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

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**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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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 CCl₄-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-nitrophenyl-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 

$$\frac{(A_o - A_e)}{A_o} \times 100$$

where \(A_o\) = 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-SMS 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. IC50 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 IC50 values are shown in Table 1. IC50 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 (IC50 value = 270.27 ± 4.67) had significantly higher activity than the pedicel extract (IC50 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), R2 values being >0.9. From the IC50 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.
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 IC50 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.

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

| Materials            | β-Glucuronidase inhibition IC50 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           | 10.64 ± 0.084                                     |
| Silymarin            | 794.62 ± 10.01                                    |

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

Notes: CF: Chloroform 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       | –                      | 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                | –                    | –             | –                 | 1.97 ± 0.08            |                   |
| 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-methoxy 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          | –             | –                 | 2.38 ± 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)
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. pubescens* is very active against the enzyme \( \beta \)-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.

Funding
Bratati De, acknowledges financial support from Department of Science and Technology (Govt. of West Bengal) [Sanction letter number 494 (Sanct.)/ST/P/ S&T/F/1G-S/11/2011] and DST-FIST programme, Govt. of India [grant number SR/FST/LSI-459/2010].

Competing interests
The authors declare no competing interest.

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Citation information
Cite this article as: Bioactivity-guided fractionation to identify \( \beta \)-glucuronidase inhibitors in *Nymphaea pubescens* flower extract, Jayashree Acharya & Bratati De, Cogent Food & Agriculture (2016), 2: 1134379.

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