the first report on the antioxidant properties of extracts from *I aquatica* leaves. It was observed that extraction with methanol gave a higher yield, and the extract also showed higher antioxidant activity.

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