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Evaluation of antioxidant activity of *Stuckenia pectinata* L.

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The aim of this study was to determine the antioxidant activity of *Stuckenia pectinata* L. Dried leaves and stems of *Stuckenia pectinata* L. were extracted with 60% methanol, 40% ethanol, and water. Total phenolic content, total flavonoid content, and antioxidant activity were evaluated using CUPRAC, H_2O_2, ABTS, FRAP and DPPH methods. The relative antioxidant capacity index (RACI) was applied for a more comprehensive comparison. The phenolic and flavonoid contents were found to depend on the extraction solvent. Both aqueous and 40 % ethanolic extracts had lower levels of phenolic acids and flavonoids compared to the 60 % methanolic extracts. The 40% ethanolic extracts showed the highest antioxidant activity by CUPRAC, H_2O_2, ABTS, and FRAP methods. The highest activity was recorded in CUPRAC assay, followed by ABTS assay and H_2O_2 assay. The DPPH method showed very low activity. No significant differences were observed between leaf and stem extracts. *Stuckenia pectinata* as a biosorbent for the removal of inorganic pollutants from aquatic environments significantly increased the reduction of nitrogen compounds concentration (85.71% reduction of N-NO_2 and 83.87% of NO_2) when adding leaves of the plant. This confirms the applicability of the plant to be used as a natural adsorbent.

Keywords: Aquatic ecosystem, *Stuckenia pectinata* L., total phenolic content, total flavonoid content, antioxidant activity

INTRODUCTION

Reactive oxygen species (ROS) is a collective term used for oxygen-containing free radicals, depending on their reactivity and oxidizing ability [1]. ROS are involved in many physiological processes. As a result, there is a need to explore substances with free radical scavenging and antioxidant activity [2]. Antioxidants are employed to protect biomolecules from the damaging effects of ROS [1]. At present, there are keen interests and wide-spread research on exogenous antioxidants from natural sources, perhaps, due to the fact that they are less expensive, readily available and believed to have lesser side effects when compared to their synthetic counterparts [2]. Phenolic acids and flavonoids are polyphenolic compounds that may have beneficial health effects because of their antioxidant properties and their inhibitory role in various stages of tumor development in animal studies [3]. There are various methods for determining antioxidant activity, which are generally classified into 2 assays based on their mode of action mechanisms, namely electron transfer mechanism (ET) and hydrogen atom transfer mechanism (HAT) assays [4, 5]. In addition to their role in environmental remediation, aquatic macrophytes have also attracted interest for their potential as sources of bioactive compounds with antioxidant properties. Aquatic plants have been the subject of considerable research in phytoremediation as they have demonstrated the ability to remove a wide range of contaminants in water bodies [6]. Furthermore, the abundance of macrophyte species and the beneficial substances they possess have been widely documented and effectively applied as raw materials for biofertilizer, animal feed or therapeutic agents [7]. *Stuckenia pectinata* L., a synonym of *Potamogeton pectinatus* L., is a submersed perennial macrophyte also called sago pond grass, fennel and ribbon grass [8]. The species is cosmopolitan and is a food source for many species of waterfowl [9], as well as shelter for amphibians, reptiles, fish, and mammals [10], but can also create problems in irrigation canals and recreational areas [11,12]. *Stuckenia pectinata* L., possesses a variety of beneficial physiological characteristics - its ability to grow in oligotrophic to eutrophic waters, and its ability to improve water quality by removing nutrients and heavy metals, makes it an important tool for the purification of aquatic ecosystems [13, 14]. In a study related to the accumulation of As, Pb, Zn by different macrophyte species, *Stuckenia pectinata* L., demonstrated the easiest accumulation of these elements from the river substrate, whereby the authors proved that the species can help to restore areas exposed to metal and metalloid pollution [15]. Macrophyte possesses natural antioxidant properties, and is suitable for use as an additive in the food and pharmaceutical industries.

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Furthermore, phytochemical screening of this species indicates the presence of important medicinal and active substances such as flavonoids, saponins, alkaloids, terpenes and glycosides in *Stuckenia pectinata* L. which can be used as natural preservative in the processing of food raw materials [16].

The aim of this study was to determine the total phenolic content, total flavonoid content and antioxidant activity of *Stuckenia pectinata* L. collected from Burgas Lake, Bulgaria. Also, a preliminary study of macrophyte adsorption was conducted to reduce the concentration of various pollutants in water samples from the lake. The species *Stuckenia pectinata* L. was selected due to its high tolerance to pollutants and adverse environmental conditions, as well as the content of potentially useful bioactive compounds of high value.

MATERIALS AND METHODS

Sampling of Stuckenia pectinata L.

Samples of *Stuckenia pectinata* L. were collected in May-October 2024 from three points in Burgas Lake (1. Burgas Lake - east N 42.483257 E 27.438163; 2. Burgas Lake - west N 42.493123 E 27.344237; 3. Burgas Lake - center N 42.51058 E 27.367608). The collected samples were pre-rinsed several times with tap water to remove any dirt, sand particles and epiphyton. The samples were left on filter paper and dried at 25°C in the dark for 15 days [16]. The dried plant material was divided into parts, leaves and stems, and then cut into different fractions in the range of 1-5 mm for the adsorption experiments. The water samples from Burgas Lake were pre-filtered through 0.45 µm syringe filters to eliminate solid particles and ensure sample homogeneity [17]. The adsorption experiment was carried out in 250 ml Erlenmeyer flasks, the adsorbent (leaves and stems of *Stuckenia pectinata*) was prepared in an amount of 0.25 mg per 125 ml of water sample and was added to water-permeable bags measuring 4.5 × 6 cm and about 20-40 µm thick [18, 19], which were immersed in the water samples. The adsorption process was carried out at an optimal contact time of 120 min, based on previous studies showing that during this period the adsorption reached significant values without any signs of saturation of the adsorbent [20]. The samples were analyzed before and after adsorption for selected physicochemical parameters. For the experiments on the antioxidant activity of macrophytes, the plant material was ground in a grinder to obtain a uniform powder, which was passed through a 2 mm sieve [21, 22]. After that, the powdered dried leaves and stems of *Stuckenia pectinata* L. were extracted using 60% methanol, 40% ethanol, and distilled water under the following extraction conditions: 0.2 g of *Stuckenia pectinata* L. powder was mixed with 10 mL of solvent. The extraction was carried out for 30 min at room temperature on an orbital mechanical shaker at 60 revolutions per minute (rpm). The extracts (60 % methanolic, 40 % ethanolic and aqueous) were filtered by a 0.45 µm syringe filter and were used for further analysis – determination of total phenolic content, total flavonoid content and antioxidant activity by DPPH-assay, ABTS-assay, H_2O_2-assay, FRAP-assay and CUPRAC-assay.

Reagents and instruments

All chemicals were of analytical grade quality and were purchased from Honeywell and Sigma Aldrich (USA). The main physicochemical parameters (pH, dissolved oxygen, temperature, salinity, TDS (total dissolved solids), conductivity), were measured with a WTW Multi 3630 IDS portable multimeter, the indicators N-NO_2 - nitrite nitrogen, NO_2 - nitrite, were measured with ready-to-use Hach® LCK cuvette tests with DR 3900 spectrophotometer (Hach). Assays for total phenolic content, total flavonoid content and antioxidant activity were performed with spectrophotometer Spectroquant Pharo 300, UV/Vis (Merck, USA).

Determination of total phenolic content (TPC) and total flavonoid content (TFC)

The TPC of the extracts was assessed using the Folin-Ciocalteu method (FC method). Absorbance was measured at 765 nm. The concentration of the phenolic compounds in the extracts was calculated using gallic acid as standard with linear regression of gallic acid calibration curve: y=0.0349 +0.10095x, with r^2=0.9892. The results were expressed as mg gallic acid equivalents per g extract (mg GAE/g) TFC was assessed using the method described by Kirkova *et al.* [23]. Absorbance was measured at 440 nm against the blank – methanol. The concentration of the flavonoids in the extracts was calculated using rutin as a standard with linear regression of calibration curve: y=0.0722+0.02564x and r^2=0.9993. The results were expressed as mg rutin equivalents per g extract (mg RE/g) [23].

DPPH assay

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging capabilities of the extracts were evaluated using the free radical method described by Kirkova *et al*. [23]. The absorbance was measured at 515 nm against methanol as a blank [23].

ABTS assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) scavenging activities of the extracts were evaluated according to the original method of Re *et al.*, 1999 with slight modifications [25]. The absorbance at 734 nm was measured for each sample relative to methanol [25].

H_2O_2 assay

The hydrogen peroxide scavenging activity (HPSA) of the extracts was determined using 0.2 M phosphate buffer (PB, pH = 7.4) and H_2O_2 (2 mM dissolved in PB). A reaction mixture containing 0.1 mL of plant extract, 0.6 mL of H_2O_2 and 3.3 mL of PB was prepared in a test tube. After incubation in the dark for 10 min, the absorbance was measured at 230 nm for each sample [24].

FRAP assay

The ferric reducing antioxidant power (FRAP) of the extracts was measured using the method of Benzie with a slight modification reported by Docheva *et al.*, 2020 [26, 27]. Absorbance was measured at 593 nm against a blank sample which consisted of 0.05 ml of the respective extraction solvent and 0.15 ml of distilled water mixed with 1.5 ml of FRAP reagent.

CUPRAC assay

The cupric ions (Cu^{2+}) reducing power of the extracts was determined using method described by Apak *et al*., 2006 [28] with slight modifications. The absorbance was measured at 450 nm after incubation in the dark for 30 min [23, 27].

The antioxidant activity was determined using Trolox as a standard, and the results were expressed as mM TE/g. The linear regression equations for the Trolox standard curve were as follows:

CUPRAC y=0.0349+0.10095x, with r^2=0.9892,
H_2O_2 y=0.0349+0.10095x, with r^2=0.9892,
ABTS y=0.0349+0.10095x, with r^2=0.9892,
FRAP y=0.0349+0.10095x, with r^2=0.9892,
DPPH y=0.0349 +0.10095x, with r^2=0.9892.

Relative antioxidant capacity index (RACI)

RACI was calculated by assigning equal weights to all applied assays, according to Petrovic *et al.*, 2016 [28]:

$$RACI=(sum-ave)/stdv, \quad (1)$$

where: *sum* - sum of all applied assays (CUPRAC, FRAP, H_2O_2, ABTS); *ave* - average of all applied assays (CUPRAC, FRAP, H_2O_2, ABTS); *stdv* – standard deviation of all applied assays (CUPRAC, FRAP, H_2O_2, ABTS).

Statistical analysis and quantitative calculations

All observations were taken in triplicate and the data were expressed in mean ± standard deviation. One-way ANOVA and Duncan`s test were employed to identify differences in the means of each group. Statistics were analyzed using SPSS Statistics (version 25, IBM Corp.) and significance was declared at $p<0.05$. Correlation was performed using MS Excel correlation data analysis. Calculation of percent removal of nitrate ($N\text{-}NO_3$, NO_3), ammonium ($N\text{-}NH_4$, NH_4) and nitrite ($N\text{-}NO_2$, NO_2) from water samples was performed in agreement with formula 2 according to [29].

$$\%\ Removal = \frac{(C_i - C_e)}{C_i} \times 100 \quad (2)$$

where C_i and C_e are the initial and final concentrations of the contaminants in the sample.

RESULTS AND DISCUSSION

- *Total phenolic and total flavonoid content.*

Plants produce diverse phytochemicals such as lignans, tannins, phenolic acids, stilbenes, and flavonoids at different concentrations in leaves, stem, flowers, fruits and roots [30, 31]. The yield of extracted phenolic acids and flavonoids depends on the solubility of the compounds in the extraction solvents [30]. High solubility of phenolic acids and flavonoids in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents [32]. In this study 60 % methanolic, 40 % ethanolic and aqueous extracts of *Stuckenia pectinata* L. leaves and stems were prepared to examine the TPC, TFC and antioxidant activity (Table 1). The highest TPC was measured in 60 % methanolic extracts of *Stuckenia pectinata* L. (18.1±0.6 mg GAE/g – leaves extract and 12.4±0.4 mg GAE/g – stems extracts), followed by 40 % ethanolic extracts – 13.9±0.4 mg GAE/g – leaves extract and 13.0±0.4 mg GAE/g – stems extracts. The lowest TPC was detected in aqueous extracts of leaves and stems - 9.7±0.3 mg GAE/g and 9.9±0.3 mg GAE/g, respectively – Table 1.

To date, no studies have been found in the scientific literature regarding the content of phenolic acids, flavonoids, and antioxidant activity of extracts from *Stuckenia pectinata* L. (also known as *Potamogeton pectinatus* L.).

Table 1. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of *Stuckenia pectinata* L. extracts

Stuckenia pectinata L.	TPC*, mg GAE/g	TFC*, mg RE/g	Antioxidant activity**, mM TE/g				
			CUPRAC	FRAP	H_2O_2	ABTS	DPPH
60% Methanol							
Leaves	18.1±0.6	9.1±0.2	403.4±28.1^{d}	95.0±3.8^{ab}	135.0±9.9^{cd}	118.9±8.7^{b}	2.3±0.03^{a}
Stems	12.4±0.4	4.3±0.1	405.1±28.1^{d}	93.0±3.8^{a}	88.4±6.5^{a}	115.1±8.7^{b}	2.6±0.03^{a}
40% Ethanol							
Leaves	13.9±0.4	10.6±0.3	548.1±38.2^{f}	105.9±4.1^{ab}	135.6±9.9^{cd}	134.2±9.8^{cd}	2.2±0.03^{a}
Stems	13.0±0.4	5.5±0.1	546.4±38.2^{f}	90.6±3.8^{ab}	112.4±8.3^{c}	124.9±9.1^{c}	2.4±0.03^{a}
Distilled water							
Leaves	9.7±0.3	10.2±0.3	187.7±13.0^{cd}	58.3±2.4^{a}	118.9±8.35^{b}	96.1±6.9^{ab}	n.d.
Stems	9.9±0.3	7.9±0.2	254.0±15.3^{cd}	47.5±2.1^{a}	103.4±8.2^{b}	87.9±6.7^{a}	n.d.

*Each value is a mean ± SD (n = 3). **Values in the same column followed by a different letter (a-f) are significantly different ($p< 0.05$). n.d. - not determined.

Haroon and Daboor (2009) [33] investigated the TPC and TFC in the Egyptian macrophyte species *Potamogeton nodosus*. They found the highest TPC in the methanol extract (19.313 mg GA/g) while the lowest content was observed in the chloroform extract (2.271 mg GA/g). The TFC in the methanol extract was up to 17.885 mg RU/g. In addition, Jelena *et al.* (2014) found the highest values of TPC and TFC in ethyl acetate extract (28.45 mg GA/g and 102.09 mg RU/g respectively) and the lowest in ethanol extract [33, 34]. Some of these results were higher than those, observed in our study. This could be attributed to various factors, such as plant growth form, location where the plant was growing, time of samples collection, environmental conditions in the sampling area. Results of the present study showed that among all extracts, the aqueous methanol and aqueous ethanol extracts had the highest TPC. This may be due to the fact that phenolics are often extracted in higher amounts in polar solvents such as aqueous methanol/ethanol [35]. Amounts of TFC in all leaves extracts were higher compared to the stem extracts, regardless of the solvent used. The TFC in leaves varied between 9.1±0.2 mg RE/g (60 % methanol) and 10.6±0.3 mg RE/g (40 % ethanol), while in steams - 4.3±0.1 RE/g (60 % methanol) and 7.9±0.2 mg RE/g (water). The differences in the extract yields of the tested materials in the present analysis might be ascribed to the different availability of extractable components, resulting from the varied chemical composition of plants [35].

- *Antioxidant activity (AOA):* The antioxidant activity of *Stuckenia pectinata* L. extracts was evaluated using five methods employing different mechanisms of action: H_2O_2, ABTS, and DPPH assays employ a hydrogen atom transfer mechanism (HAT), while CUPRAC, FRAP assays utilize an electron transfer mechanism (ET). These methods were selected, because they are rapid, robust, and accurate for systematical assessing of the total antioxidant capacity of extracts from *Stuckenia pectinata* L. on a large scale [36]. The combination of these methods allows a more complete characterization of the antioxidant properties of the *Stuckenia pectinata* L. extracts due to the different mechanisms involved in radicals - $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and H_2O_2, and the ability of these extracts to reduce Fe^{+3} to Fe^{+2} (FRAP method) and Cu^{+2} to Cu^{+} (CUPRAC method). A post-hoc statistical test (Duncan`s test) was applied to assess the differences between the mean values of each extract in the group and to determine their statistical similarity [37, 38]. The antioxidant activity of *Stuckenia pectinata* L. extract including 60 % methanolic, 40 % ethanolic and distilled water is presented in Table 1. It was found that the extracts exhibited a greater ability to reduce Cu^{+2} to Cu^{+} than Fe^{+3} to Fe^{+2}. The 40% ethanolic extract showed the highest antioxidant activity across both methods, followed by the 60% methanolic extract. Distilled water extracts of *Stuckenia pectinata* L. exhibited the lowest antioxidant activity (Table 1). The antioxidant activity of the extracts of *Stuckenia pectinata* L. determined by H_2O_2 assay ranged between 88.4±6.5 mM TE/g for the 60 % methanolic extract (from steam) to 135.6±9.9 mM TE/g for the 40 % ethanolic

extract (from leaves). The antioxidant activity measured by the ABTS assay was similar, ranging from 87.9±6.7 mM TE/g (water extract from leaves) to 134.2±9.8 mM TE/g (40% ethanolic extract from leaves). The extracts of *Stuckenia pectinata* L. showed no significant activity against DPPH assay – Table 1.

- *Relative antioxidant capacity index (RACI) and phenolic antioxidant coefficients (PAC):* Relative antioxidant capacity index (RACI), calculated by assigning equal weight to all applied assays, was used to achieve more comprehensive comparison between analyzed samples, as well as applied assays [27, 39]. Due to the low values of the DPPH analysis, it is not included in the RACI calculations. The highest values of RACI were ascribed to the distilled water extract of *Stuckenia pectinata* L. leaves – 6.35 and *Stuckenia pectinata* L. stems - 4.09, which is associated with the smaller standard deviation in the values for antioxidant activity obtained by the different methods (Figure 1). The 60% methanol extracts had RACI of 3.90 (leaves) and 3.42 (stems). Despite the highest antioxidant activity of the 40% ethanol extracts obtained by the different methods, the RACI values were the lowest, which can be explained by the large standard deviation obtained for the values of the individual methods.

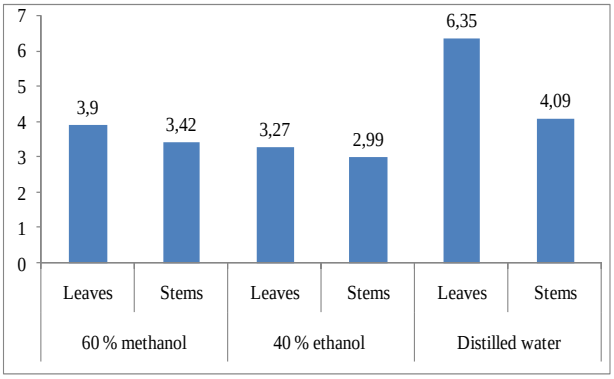

Fig. 1. RACI values

The *Stuckenia pectinata* L. extracts have higher RACI values compared to germander (1.57 and 1.33), sage (0.85), mentha (0.73), and mountain germander (0.77) [30].

- *Correlation between TPC / AOA and TFC / AOA:* The correlations between TPC / AOA and TFC / AOA of *Stuckenia pectinata* L. extracts are presented in Table 2. It is noteworthy that there is a higher correlation between TPC and AOA compared to TFC and AOA. The highest correlation was reported between TPC and FRAP assay (R^2=0.554) and TPC and ABTS assay (R^2=0.447). There were weak correlations between TPC and CUPRAC assay and TPC and H_2O_2 assay - R^2 = 0.321 and R^2 = 0.320, respectively. The best correlation was established between TFC and H_2O_2 assay - R^2 = 0.657. No correlation between TFC and CUPRAC assay, TFC and FRAP assay and TFC and ABTS assay was observed – Table 2.

Table 2. Linear regression between TPC and AOA and TFC and AOA by CUPRAC assay, FRAP assay, H_2O_2 assay and ABTS assay of extracts of *Stuckenia pectinata* L.

	Methods of antioxidant activity determination			
	CUPRAC	FRAP	H_2O_2	ABTS
TPC	0.321	0.554	0.320	0.447
TFC	0.057	0.013	0.657	0.020

- *Adsorption study with Stuckenia pectinata L.:* Before and after adsorption, the main physicochemical parameters of the samples were measured with leaves and stems of *Stuckenia pectinata* L. as follows: pH from 8.1 to 7.82 (leaves) and 7.98 (stems); Salinity, ‰ from 8.5 to 8.7 (leaves) and 8.6 (stems); Conductivity, µS/cm from 4670 to 4900 (leaves) and 4740 (stems). The leaves of *Stuckenia pectinata* L. as adsorbent offered the most significant percent removal of N-NO_2 mg/l and NO_2 mg/l 85.71% and 83.87%, respectively, and with stems 28.57% and 30.11%, respectively.

CONCLUSION

This is the very first study of *Stuckenia pectinata* L. extracts from leaves and stems, collected in Burgas Lake, Bulgaria. It may be concluded that *Stuckenia pectinata* L. is a rich source of phenolic acids (TPC) and flavonoids (TFC). The AOA of *Stuckenia pectinata* L. extracts decreased in the following order: CUPRAC assay, ABTS-assay, H_2O_2 assay, FRAP assay and DPPH assay. It is found that 40 % ethanolic extracts had the highest antioxidant activity, followed by 60 % methanolic extracts and aqueous extracts. No difference in the total phenolic content, total flavonoid content and antioxidant activity between leaf extracts and stem extracts of *Stuckenia pectinata* L. was observed. There were higher correlations between TPC and AOA compared to TFC and AOA. The aquatic macrophyte *Stuckenia pectinata* L. was used in this preliminary adsorption study. Satisfactory results were obtained in static adsorption with macrophyte leaves and the reduction of N-NO_2 mg/l and NO_2 mg/l was 85.71% and 83.87% respectively.

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