



Detection of Quercetin, Kaempferol and High Value Amino Acids from Seed Endosperm of *Nymphaea stellata* cv. *alba* L.

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ABSTRACT

Background: In this study, the seed endosperm of *Nymphaea stellata* cv. *alba* was subjected to quantitative estimation of various phyto-chemicals, as it is commonly consumed as a nontraditional, non-cereal food in rural India.

Methods: AOAC techniques were used to determine the approximate composition. UHPLC amino acid and flavonoid analyzers are used to measure the amino acids and flavonoids in seed endosperm, respectively.

Result: From standard analysis, it was evident that *Nymphaea* seed endosperm had 7.5±0.37% protein, 2.9±0.08% crude fiber and 1.8±0.21% total fat. The essential amino acids were determined in the seed endosperm by UHPLC method using pre-column derivatization method with o-phthalaldehyde- 2-mercaptoethanol. The results shown in the endosperm of *Nymphaea stellata* cv. *alba* produced the maximum amount of amino acids, aspartic acid (0.293 mg/g), glutamic acid (0.701 mg/g), serine (0.278 mg/g), histidine (0.116 mg/g), glycine (0.140 mg/g), threonine (0.149 mg/g) arginine (0.429 mg/g), alanine (0.239 mg/g), tyrosine (0.096 mg/g), cystine (0.424 mg/g), valine (0.50 mg/g), methionine (0.135 mg/g), phenylalanine (0.170 mg/g), isoleucine (0.339 mg/g) and leucine (0.34 mg/g). Furthermore, UHPLC chromatogram of quercetin and kaempferol in water lily seed endosperm revealed that the presence of quercetin 0.340 mg/kg and kaempferol 0.127 mg /kg as pure form without other derivatives of the two flavonoids like their glucoside compounds, esters, etc. The *Nymphaea stellata* cv. *Alba* seed endosperm has different beneficial composition including amino acids and flavonoids that may be of great importance to determine food quality as they helps in glucose metabolism and reduce oxidative stress caused by reactive oxygen species (ROS).

Key words: Anti-diabetic components, Arginine, Kaempferol, Quercetin, Water lily.

INTRODUCTION

Water lily (*Nymphaea stellata* cv. *alba* L.) is widely grown in wetlands belongs to all types of climate from temperate to tropical condition. It belongs to family Nymphaeaceae. India is native place of different types of *Nymphaea*. Water lily seed is very small and popped nut is not popular in India. Poor and rural people primarily enjoy its fried nuts/pop with jaggery during ceremonies such as Poush Sankranti. It develops spontaneously in abandoned wetlands and can even be grown like transplanted rice fields as weed (Saji *et al.*, 2023). *Nymphaea* seed endosperm are highly nutritious (Abelti *et al.*, 2023) like other aquatic nut endosperm, makhana (Jana *et al.*, 2022), but scientific cultivation is more easier than that of makhana (Khadatkar *et al.*, 2014). According to Gopalan *et al.* (1996) water lily seeds contain 10.0% moisture, 8.3% protein, 1.0% fat, 0.9% minerals, 4.2% fiber and 75.6% carbohydrate. The 100 g of pop nut provide 345.0 k cal energy. Among minerals, it contained 20 mg/100 g calcium and 110 mg/100 g phosphorus. So nutritionally, *Nymphaea* seeds are as important as makhana and lotus nut. Many scientist reported the lotus nut to have different flavonoids, which are anti-oxidant in nature. Flavonoids have anti-inflammatory and anti-oxidative effects also and thus may reduce against diabetic complications. It has been proven that flavonoid increases insulin uptake by modifying GLUT4 (Cho *et al.*, 2010). Recent studies have also suggested that flavonoids may have hyperglycemic activity by preventing the activity of

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enzymes that break down carbohydrates, like amylase, glucosidase and disaccharidases (Tadera *et al.*, 2006 and Pareira *et al.*, 2011). The flavonoid, quercetin, is a polyphenolic compound and the most abundant flavonoid in edible vegetables, fruit, wine and nuts. It is a major bio-flavonoid in the human diet and may produce anti-proliferative effects resulting from the modulation of either EGFR or estrogen-receptor mediated signal transduction pathways. Quercetin, hereby, preventing the production of pro-inflammatory mediators and also reduces inflammation through the lipo-oxygenase and cyclo-oxygenase pathways. In addition, it is effective against cardiovascular disease, hypercholesterolemia, rheumatic diseases, infections and cancer.

Hyperglycemia by improved insulin stimulated glucose uptake in adipocytes *via* quercetin and kaempferol was

illustrated by Fang *et al.* (2008). Kaempferol has also beneficial role on diabetes by preventing oxidative damage in pancreatic β cells (Khalid *et al.*, 2015). Seed flour of *Nymphaea* is generally used in preparation of baby foods, *kheer*, *halwas*, *murkee* and *sweets*. Flavanol glycosides in *Nymphaea* keep the head cool by helping blood circulation to the scalp and also to reduce the risk of heart disease.

As a result, in the current study, we quantified several phytochemicals such as flavonoids like quercetin and kaempferol, as well as nutrient components from water lily seed endosperm, in order to raise awareness about their use in diabetic and elderly patients. These studies provide a window for further research in this non-traditional and non-cereal aquatic food for human benefit.

MATERIALS AND METHODS

Preparation of samples

Seeds of water lily was collected from field during Nov-Dec. Fruit of *Nymphaea* is many seeded berry (approx. 2000 seeds /fruit) usually ripens during December (Plate : 1a,b,c). Seeds are collected and dried in sunlight for 3-5 days. Seeds are very minute and crashed with hammer and endosperms were separated by sieving.

Proximate composition

Nutritive properties including moisture, ash, crude fat and crude protein contents were determined by AOAC methods (A.O.A.C., 2016). Crude protein was analyzed using Kelplus Elite Ex Micro Kjeldahl method using conversion factor 6.25. Carbohydrates (by difference) % weight was determined as followed by the method of Gopalan *et al.*, (1998). Total dietary fiber (TDF) were determined by digesting the sample with α -amylase (AOAC method 991.43). All the biochemical analyses were carried out in four replications using fresh and dry *Nymphaea* kernel powder.

Amino acids

Amino acid profile was obtained by the analysis of samples through adopting procedures of Bidlingmeyer *et al.* (1987). Determination of amino acid in seed endosperm by UHPLC amino acid analyzer. The method for amino acid extraction was followed with modification. The samples were hydrolyzed with HCl 6 M at 110°C for 17 h. After hydrolysis, the acid was removed by rotary evaporation. Sample was resuspended in 25 mL of sodium citrate buffer at pH 2.2. A solution of Amino acids standards from Sigma (AAS-18) solution containing 2.5, 5.0 and 7.5 mg/L (Aspartic acid, glutamic acid, serine, histidine, glycine, L- threonine, Arginine, Alanine, Tyrosine, Cystine, Valline, Methionine, Phenylalanine, Isoleucine Leucine, Lysine, Proline) was prepared for calibration curves.

UHPLC condition

The amino acid is determined by UHPLC method (Rouba and Ulrich, 2013), Instrument :Nexera UHPLC (Shimadzu) with SIL-30AC autosampler (Shimadzu, 2007), Column: Shimpack GIST (C18), 5.0 μ m (250 mmL $\times$ 4.6 mm I.D.), Mobile Phase :A :25 mmol/L Phosphate Potassium Buffer (pH 6.9) ,B: 45/40/15 Acetonitrile/Methanol/Water, Time Program : B Conc.10% (0.01) $\rightarrow$ 11% (0.35 min) $\rightarrow$ 45% (35.00 min) $\rightarrow$ 100% (40.00-45.00 min), $\rightarrow$ 10% (60.00 min), Flow Rate : 1.0 mL/min, Column Temp. : 35°C, Injection Volume: 10 μ L, Detection: RF-20Axs Ex. at 350 nm, Em. at 450 nm, $\rightarrow$ Ex. at 266 nm, Em. at 305 nm (9.0 min), Cell Temp.: 20°C. Flow Cell: Conventional cell.

Preparation of derivatization reagents

Mercaptopropionic Acid- 3- Mercaptopropionic Acid 10 μ L in 0.1 mol/L Borate Buffer (pH 9.2) 10 mL, 2.o- Phthalaldehyde Solution L: o-Phthalaldehyde 10 mg in 0.1 mol/L Borate Buffer (pH 9.2) 5 mL.3. Fluorenyl Methyl Chloro Formate (FMOc) - Acetonitrile Solution 9-Fluorenyl Methyl



Plate 1: a. *Nymphaea stellata* cv. *alba* plant b. Fruits and c. Broken endosperms.

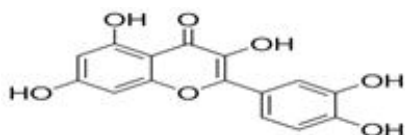
Chloro Formate 4 mg in Acetonitrile 20 ml (Einarson *et al.*, 2001).

Derivatization procedure

Mercaptopropionic Acid, OPA and sample was taken in (μ L) ratio of 45:22:7.5 and thoroughly mixed and kept for 1 min. then added 10 μ L of FMOc and sample was injected to the UHPLC using Autosampler.

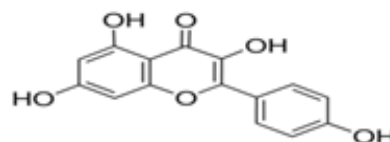
Quantification of quercetin

Structurally quercetin (Quercetin>95% (HPLC Solids), is a pentahydroxyflavone having the five hydroxy groups placed at the 3-, 3'-, 4'-, 5- and 7-positions. So, it is 3,3',4',5,6-Pentahydroxyflavone, 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one. Chemical formulae of quercetin is C₁₅H₁₀O₇ and molecular weight of 302.24. We applied standard quercetin (Q4951-10G) from Sigma-Aldrich company, Bangalore, India.



Quantification of kaempferol

Kaempferol is a tetrahydroxyflavone in which the four hydroxyl groups are located at positions 3, 5, 7 and 4'. Chemical name is 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one and 3,4',5,7-Tetrahydroxyflavone., Chemical formula and molecular weight are C₁₅H₁₀O₆ and 286.24, respectively. We purchased standard kaempferol (K0133-10MG) from Sigma-Aldrich company, Bangalore, India.



Sample preparation

Ten gram of sample was homogenized with 70% methanol. Samples were vortexed for 5 min followed by centrifugation at 4,500 rpm for 10 mins. Filter the supernatant in 0.45 μ m syringe filter. Then the samples were injected in HPLC system. Column oven temperature was 30.0°C. Injected volume was 20 μ L and flow rate was 1.0 ml/min. Mobile phase A was 6% acetic acid in 2 mM sodium acetate aqueous solution (v/v, final pH 2.55). On the other hand,

Table 1: Proximate composition of seed endosperm of *Nymphaea stellata* cv *alba*.

	Moisture (%)	Total ash (%)	Total fat (%)	Protein (%)	Crud fibre (%)	Carbohydrates (%)
Water Lily	11.55 $\pm$ 0.75	0.67 $\pm$ 0.09	1.8 $\pm$ 0.21	7.5 $\pm$ 0.37	2.9 $\pm$ 0.08	74.5 $\pm$ 0.46

Results were expressed as the mean of four measurements SD.

Table 2: UHPLC validation data for amino acid content in *Nymphaea stellata* cv. *alba*.

Name	R.T (min)	Area	Concentration (mg/g) mg (mg/g)
Aspartic Acid	3.317	940213	0.293
Glutamic Acid	4.355	1919979	0.701
Serine	8.73	1369209	0.278
Histidine	10.78	81227	0.116
Glycine	11.93	1123556	0.140
Threonine	12.314	702935	0.149
Arginine	13.643	1413529	0.429
Alanine	15.989	1402644	0.239
Tyrosine	19.795	276868	0.096
Cystine	26.306	992475	0.424
Valine	26.93	264645	0.050
Methionine	31.355	537623	0.135
Phenylalanine	32.012	723463	0.170
Isoleucine	34.139	1653681	0.339
Leucine	35.509	127903	0.074
Lysine	37.387	ND	ND
Proline	39.256	ND	ND

Note: ND- Not Detected.

the mobile phase B was Aceto-nitrile. The time gradient was, 0.01, 45.0, 60.0, 65.0, 70.0, 75.0 and 80.0 minutes (stop). Post run time was 5.0 minutes (Tsao and Yang, 2003). Before sample loading we run two standards separately with above mention column condition.

Statistical analysis

The analysis of variance (ANOVA) was employed to compare means between genotypes/samples through the Fisher protected LSD with a significance level of 0.05%. Results were expressed as the mean of four (sample) measurements (replications) $\pm$ SD. The linearity was established using three concentrations of amino acid standards. The data of peak area vs. amino acid concentration were treated by linear least squares regression analysis. The linearity data obtained should obey the equation:

$$y = bx + a$$

Where a is zero within the 95% confidence limits and the coefficient of determination (R^2) is greater than 0.984 (Armagan *et al.*, 2013 and Perucho *et al.*, 2015). Another linearity standard curves were created for two flavonoids.

RESULTS AND DISCUSSION

Proximate composition

Data pertaining to Table 1 revealed that *Nymphaea* seed endosperm contained $7.5 \pm 0.37\%$ protein, $2.9 \pm 0.08\%$ crude fiber, $1.8 \pm 0.21\%$ total fat and $74.5 \pm 0.46\%$ carbohydrates. Similar results were also obtained by Gopalan *et al.* (1998) and Jana *et al.* (2021). According to Gopalan *et al.* (1996) water lily seeds contain 10.0% moisture 8.3% protein 1.0% fat 0.9-minerals 4.2% fibre and carbohydrate 75.6%. Jana *et al.* (2021) also reported that water lily nut contains $10.19 \pm 0.92\%$ moisture, $8.0 \pm 0.04\%$ protein and $74.9 \pm 0.87\%$ carbohydrates. The appreciable amount of protein and crude fibre and low amount of fat is appropriate for good health.

Table 3: UHPLC validation data for flavonoid content in *Nymphaea stellata* cv *alba*.

Name	RT (min)	Area	Results (mg/kg)
Quercetin	64.50	1565.0	0.340
Kaempferol	67.90	895.0	0.127

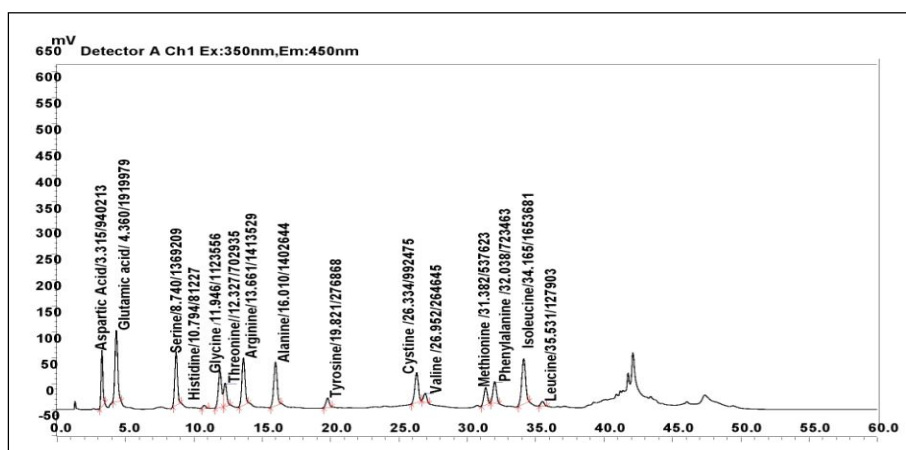


Fig 1: UHPLC chromatogram of amino of water lily seed endosperm.

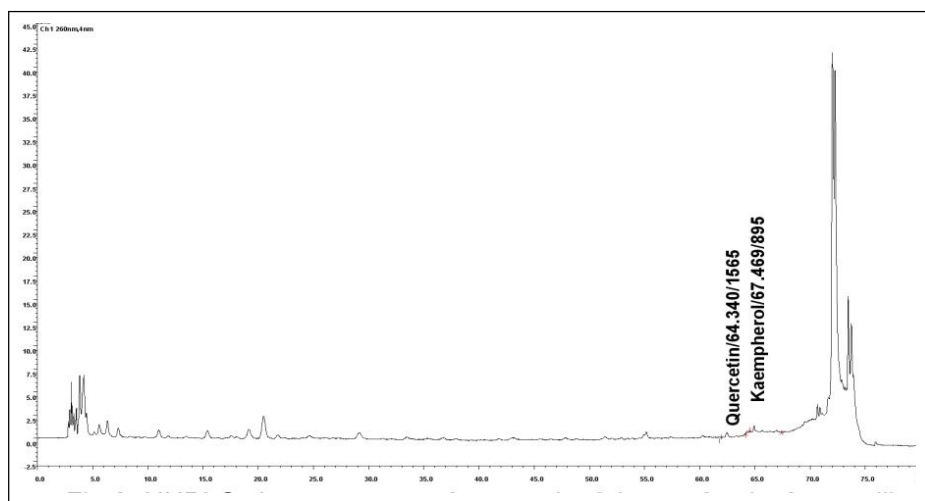


Fig 2: UHPLC chromatogram of quercetin and kaempferol of water lily seed endosperm.

Amino acids

The results shown in the *Nymphaea stellata* cv. *alba* produced the maximum amount of amino acids, aspartic acid (0.293 mg/gm), glutamic Acid (0.701 mg/gm), serine (0.278 mg/gm), histidine (0.116 mg/gm), glycine (0.140 mg/gm), threonine (0.149 mg/gm), arginine (0.429 mg/gm), alanine (0.239 mg/gm), tyrosine (0.096 mg/gm), cystine (0.424 mg/gm), valine (0.50 mg/gm), methionine (0.135 mg/gm), phenylalanine (0.170 mg/gm), isoleucine (0.339 mg/gm) and leucine (0.34 mg/gm) (Table 2). Similar type of results regarding different amino acids presence *i.e.* composition in makhana was confirmed by Jana and Idris (2018). This is the first ever amino acid profile developed in *Nymphaea* seed endosperm (Fig 1). Research results from different experiments have shown that arginine can reduce insulin resistance, That is, insulin sensitivity can be increased (Krisna Mohan and Das, 1998). The amino acid arginine is of great importance for the sensitivity of body cells to insulin. Arginine is a precursor of nitrogen oxide, a transmissible substance that has a direct effect on insulin sensitivity. L-arginine improves peripheral and hepatic insulin sensitivity in type 2 diabetic patients (Piatti *et al.*, 2001). In addition, arginine significantly expands blood vessels and reduces BP.

Flavonoids

The flavonoid, quercetin, is a polyphenolic compound and the most abundant flavonoid in edible vegetables, fruit, wine and nuts. In the present experiment, flavonoids quercetin and kaempferol were estimated by using UHPLC chromatography. In the present experiment, the retention time for quercetin and kaempferol was 64.5 and 67.9 min., respectively. Furthermore, the peak area was 1565 units and 895 units, respectively. The presence of quercetin 0.340 mg/kg and kaempferol 0.127 mg/kg as pure form (Table 3) without other derivatives of the two flavonoids was revealed by a UHPLC chromatogram of *Nymphaea* seed endosperm. Other glucoside compounds and esters may be present in the seed endosperm as many other peaks were visible in Fig 2. This research is first of its kind. These are pure flavonoids. The seeds may contain their other derivatives like quercetin and kaempferol glucosides and glucuronides, esters compounds. With the help of modification of GLUT4, flavonoid has been proven to promote insulin uptake Cho *et al.* (2010). According to recent research, flavonoid may exhibit anti-hyperglycemic activity by inhibiting carbohydrate hydrolyzing enzymes such as amylase, glucosidase and disaccharidases (Tadera *et al.*, 2006 and Pareira *et al.*, 2011). From this study, we confirmed first time that *nymphaea* seed endosperm contains anti-diabetic flavonoids like quercetin and kaempferol in appreciable amount. The confirmation of the results corroborated by Zhu *et al.*, (2017) as another aquatic nut *i.e.* lotus nut that had similar flavonoids. *Nymphaea* petal contain significant amount of quercetin and kaempferol compounds (Jambor and Skrzypczak 1991) by previous study. In general, kaempferol can potentially ameliorate glycoprotein abnormalities related to the risk of diabetes mellitus (Fang

et al., 2008, Lee *et al.*, 2010). These two flavonoids increase insulin sensitivity and act as free radical scavengers and the amino acids in seeds are a good health indicator.

CONCLUSION

Thus, the *Nymphaea* endosperm is very nutritive food and useful for diabetic patient due to having flavonoids like quercetin and kaempferol along with high value amino acids Arginine, Histidine, Isoleucine and leucine. This research is first of its kind and may open a new window for further research.

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Conflict of statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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