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FOOD SCIENCE & TECHNOLOGY | RESEARCH ARTICLE

Bioactivity-guided fractionation to identify β-glucuronidase inhibitors in *Nymphaea pubescens* flower extract

Jayashree Acharya[1] and Bratati De[1]*

Abstract: The plant *Nymphaea pubescens* Willd. (Family: Nymphaeaceae) is edible having medicinal importance. The objective of the study was to analyze the potential hepatoprotective properties of the flowers and pedicels of *N. pubescens* by inhibiting the enzyme β-glucuronidase. Crude methanol extracts of flower and pedicel as well as chloroform, ethyl acetate, and aqueous fractions of the flower extract were tested for their activities against the enzyme *in vitro*. The extracts and the fractions were analyzed by GC–MS to identify metabolites present in them. Flower (IC_{50} value = 270.27 ± 4.67 µg/ml) and pedicel (IC_{50} value = 868.46 ± 28.21 µg/ml) extracts have shown to inhibit the β-glucuronidase activity. Chloroform (IC_{50} value = 147.16 ± 6.68 µg/ml), ethyl acetate (IC_{50} value = 183.94 ± 2.37 µg/ml), and aqueous (IC_{50} value = 339.43 ± 5.34 µg/ml) fractions showed significantly stronger activity than that of silymarin (IC_{50} value = 792.62 ± 10.01 µg/ml), the known inhibitor of the enzyme. GC–MS-based analysis of the flower extract and solvent fractions led to the identification of kaempferol having 79-fold stronger activity than that of silymarin, IC_{50} value of kaempferol being 10.44 ± 0.084 µg/ml or 0.0037 mM ± 0.0001.

Subjects: Food Analysis; Food Chemistry; Fruit & Vegetables

Keywords: kaempferol; enzyme assay; hepatoprotection

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Bratati De, professor, is interested in studying bioactive compounds of medicinal and food plants. She is working for dereplication of bioactive constituents of plants using plant metabolomics approach.

PUBLIC INTEREST STATEMENT

The flower and pedicel of the aquatic edible plant *Nymphaea pubescens* Willd, familiar as water lily in English and sapla-shaluk in Bengali, have medicinal importance. The flowers are cardio-tonic and astringent. The whole plant or parts of the plant are used in folk medicine and siddha for curing diabetes, bleeding piles, dyspepsia, jaundice, and eye disorders. Present work was carried out to justify, partially, the health beneficial effect scientifically. The study supports the hepatoprotective potential of the flower and pedicel extracts. A plant metabolite having such activity was also identified. After further study, this aquatic edible plant has the potential to be utilized as a novel hepatoprotective food cum medicinal plant.

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1. Introduction

Nymphaea pubescens Willd. (Family: Nymphaeaceae) is an aquatic plant familiar as water lily in English and sapla-shaluk in Bengali. It is a rhizomatous herb, long-petioled with cordate and ovate to orbiculate leaves. Flowers are large, solitary with long pedicel (Rushender, Eerika, Madhusudhanan, & Konda, 2012). Flowers and pedicel are edible and in addition have medicinal values. The flowers are cardio-tonic and astringent. The seeds are known to be a cooling agent, aphrodisiac, sweet, constipating, stomachic, and restorative (Muthulingam, 2010). The whole plant or parts of the plant are used in folk medicine and siddha for curing diabetes, bleeding piles, dyspepsia, jaundice, and eye disorders (Selvakumari & Shantha, 2010). Recently, antioxidant and hepatoprotective activity of *N. pubescens* flower on CCl_4-induced hepatotoxicity model in rats have been reported (Debnath, Ghosh, & Hazra, 2013). However, the mechanism of hepatoprotective action is not yet known. β-Glucuronidase inhibitors are suggested as potential hepatoprotective agents (Shim, Kim, & Kim, 2000). The enzyme β-glucuronidase catalyzes the hydrolysis of β-glucuronide conjugates of endogenous and exogenous compounds in the body (Shim et al., 2000). In mammals, glucuronidation is a major detoxification process. Glucuronides of metabolic wastes, xenobiotics are then excreted from the body, unless hydrolyzed by the intestinal β-glucuronidase (Fior & Gerola, 2009). Serum β-glucuronidase activity is inversely related to plant food intakes in humans (Lampe, Li, Potter, & King, 2002). Several classes of β-glucuronidase inhibitors, including plant derivatives, are D-glucaric acid, silymarin, tectorigenin, 18-β-glycyrrhetinic acid (Kim, Shim, Kim, & Jang, 1999; Shim et al., 2000; Walaszek et al., 1997). To our knowledge, there is no report on the β-glucuronidase inhibition property of the plant *N. pubescens*. The property of the plant extract to inhibit this enzyme would further scientifically validate the previously reported hepatoprotective action of this plant. So the present study aims to assess the β-glucuronidase inhibitory property of the crude extracts and fractions of the flower and pedicel of *N. pubescens* and also to explore the phytoconstituents present in these parts of the plant in order to identify β-glucuronidase inhibitor(s).

2. Materials and methods

2.1. Plant material

Whole plant specimens were collected from local market, Chandannagar, India in October, 2011 (Voucher No. 33232) and were identified by Ambarish Mukherjee, Professor of Botany, Burdwan University, India. Flowers and pedicels were shed dried separately at normal temperature and powdered using a grinder.

2.2. Extraction and fractionation

The dried powdered flower (186 g) was refluxed with methanol (300 ml) for 5 h. The filtrate was evaporated to dryness to obtain crude extract (17.2 g). The dried pedicel (146 g), extracted similarly, yielded 19.6 g crude extract. The crude flower extract was dissolved in methanol:water: 70:30. Methanol was evaporated. The residual aqueous extract was fractionated for chloroform extract as well as for ethyl acetate extract to obtain chloroform, ethyl acetate, and aqueous fractions, respectively. Each fraction was evaporated to dryness.

2.3. β-Glucuronidase inhibition assay

β-Glucuronidase inhibition assay was carried out following the method of Kim et al. (1999). β-glucuronidase (100 µl) from bovine liver (9864 units/ml in 0.1 M phosphate buffer, pH 7.0) and flower extract in 0.1 M phosphate buffer (pH 7.0) (340 µl) were pre-incubated at 37°C for 15 min. Following the pre-incubation, 60 µl of p-nitrophenly-β-D-glucuronide (3.15 mg/ml in 0.1 M phosphate buffer, pH 7.0) was added and incubated for 50 min at 37°C. The absorbance was measured at 405 nm spectrophotometrically. The percentage of β-glucuronidase inhibitory property was calculated as $[(A_o - A_e)/A_o] \times 100$ (A_o = absorbance without extract; A_e = absorbance with extract).

2.4. Gas chromatography–mass spectrometry

GC–MS analysis was carried out following the method of Kind et al. (2009) after modification. HP-5MS capillary column (Agilent J & W; GC Columns (USA) (length 30 m plus Duraguard 10 m, diameter

0.25 mm narrow bore, film 0.25 µm) was used. The analysis was performed under the following oven temperature programme: Injection in sandwich mode with fast plunger speed without viscosity delay or dwell time, oven ramp 60°C (1 min hold) to 325°C at 10°C/min, 10-min hold before cool down, 37.5-min run time. The injection temperature was set at 250°C; the MS transfer line at 290°C and the ion source at 230°C. Helium was used as the carrier gas at a constant flow rate of 0.723 ml/min (carrier linear velocity 31.141 cm/s). The dried extracts were derivatized after using methoxyamine hydrochloride (20 mg/ml in Pyridine) and subsequently with N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) to increase volatility of metabolites. 2 µl FAME (Fatty Acid Methyl Esters) markers [a mixture of internal Retention Index (RI) markers prepared using fatty acid methyl esters of C8, C10, C12, C14, C16, C18, C20, C22, C24, and C26 linear chain length, dissolved in chloroform (HPLC) at a concentration of 0.8 mg/ml (C8-C16) and 0.4 mg/ml (C18-C26)] was added (Kind et al., 2009). Derivatized samples (1 µl) were injected via the split mode (Split ratio 1:5) into the GC column. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute and identify chromatographic peaks. Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra and retention times with entries of mass spectra and retention time in Agilent Fiehn Library. The relative response ratios of all the metabolites were calculated after normalizing the peak areas of the compounds by extract dry weight.

2.5. Statistical analysis

All the experiments were performed at least thrice. Mathematical calculations (means and standard deviations) were calculated from replicas within the experiments and analyses have been done using Microsoft Excel 2007. Difference in values ≤0.05 was considered as significant. IC_{50} value (the concentration of the extract required to inhibit the enzyme activity by 50%) was calculated using the regression equation obtained from the percentage inhibitions against concentrations.

3. Result and discussion

The crude extracts of flower and pedicel inhibited the enzyme β-glucuronidase in a dose-dependent manner (Figure 1). The IC_{50} values are shown in Table 1. IC_{50} value for inhibition of β-glucuronidase by silymarin, the known β-glucuronidase inhibitor was 794.62 ± 10.01 µg/ml. This comparative study indicated that both flower and pedicel extracts were active against the enzyme. Flower extract (IC_{50} value = 270.27 ± 4.67) had significantly higher activity than the pedicel extract (IC_{50} value = 868.46 ± 28.21). Flower extract had threefold stronger activity than that of silymarin. Therefore, the crude flower extract was further fractionated into three fractions; chloroform soluble fraction, ethyl acetate soluble fraction, and aqueous fraction. The fractions were further analyzed to assess their activities against the enzyme β-glucuronidase. All the three fractions inhibited the enzyme in concentration dependent manner (Figure 2), R^2 values being >0.9. From the IC_{50} values, it was noted that all the fractions had activity (Table 1) significantly higher than that of silymarin. The activity of chloroform fraction was fivefold higher than that of silymarin. The activities of ethyl acetate soluble fraction and aqueous fraction, compared to silymarin, were four and twofold higher, respectively.

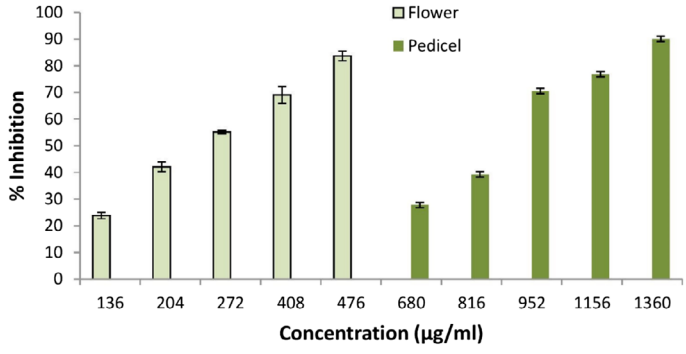

Figure 1. β-Glucuronidase inhibition by different concentrations of flower and pedicel extracts.

Table 1. β-Glucuronidase inhibition properties of crude extracts, fractions

Materials	β-Glucuronidase inhibition IC_{50} value (μg/ml ± sd)
Pedicel extract	868.46 ± 28.21
Flower extract	270.27 ± 4.67
Ethyl acetate fraction	183.94 ± 2.37
Chloroform fraction	147.16 ± 6.68
Aqueous fraction	339.43 ± 5.34
Kaempferol	10.44 ± 0.084
Silymarin	794.62 ± 10.01

Gas chromatography–Mass spectrometric (GC–MS) analysis was performed in an attempt to identify active constituents of the crude extracts and fractions. The metabolites which could be identified through GC–MS analysis of crude extract of pedicel belonged to such categories as organic acids (5), sugar and sugar alcohols (4), phenols (3), fatty acids (3), and other metabolites (porphine and indole-3-acetamide) (Table 2). The crude extract of the flower contained metabolites belonging to organic acids (6), amino acids (4), sugar and sugar alcohols (6), phenols (5), fatty acids (7) (Table 2). Some extra phenolic components, in addition to the components identified in the crude flower extract, were detected from the fractions of the flower extract (Table 2). The identified compounds in the chloroform fraction were phenols (7), organic acids (3) and fatty acids (3), sugar and sugar-derived alcohol (4), and indole-3-acetamide. Eighteen compounds could be identified from the ethyl acetate fraction. Those were organic acids (6), sugar and sugar alcohols (4), fatty acids (3), phenols (5). Metabolites identified from aqueous fraction belonged to organic acids (5), amino acids (6), sugar and sugar alcohol (6), fatty acids (3), phenols (3) (Table 2).

Thus, the flower of *N. pubescens* appear to be a source of no less than 11 phenolic metabolites (alizarin, p-anisic acid, benzoic acid, 3,4-dihydroxybenzoic acid, ferulic acid, gallic acid, 4-hydroxy benzoic acid, 4-hydroxy-3-methoxy benzoic acid, quinic acid, shikimic acid, and the flavonoid kaempferol). Phenolic compounds have been reported to be inhibitors of a number of enzymes (Quesada et al., 1996; Rohn, Rawel, & Kroll, 2002; Sarikaya, Sisecioglu, Cankaya, Gulcin, & Ozdemir, 2014). None of these phenolic compounds have been reported so far to have inhibitory action against β-glucuronidase. Thus, five of the phenolic metabolites available in the laboratory (quinic acid, 3,4-dihydroxybenzoic acid and 4-hydroxy-3-methoxy benzoic acid, p-anisic acid, and kaempferol) were tested against the enzyme. Kaempferol inhibited the enzyme β-glucuronidase. Whereas the other phenolic compounds tested did not inhibit the enzyme activity. The dose-dependent inhibition activity of kaempferol is shown in Figure 3. High activity of this flavonoid was indicated by its IC_{50} value 0.0037 mM ± 0.0001 (10.44 μg/ml ± 0.04) (Table 1). The activity was found to be 79-fold higher than that of silymarin. Other fractions, where kaempferol could not be detected, were also highly active. Thus, synergism of different metabolites present in the extracts could also be a possibility. Therefore, further study is required to identify existence of other active constituents and to test the *in vivo* efficacy of these active constituents.

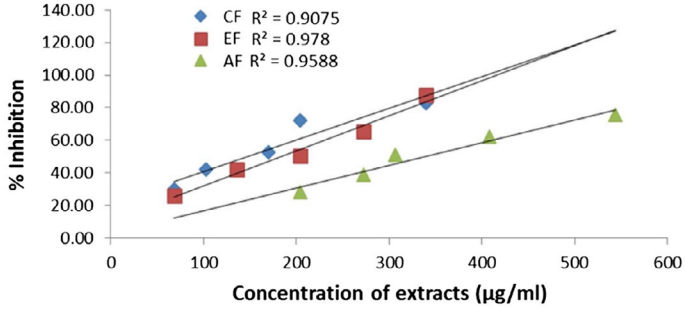

Figure 2. β-Glucuronidase inhibition by fractions of flower extract.

Notes: CF: Chlorofom fraction; EF: Ethyl acetate fraction; AF: Aqueous fraction.

Table 2. Metabolites detected in crude extracts and fractions

Metabolites		Response ratio (Mean ± sd)				
		Crude pedicel extract	Crude flower extract	Flower extract		
				Chloroform fraction	Ethyl acetate fraction	Aqueous fraction
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Organic acids	Glyceric acid	1.12 ± 0.12	1.37 ± 0.26	–	0.79 ± 0.15	1.74 ± 0.09
	Glycolic acid	2.81 ± 0.04	2.87 ± 0.10	2.78 ± 0.08	2.75 ± 0.06	2.74 ± 0.03
	Gluconic acid	1.43 ± 0.16	1.35 ± 0.11	–	–	–
	L(+)lactic acid	1.43 ± 0.06	–	2.59 ± 0.07	1.87 ± 0.11	1.70 ± 0.06
	D-malic acid	1.75 ± 0.08	1.45 ± 0.26	–	1.18 ± 0.15	2.06 ± 0.03
	Malonic acid	–	–	1.20 ± 0.42	–	–
	Oxalic acid	–	1.33 ± 0.56	–	–	–
	Succinic acid	–	0.56 ± 0.35	–	1.90 ± 0.15	–
	Uric acid	–	–	–	0.95 ± 0.14	0.84 ± 0.11
Inorganic acid	Phosphoric acid	1.07 ± 0.07	1.97 ± 0.14	–	–	2.53 ± 0.08
Amino acids	Beta alanine	–	–	–	–	1.60 ± 0.03
	L-glutamic acid	–	1.47 ± 0.25	–	–	1.97 ± 0.08
	L-pyroglutamic acid	–	–	1.37 ± 0.22	–	0.95 ± 0.24
	Glycine	–	0.37 ± 0.24	–	–	0.84 ± 0.14
	DL-isoleucine	–	0.14 ± 0.40	–	–	1.06 ± 0.12
	L-valine	–	0.81 ± 0.16	–	–	1.69 ± 0.05
Sugar and sugar alcohols	Glycerol	2.48 ± 0.15	2.62 ± 0.10	1.80 ± 0.06	2.17 ± 0.10	2.89 ± 0.03
	D-mannitol		2.59 ± 0.08	1.50 ± 0.04	1.65 ± 0.49	2.84 ± 0.01
	D-sorbitol	1.84 ± 0.07				
	Sucrose	1.08 ± 0.11	1.96 ± 0.56	1.76 ± 0.04	2.00 ± 0.70	1.41 ± 0.14
	D-(+) trehalose		2.00 ± 0.07			2.24 ± 0.03
	D-threitol	2.27 ± 0.07	1.28 ± 0.07	1.35 ± 0.10	1.18 ± 0.49	1.56 ± 0.03
	Xylitol		1.97 ± 0.64	–	–	3.14 ± 0.01
Phenols	Alizarin	–	0.39 ± 0.18	0.26 ± 0.07	–	–
	P-anisic acid	–	–	1.79 ± 0.11	–	–
	Benzoic acid	–	–	1.84 ± 0.09	–	–
	3,4 dihydroxy benzoic acid	–	–	–	1.50 ± 0.02	–
	Ferulic acid	–	–	1.84 ± 0.07	–	–
	Gallic acid	2.20 ± 0.04	2.98 ± 0.05	2.53 ± 0.06	4.02 ± 0.01	2.96 ± 0.07
	4-hydroxy benzoic acid	–	–	–	1.51 ± 0.07	–
	4-hydroxy-3-me-thoxy -benzoic acid	–	–	1.07- ± 0.21	–	–
	Kaempferol	–	0.46 ± 0.36	–	1.71 ± 0.14	–
	Quinic acid	2.05 ± 0.03	2.12 ± 0.09	–		2.18 ± 0.01
	Shikimic acid	2.21 ± 0.01	2.61 ± 0.10	1.21 ± 0.46	1.71 ± 0.03	2.62 ± 0.03
Fatty acids	4-guanidinobutyric acid	1.56 ± 0.13	1.65 ± 0.35	–	–	2.13 ± 0.14
	1-Hexadecanol	–	0.17 ± 0.11	–	–	–

(*Continued*)

Table 2. (*Continued*)

Metabolites		Response ratio (Mean ± sd)				
		Crude pedicel extract	Crude flower extract	Flower extract		
				Chloroform fraction	Ethyl acetate fraction	Aqueous fraction
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
	6-hydroxy hexanoic acid	–	–	–	1.65 ± 0.17	–
	Lauric acid	–	1.43 ± 0.10	–	–	–
	Linoleic acid	–		1.65 ± 0.08	–	–
	Myristic acid	–	1.24 ± 0.68	–	–	–
	Oleic acid	–	0.83 ± 0.32	–	–	–
	Palimitic acid	2.48 ± 0.09	2.43 ± 0.49	2.84 ± 0.12	2.30 ± 0.04	2.38 ± 0.28
	Stearic acid	2.45 ± 0.07	2.78 ± 0.32	2.97 ± 0.14	2.36 ± 0.07	2.70 ± 0.04
Others	Indole-3-acetamide	–	–	0.97 ± 0.07	–	–
	Porphine	1.63 ± 0.12	1.47 ± 0.29	–	–	–

Note: – not detected.

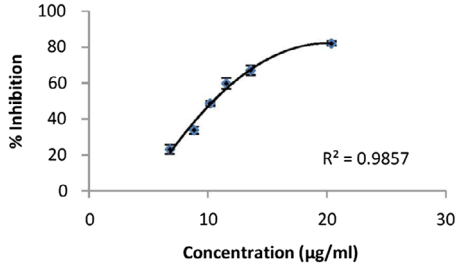

Figure 3. β-Glucuronidase inhibition by kaempferol.

4. Conclusions

Edible plants also have medicinal properties. Scientific proof of such medicinal properties would encourage people to consume such food plants for beneficial effect on health. Such edible plants may also be considered for search of drugs with lesser side effects and better activity. Present findings suggest that the edible flower of *N. pubescensis* is very active against the enzyme β-glucuronidase. Kaempferol, detected in the crude extract, had >79-fold stronger activity than that of silymarin. After further *in vivo* study, the plant has the potential to be utilized as a novel hepatoprotective food cum medicinal plant.

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Competing interests
The authors declare no competing interest.

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