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CONTENT OF PHENOLIC ACIDS AND TOTAL PHENOLICS IN SEVERAL AQUATIC PLANT SPECIES

L. DJURDJEVIĆ

Institute for Biological Research "Siniša Stanković",
11060 Belgrade, Yugoslavia

Abstract - According to the content of free phenolic compounds, the five stagnant water plant species examined in the present work can be arranged in the following order: *Nymphea alba>Lemna minor>Salvinia natans>Trapa natans>Ceratophyllum demersum.* These plants contained more of free *p*-coumaric, ferulic, vanillic and syringic acid comparing to bound forms (ester linked) of these compounds. In addition to these phenolic acids, in the bog sediment *p*-hydroxyenzoic acid was detected mainly in its bound form. Dissolved phenolics and vanillic acid were identified in the bog water, as well.

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INTRODUCTION

Within the scope of allelopathic investigations performed so far, much attention has been paid to interrelationship of terrestrial plant species. Phenolic compounds identified as the most frequently found allelochemical products were shown to act inhibiting numerous physiological processes leading to a suppression of some plant species within a phytocoenosis (Whittaker 1970; Chou and Muller 1972; Rice 1974; Lodhi 1976, 1978; Colton and Einhellig 1980; Li *et al.* 1992). However, similar data on aquatic plants of stagnant waters are rather scarce in the available literature. It has been reported earlier that some aquatic plants in Yugoslavia are conspicuously dominant forming submerged meadows which cover rather large areas (Micevski 1962, 1963, 1969; Rauš *et al.* 1978). Due to their submerged and emersed vegetation, bogs represent suitable sites for alleopathic examinations. Since aquatic plants are either completely or partially submerged allelochemicals must be trasmitted from donor (emitting) plant to acceptor (affected) plant through either their rhizosphere soil or water. In the present work content of phenolic acids (PAs) and total phenolics (TP) in five aquatic plant species, sediment and water samples of a bog ecosystem was determined.

MATERIAL AND METHODS

Plant species

The bog examined throughout the present work is situated along the Danube in the vicinity of Belgrade (opposite shore of Višnjička banja locality). It is inhabited by several emersed (*Lemna minor, Nymphaea alba, Salvinia natans* and *Trapa natans)* and one submerged (*Ceratophyllum demersum*) plant species. Phenolic compounds were determined either in whole plants (*Lemna minor, Salvinia natans* and *Ceratophyllum demersum*) or in the leaves (*Nymphea alba* and *Trapa natans*). The samples (500 *g* each) were dried, pulverized and sieved (mesh 35).

Analysis of phenolic compounds

Phenolic acids and total phenolic compounds were extracted from 2 *g* of dry plant material with 80% boiling ethanol solution followed by ethyl acetate (3 x 30 mL, 8 *h*). Pooled ethanol and ethyl actetate extracts were evaporated to the aqueous phase. After that, pH was adjusted to 2.0 with 2 N HCl, free phenolics transferred to ethyl acetate and evaporated to dryness. Dry residue was dissolved in 4 mL of 80% ethanol solution and maintained at -20 °C till the analysis. For the extraction of bound phenolics (esterified forms) dry plant material remained after the extraction of free phenolics was boiled in 2 N HCl during 60 min and the extract was transferred into ethyl acetate. The mixture was evaporated and the dry residue dissolved in 4 mL of 80% ethanol was kept at -20 °C before the analysis (Mijdla *et al.* 1975).

Aliquots of the bog water (3.0 L) were evaporated to the volume of 50 mL. After 60 min of hydrolysis in 2N HCl total phenolics and phenolic acids were transferred into ethyl acetate and the samples were evaporated to dryness. Dry residue was dissolved in 4 mL of 80% ethanol solution and kept as above.

Samples of the sediment (2 *kg*) were collected at littoral, mid- and the deepest parts of the bog and dried. Free phenolics were extracted from 30 *g* of dry sediment with boiling ethyl acetate (30 x 50 mL) for 24 *h*. For the extraction of bound phenolics (esterified forms) the residues remaining upon the first extraction were resuspended in 15 mL of 2 N NaOH and boiled for 24 *h*. After that, pH of the aqueous phase was adjusted to 2.0 with concentrated HCl, the mixture was transferred to ethyl acetate and evaporated. Dry residues were dissolved in 4 mL of 80% ethanol and maintained at -20 °C until the analysis (Hennequin and Juste 1967; Katase 1981a,b).

Identification and quantification of phenolic compounds

Content of total phenolics (free and bound) was determined spectrophotometrically using Folin-Ciocalteu reagent (Feldman and Hanks 1968). Ferulic acid was employed as a reference. For both qualitative and quantitative analyses, ascendent two-dimensional paper chromatography was applied using the mixture of isopropyl alcohol, ethyl acetate, NH_4OH and H_2O (30:50:1:19) and 2% aqueous (v/v) acetic acid as developers for the first and the second dimension, respectively. Dried chromatograms were sprayed with *p*-nitroaniline and 20% Na_2CO_3. The spots of phenolic acids were eluted with 45% ethanol solution and absorbancies were measured at the corresponding wavelenghts at a Shimadzu UV 160 spectrophotometer (Mijdla *et al.* 1975).This method provides a satisfactory separation of five PA, two of which represent cinnamic acid derivatives (ferulic acid-4-hydroxy-3-methoxycinnamic and *p*-coumaric acid-*trans*-4-hydroxycinnamic acid) and three represent benzoic acid derivatives (*p*-hydroxybenzoic acid, vanillic acid-4-hydroxy-3-methoxy-benzoic acid and syringic-3,5-dimethoxy-4-hydroxybenzoic acid). All analyses were done in triplicates in three independent experiments.

RESULTS AND DISCUSSION

Four of the five examined species are angiosperms and only one, *Salvinia natans*, belongs to pteridophytes. Content of total free phenolic compounds exceeded that of their bound forms with the exception of *Trapa natans* where an opposite situation was recorded. Based on the content of total free phenolics the above aquatic plant species can be arranged in the following order: *Nymphea alba* > *Lemna minor* > *Salvinia natans* > *Trapa natans* > *Ceratophyllum demersum* (Table 1). Ratio of free and bound phenolic compound forms was the highest in *Lemna minor* (4.42) and the lowest in *Trapa natans* (0.66).

Table 1. Content of total phenolics and phenolic acids in several aquatic plants

		Salvinia natans	*Trapa natans*	*Lemna minor*	*Ceratophyllum demersum*	*Nymphaea alba*
Total Phenolics (*mg/g*)	free	12.50	9.77	15.18	3.80	16.86
	±	1.05	0.94	1.18	0.24	0.97
	bound	4.65	14.69	3.43	1.34	9.60
	±	0.19	0.27	0.07	0.10	0.95
	total	17.15	24.46	18.61	5.14	26.46
PAs (μg/g)						
p-coumaric	free	-	201.01	93.27	84.10	87.31
	±	-	19.49	9.78	7.45	8.91
	bound	-	45.32	-	-	-
	±	-	3.87	-	-	-
Ferulic	free	-	194.32	155.92	238.52	113.27
	±	-	20.91	17.06	21.44	10.97
	bound	-	-	-	-	-
	±	-	-	-	-	-
p-hydroxy-benzoic	free	-	-	tr.	-	-
	±	-	-	-	-	-
	bound	-	-	24.71	-	-
	±	-	-	3.02	-	-
Vanillic	free	73.63	92.98	76.39	50.76	94.37
	±	6.42	9.07	8.65	4.62	8.97
	bound	15.44	23.70	24.37	15.16	24.02
	±	1.42	1.98	2.07	1.91	2.07
Syringic	free	-	154.76	98.33	253.69	-
	±	-	16.82	10.36	20.91	-
	bound	-	33.24	-	32.67	-
	±	-	2.74	-	2.98	-
Total	free + bound	89.07	745.33	472.99	674.90	318.97

A pteridophyte *Salvinia natans* contained only vanillic acid, predominantly as a free form. In the remaining species free forms of *p*-coumaric, ferulic, vanillic and syringic acid were dominant. Ferullic acid represents a dominant phenolic compound making 30-38% of total phenolic acids in all plant species examined, with the exception of *S. natans*. Only bound form of vanillic acid was common to all five plant species. In addition to the above phenolic acids, *Lemna minor* was found to contain *p*-hydroxybenzoic acid which was not detected in other plants examined.

According to the data reported so far, different phenolic compounds were identified in aquatic plants, *e.g.* tellimagrandin II in *Myriophyllum spicatum* (Gross *et al.* 1996), gallotannin and two ellagitannins in the root of *Nuphar variegatum* (Nishizawa *et al.* 1990), orientin, vitexin, apigenin and luteolin in the chloroplasts of *Spirodela intermedia* and *S. polyrhiza*, Lemnaceae (Saunders and McClure 1976). In the leaves of a marine phanerogam *Posidonia oceanica* *p*-coumaric, ferulic, *p*-hydroxybenzoic, cinnamic, sinapic and *p*-anisinic acid were recorded together with some other phenolic compounds (Cuny *et al.* 1994, 1995), ferulic acid being the most abundant among phenolic acids. An intestine content of some cyprinid fishes composed of microalgae, contained 71.98-105.06 μg/g of free and 15.88-32.26 μg/g of bound phenolics. Phenolic acids found were: *p*-hydroxybenzoic and vanillic acid in free and bound form (Djurdjević *et al*. 1997).

Littoral samples of the bog sediment contained several fold lower amounts of total free phenolics in comparison with those taken from the mid- and the deepest parts of the bog (Table 2). Content of bound forms of the phenolic compounds was several fold higher than that of free forms. Bound forms were unevenly distributed in the bog sediment, littoral and mid-part samples containing only vanillic acid while in those from the deepest parts of the bog *p*-coumaric, ferulic, vanillic and syringic acid were detected.

Phenolic acids were identified in different types of soil. Soil from different forest communities was found to contain several phenolic acids in a concentration range from 0.09-1,100 μg/g (Lodhi 1975; Kuiters and Denneman 1987). Peat bog soil is characterized by significantly higher levels of phenolic acids, bound forms being predominant (Katase 1981a,b; Katase and Kondo 1984). Katase (1983) found in a pond sediment *p*-coumaric, ferulic, *p*-hydroxybenzoic and vanillic acid both as free and bound forms in concentrations similar to those observed in the present work. Levels of phenolic compounds in bog sediments are significant for emerged plants such as *Nymphea alba* and *Trapa natans* with the roots developed in the sludge. Inhibitory effects of phenolics accumulated in soil on plants have been repeatedly demonstrated (Whittaker 1970; Chou and Muller 1972; Rice 1974; Lodhi 1976, 1978).

Table 2. Content of total phenolics and phenolic acids in the bog sediment.

		littoral part	mid-part	deepest part
Total phenolics (μg/g)	free	3.92	17.55	10.74
	±	0.59	1.42	0.96
	bound	251.82	337.75	224.42
	±	20.98	30.04	18.67
	total	255.74	355.30	235.16
PAs (μg/g)				
p-coumaric	free	tr.	-	-
	±	-	-	-
	bound	-	-	7.94
	±	-	-	0.73
ferulic	free	-	-	tr.
	±	-	-	-
	bound	-	-	10.89
	±	-	-	0.12
p-hydroxy-benzoic	free	0.75	0.75	0.87
	±	0.09	0.10	0.11
	bound	-	tr.	-
	±	-		-
vanillic	free	0.68	0.87	0.76
	±	0.07	0.08	0.10
	bound	34.58	8.86	4.81
	±	2.46	0.91	0.37
syringic	free	-	-	tr.
	±	-	-	-
	bound	-	-	11.06
	±	-	-	0.92
total	free + bound	36.01	10.48	36.33

Samples of the bog water in which aquatic plants are either completely or partially submerged contained 785.28 μg/L of total phenolics, while among phenolic acids only vanillic acid was identified in a concentration of 52.54 μg/L. Similar to terrestrial plants, aquatic plants release phenolic compounds into their environment. *Myriophyllum spicatum* L. propagated fast in the eastern United States and Canada after its introduction from

Europe during the end of the last century. It may displace the native vegetation and milfoil-dominated lakes usually have low phytoplankton densities. It was recently reported that *Myriophyllum spicatum* releases polyphenols acting as inhibitors of cyanobacteria, chlorophytes and diatoms. A part of the inhibitory activity is due to complexation and inactivation of algal extracellular enzymes by hydrolysable polyphenols from *M. spicatum* (Gross *et al.* 1996).

The presence of phenolic compounds in aquatic plants, as well as in the samples of both the bog sediment and water together with the dominance of one to two plant species suggest allelopathic influence of the released phenolics on other plant species within the aquatic ecosystem examined. Further studies along this line are in progress.

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ФЕНОЛНЕ КИСЕЛИНЕ И УКУПНИ ФЕНОЛИ НЕКИХ ВОДЕНИХ БИЉАКА

Л. ЂУРЂЕВИЋ

Инсѿиѿуѿ за биолошка исѿраживања "Синиша Сѿанковић",
11060 Београд, Југославија

Рад се бави проучавањем фенолних једињења барских биљака, с обзиром на досадашња недовољна алелопатска проучавања водених екосистема. Према количини укупних слободних фенола којих има више од везаних, може се успоставити следећи низ водених биљака: *Nymphea alba*>*Lemna minor*>*Salvinia natans*>->*Trapa natans*>*Ceratophyllum demersum.* У ткивима проучаваних врста биљака преовлађују слободни облици *p*-кумаринске, ферулинске, ванилинске, и сирингинске киселине. Муљ узет из приобалног, средњег и најдубљег дела баре садржи исте фенолне киселине, претежно у везаном облику. Растворена фенолна једињења детектована су и у барској води. Присуство фенолних једињења у воденим биљкама, барском муљу и води и доминација 1-2 биљне врсте, указују на алелопатски утицај њихових фенолних једињења на остале биљке у воденом екосистему, што треба даљим истраживањима потврдити.