ANALYSIS OF BENZOIC AND CINNAMIC ACID DERIVATIVES OF SOME MEDICINAL PLANTS IN SERBIA

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Abstract - Natural phenolics, which are ubiquitously distributed in plants, have been reported as functional factors in phytotherapy. We have examined phenolic compounds in the leaves and inflorescences of five significant medicinal plants of different plant families: *Salvia officinalis (*Lamiaceae*); Achillea clypeolata (*Asteraceae*); Nymphaea alba (*Nymphaeaceae*); Rumex acetosella (*Polygonaceae*)* and *Allium ursinum (*Alliaceae*)*. The examined species were rich in total phenolics (up to 30.88 mg/g dry weight). According to their total phenolics contents, the plants can be arranged in the following order: *A. clypeolata>N. alba>S. officinalis>R. acetosella>A. ursinum.* Free phenolics prevailed in all species in comparison to the bound forms (63.72-82.68% of total phenolics). The highest content of total free phenolics was measured in the tissues of *A. clypeolata* and *N. alba*, and the lowest in *A. ursinum*. Five phenolic acids were isolated and measured. *p*-Coumaric and ferulic acids as derivatives of cinnamic acid prevailed in the leaves of *R. acetosella* and *A. ursinum* (up to 4.81%).

Key words: Achillea clypeolata, Allium ursinum, biodiversity, *Nymphaea alba,* phenolic acids, *Rumex acetosella, Salvia officinalis,* secondary metabolites, total phenolics

INTRODUCTION

Using a variety of plants in their diet throughout history, humans have observed some of their medicinal properties. As there was no knowledge about the causes of disease and how to make use of healing plants, everything was based on experience. However, with advances in science specific reasons for using herbs for specific diseases have been discovered. Until the advent of "Medical wisdom on all things" in the sixteenth century, plants were a basis of treatment and prophylaxis (Parojčić and Stupar, 2003). The use of medicinal plants was also known in the ancient civilizations of Babylon, Egypt, Greece and Rome (Anatidae, 1992). The most important historical sources are the Sumerian clay tablet from Nippur (about 5000 years old), the Chinese Book of Herbs and Roots (Che-Nung-Pents' ao-ching, written about 2800 BCE), Ebers Papyrus (written in Thebes around 1550 BCE), the Bible, the Talmud, the Hindu Vedas, the Homeric epics "Iliad" and "Odyssey", and Dioscorides' De Materia Medica. In the Middle Ages, Serbs inherited from Byzantium medical advances. The most important records about the use of herbs in phytotherapy are in the Hodosh Codex (14th Century), and the Chilandar Medical Codex, (15-16th Century) (Jarić et al.).

The curative properties of plants are derived from a number of different compounds, among which phenolics, as secondary metabolites, are the most numerous. Several thousand phenolic compounds have been discovered so far in numerous plant species, and represent a widely distributed group of secondary plant metabolites, which are formed from the shikimate and acetate-malonate pathways (Harborne 1980). They are located in all plant organs, both as free forms and associated with other compounds, such as lignin (Bate-Smith 1969; Saunders and McClure 1976; Harborne 1980) and polysaccharides of cell walls (Whitmore 1976; Lam et al., 1994).

Phenolics have significant physiological and ecological roles as allelopathic agents that can be autotoxic or affect the growth of other plants in the environment (Muller, 1960; Harborne, 1980, Rice, 1974). Additionally, phenolics have an important role in therapeutic and pharmaceutical applications as they appear to be involved in the defense against invading pathogens, including bacteria (Rocha et al., 1995; Nelson et al., 1997), fungi (Garcia et al., 1995) and viruses (Lin et al., 1997). These compounds have also been shown to possess antimutagenic, anticarcinogenic, antiglycemic, anti-inflammatory, antiradical and antioxidative beneficial properties (Gordon and An 1995; Frankel et al., 1995; Siess et al., 1997; Goldbohm et al., 1997; Friedman, 1997). Epidemiological studies suggest that the consumption of flavonoids is effective in lowering the risk of coronary heart disease (Rice-Evans and Miller, 1996; Hollman et al. 1996). Some dietary phenolic compounds such as flavonoids may affect autoimmunity (Nakagami et al., 1995).

Literature data on phenolic acids as secondary metabolites of medical plants in comparison to other groups of phenolic compounds are rather sparse. The aim of this study was to analyze the content of total phenolics, cinnamic (*p*-coumaric and ferulic acid) and benzoic acid derivatives (*p*-hydroxybenzoic, vanillic and syringic acid) in the leaves and inflorescences of five significant medical plants of different plant families: *Salvia officinalis (Lamiaceae); Achillea clypeolata (Asteraceae); Nymphaea alba (Nymphaeaceae); Rumex acetosella (Polygonaceae)* and *Allium ursinum (Alliaceae)*. The use of the five examined species and their chemical compounds in traditional medicine and phytotherapeutic practice, and their physiological and ecological importance, have been discussed.

MATERIALS AND METHODS

Sampling of plant material

Plants were collected during flourishing. The same quantities (100 g) of plant material of *Rumex acetosella* (leaves and inflorescences), *Nymphaea alba* (leaves), *Salvia officinalis* (leaves), *Achillea clypeolata* (inflorescences) and *Allium ursinum* (leaves) were air-dried, milled and sieved (pore size = 2 mm) before the analyses of phenolic compounds.

Extraction of plant phenolics

Both phenolic acids and total phenolic compounds were extracted from 3 x 2 g aliquots of dry plant material with an 80% (v/v) boiling aqueous methanol solution (4 h), followed by ethyl acetate (4 h). Pooled methanol and ethyl acetate extracts were evaporated, dissolved in water adjusted to pH 2.0 with 2 N HCl, and transferred to ethyl acetate. The mixture was evaporated to dryness and the residue dissolved in 4 mL of an 80% (v/v) methanol solution (free phenolics), and used for HPLC analysis or stored at -20°C until use. Bound phenolics were prepared by boiling the ground plant material remaining after the extraction with methanol and ethyl acetate in 2 N HCl for 60 min, and transferred to ethyl acetate. The ethyl acetate phase was evaporated to dryness and diluted in 4 mL of an 80% (v/v) methanol solution and used for HPLC analysis or stored at -20 ^{0}C until use. A detailed procedure for phenolic acid extraction and determination is described by Djurdjević et al. (2007).

Determination of total phenolics

Total phenolics (free and bound) were measured by a spectrophotometric method (a Shimadzu UV 160 spectrophotometer), using the Folin-Ciocalteu reagent, as suggested by Feldman and Hanks (1968). Total phenolics were analyzed in samples taken in three replicates for each extract (n=9 for each plant species). A standard curve was constructed with different concentrations of ferulic acid.

Determination of phenolic acids by HPLC analysis

Phenolic acids were detected between 210 and 360 nm using a Hewlett Packard diode array detector (HP 1100 HPLC system). The separation was achieved with a Nucleosil 100-5 C_{18} column; 5 µm; 4.0x250 mm (Agilent Technologies, U.S.A.). The following step-gradient of acetonitrile in water was used: 15% acetonitrile (5 min), 30% acetonitrile (20 min), 40% acetonitrile (25 min), 60% acetonitrile (30 min), 60% acetonitrile (35 min) and 100% acetonitrile (45 min, isocratic). In order to avoid tailing of the phenolic acids, 0.05% *o*-phosphoric acid was added to the solvents. The flow rate was 1 mL/min and the injection volume 5 µL. Phenolic acids were identified by comparing the retention times and absorption maxima of peaks obtained with analytically pure standards. For this purpose, *p*-hydroxybenzoic and syringic acids (Acros organics, USA), ferulic, vanillic and *p*-coumaric acid (Serva, Germany) were used. Units of phenolic acids were expressed in µg per gram dry weight. Phenolic acids were analyzed in samples taken in three replicates for each extract (n=9 for each plant species).

RESULTS

All five species were rich in total phenolics. The highest amounts of total phenolics were measured in inflorescences of *A. clypeolata* (30.88 mg/g dry weight), and the lowest in leaves of *A. ursinum* (4.34 mg/g). According to the total phenolics content, the examined medicinal plants can be arranged as follows: *A. clypeolata*>*N. alba*>*S. officinalis*>*R. acetosella*>*A. ursinum*. Free phenolics predominated in all species in comparison to bound forms (63.72-82.68% of total phenolics). The highest content of total free phenolics was measured in the tissues of *A. clypeolata* and *N. alba*: the lowest content was in *A. ursinum* (Tab. 1.).

Five phenolic acids were isolated. Free phenolic acids predominated over bound species in the leaves of *R. acetosella* and *N. alba* (up to 6.42%), whereas bound acids predominated in the leaves of *A. ursinum* (up to 12.3%). *p*-Coumaric and ferulic acids as derivatives of cinnamic acid, prevailed in the leaves of *R. acetosella* and *A. ursinum* (up to 4.81%). Also, derivatives of benzoic acid (*p*-hydroxybenzoic, vanillic and syringic) were also detected in these two species (up to 2.80%). In the tissues of *S. officinalis* and *A. clypeolata*, free and bound *p*-coumaric and ferulic acids were present in traces. However, bound forms of *p*-hydroxybenzoic, vanillic and syringic acid were detected (42.57µg/g; Table 2 and 3). Two aromatic species, *S. officinalis* and *A. clypeolata*, known for their high content of essential oils, contain a very small percentage of free phenolic acids in relation to total phenols (0.03%), in contrast to *R. acetosella* and *A. ursinum,* which display 5.31% and 2.76% of free phenolic acids. With respect to the amount of free phenolic acids in tissues, the five species of medicinal herbs can be arranged as follows: *Rumex acetosella*> *Nymphaea alba*> *Allium ursinum*> *Achillea clypeolata*> *Salvia officinalis*.

DISCUSSION

The presented results reveal a considerable variability of the examined medical plants in their total phenolics contents and in the chemical composition of phenolic acids. A characteristic chemical feature of many species belonging to the family *Lamiaceae* is the secretion of different phenolics on the leaf surface (Grayer et al. 1996; Tomas-Barberan and Wollenweber 1990). As an established aromatic species with a high content of essential oils (Tucakov 1978; Jančić 1995; Todorova 1998), *S. officinalis* also displayed a high content of total phenolics and a low content of phenolic acids. Recently, Santos-Gomes et al. (2003) identified twelve phenolic compounds in *S. officinalis* tissues: gallic acid, 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, caffeic acid, rosmarinic acid, hesperetin, epirosmanol, hispidulin, genkwanin, carnosol, carnosic acid, and methyl carnosate. Zheng and Wang identified the following compounds in the tissues of *S. officinalis*: vanillic, caffeic and rosmarinic acid, luteolin, hispidulin, cirsimaritin and apigenin. Among the identified phenolic compounds, rosmarinic acid was predominant (Zheng and Wang, 2001). In ad-

Table 1. Amount of total free and bound phenolics in five medicinal plants from Serbia

		Rumex acetosella	*Nymphaea alba*	*Salvia officinalis*	*Achillea clypeolata*	*Allium ursinum*
Total phenolics (mg/g)	free	8.45	16.86	9.46	25.33	3.24
	±	0.757	0.97	1.38	2.42	0.29
	bound	1.77	9.60	3.64	5.55	1.10
	±	0.12	0.95	0.41	0.65	0.17
	total	10.22	26.46	13.10	30.88	4.34

Table 2. Content of free phenolic acids

PAs (μg/g)	*Rumex acetosella*	*Nymphaea alba*	*Salvia officinalis*	*Achillea clypeolata*	*Allium ursinum*
p- coumaric	158.45	87.31	tr.	tr.	tr.
	±12.34	±8.91	-	-	-
ferulic	254.14	113.27	tr.	tr.	58.27
	±23.11	±10.97	-	-	±2.98
p-hydroxy	24.45	-	-	-	-
benzoic	±2.11	-	-	-	-
vanillic	105.33	94.37	2.34	3.54	61.48
	±11.78	±8.97	±0.3	±0.38	±6.78
syringic	-	-	1.75	4.35	-
			±0.21	±0.39	
Total PAs	542.37	294.95	4.09	7.89	119.75

Table 3. Content of bound phenolic acids

PAs (μg/g)	*Rumex acetosella*	*Nymphaea alba*	*Salvia officinalis*	*Achillea clypeolata*	*Allium ursinum*
p- coumaric	34.43	tr.	tr.	tr.	25.43
	±3.60	-	-	-	±1.45
ferulic	44.75	tr.	tr.	tr.	49.82
	±3.96	-	-	-	±3.55
p-hydroxy	0.96	-	-	-	-
benzoic	±0.01	-	-	-	-
vanillic	11.53	24.02	11.33	34.88	60.05
	±0.97	±2.07	±1.95	±3.89	±7.02
syringic	-	-	15.13	42.57	-
	-	-	±2.11	±4.97	-
Total PAs	91.67	24.02	26.46	77.45	135.30

dition, two phenolic acids and five flavonoids have been detected and identified in *Salvia triloba* tissues (El-Sayed et al. 2001). In the leaves of *S. officinalis*, we detected *p*-coumaric and ferulic acid (free and bound forms) in traces, and bound vanillic and syringic acid as derivatives of benzoic acid in higher amounts. Owing to its antimicrobial activity against various bacteria, yeasts and fungi involved in food poisoning and food spoilage (Chipely, 1993), benzoic acid is widely used in the food industry as a preservative in acid foods,

The aromatic species *A. clypeolata* also has a high content of total phenolics and a low content of phenolic acids. In the eastern and southern regions of Serbia where it grows, it is more valued as a medicinal plant than *A. millefolium*. Medical use is based on an increased content of essential oils (Tucakov, 1978; Todorova et al., 1998). Most of the 21 species of *Achillea* sect. mainly accumulate methyl ethers of the flavonols 6-hydroxykaempferol and quercetagetin, predominantly as 4´-methylated derivatives (Valant-Vetschera and Wollenweber, 1996). According to our results, *A. clypeolata* assumed first place in the content of total phenolics.

In the leaves of *A. ursinum* we measured the lowest content of total phenolics (only 4.34 mg/g). Carotenuto et al. (1996) isolated from *A. ursinum* three new flavonoid glycosides, identified as kaempferol derivatives. Different cultivars of the colored onion (*Allium cepa* L.) were reported to contain up to 30.66 mg/g of total quercetins (Patil and Pike, 1995; Patil et al. 1995). A phytochemical investigation of the extracts obtained from the bulbs of leek, *Allium porrum* L., has led to the isolation of five flavonoid glycosides based on the kaempferol aglycone (Fattorusso et al., 2001). In comparison to *A. ursinum* that had the lowest content of total phenolics, in the leaves of *N. alba* we found more than 6-fold higher, and in *R. acetosella* over 2-fold higher amounts of total phenolics.

The territory of Serbia is characterized by great biodiversity, exemplified by a great wealth of plant species suitable for use in phytotherapy. The use of medicinal plants for the treatment of many diseases has a long tradition that has held up to the present day (Tucakov 1978; Jančić et al., 1995; Parojčić and Stupar, 2003; Jaric et al., 2007, 2011).

CONCLUSIONS

The obtained results revealed a considerable variability in the total phenolics content and chemical composition of phenolic acids. In view of the high content of highly soluble total phenolics and phenolic acids, the examined species are suitable for use in folk medicine and phytotherapy. Based on the content of secondary metabolites, the five plants can serve as significant sources of phenolics and phenolic acids in medical use.

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