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SEEDS AND FLOWERS OF CHESTNUT TREES IN URBAN AREAS: A MUNICIPAL WASTE OR A RAW MATERIAL?

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ABSTRACT

This study deals with the examination of total aescin content in flowers and seeds of *Aesculus hippocastanum* L. and *Aesculus x carnea* Hayne (Hippocastanaceae), collected in urban areas, as well as the content of Pb, Cd and Hg as an indicator of potential aerial pollution. Aescin identification was performed by TLC. Total aescin content was determined by UV-VIS spectrophotometry and HPTLC densitometry. The contents of Pb, Cd and Hg was determined by AAS. The concentration of aescin varied in a wide range between 0.17% (*A. hippocastanum* hulls) and 2.95% (*A. hippocastanum* cotyledons), as determined by spectrophotometric assay. Slightly lower results were recorded in HPTLC densitometry assay, ranging from 0,10% (the hulls of both plant species) to 2.39% (*A. x carnea* cotyledons). The extractable matter yield was between 5% and 26%, with a high share of total aescin (5% - 13%, depending on the source). The levels of Pb, Cd and Hg both in the plant material and the extracts remained low, indicating that the health safety of the plant specimens was not compromised. Our results suggest that the seeds and flowers of Chestnut trees in urban areas could be considered as a raw material for chemical and pharmaceutical industry in Serbia.

Key words: *Aesculus hippocastanum* L., *Aesculus x carnea* Hayne, aescin, health safety, spectrophotometry, HPTLC densitometry, AAS.

INTRODUCTION

Aesculus hippocastanum L. (Hippocastanaceae) is a large deciduous tree, commonly known as horse chestnut. As an arctotertiary relict of Balkan Peninsula, it is native to small areas in southeastern Europe, e.g. in the Drim River basin, as well as to mixed forests regions in northern Greece, Albania, the Former Yugoslav Republic of Macedonia, Serbia and Bulgaria. As an ornamental tree, it was first introduced in the Byzantine Empire, whereas the first reports for Western Europe date back to 16th century. Today, it is widely cultivated throughout the temperate climate for its decorative qualities [1-3].

On the other hand, red horse chestnut, *Aesculus x carnea* Hayne (synonyms: *A. rubra* Poir., or *A. rubicunola* Loisl.), is an artificial hybrid between the red buckeye (*A. pavia* L.) and the common horse chestnut. The origin of this hybrid is not known, but it probably first appeared in Germany, in 19th century. This is a medium-size tree, intermediate between the parent species in most respects, but inheriting the red flower colour from *A. pavia*. It is also a popular tree in large gardens and parks [1].

The seeds of horse chestnut have a record of regular use, both in human and veterinary traditional medicine, as an analgesic, antipyretic, narcotic, tonic, and vasoconstrictor. Its seeds are largely recommended by herbalists for a range of diseases, such as backache, sunburns, neuralgia, rheumatism, whooping cough and hemorrhoids. Today, standardised horse chestnut seed extract is one of the most widely prescribed herbal medications for chronic venous insufficiency, which is characterised by swollen legs, varicose veins, a feeling of heaviness, pain, tiredness, itching, tension and cramps in the calves. It reduces vascular fragility by acting upon the connective tissue barrier between the blood vessels and the tissues, thereby inhibiting exudation and oedema development [4-6]. In the same time, chemical inventory and/or specific medicinal use of Red Horse Chestnut are not listed by available reference data.

The principal constituent of horse chestnut seed extract is aescin, a mixture of triterpenoid saponin glycosides. Aescin decreases the transcapillary filtration of water and proteins [4]. It has been used to treat a wide variety of inflammatory conditions, to reduce post-operative and post-traumatic soft tissue swelling, associated with bruises, fractures, acute thrombophlebitis and brain trauma [7,8]. Therefore, horse chestnut seeds, as a biological source of aescin, can be considered as a valuable raw material for chemical and pharmaceutical industry, with a whole array of possible applications in medicine, cosmetology and dermatology.

Having in mind the importance of horse chestnut seeds as a raw material for pharmaceutical industry, and the facts that, at least in Serbia, there are no chestnut plantations and only a limited part of market demands can be supplied from domestic natural resources, we applied a set of routine pharmaceutical tests in

order to assess the quality and health safety of chestnut plant material collected in urban areas. The main objective of this paper is, therefore, to investigate a possibility of establishing a sustainable practice other than „collect-and-burn“, as the potentials of the *employment* of chestnut waste plant material from urban areas, not only of its *disposal*, are far from being explored.

MATERIALS AND METHODS

General experimental conditions

All spectrophotometric measurements were performed on Evolution 300 UV-VIS spectrophotometer (Thermo Scientific). HPTLC densitometry was done using Camag TLC-Scanner II, controlled by CATS software (Camag, Muttenz, Switzerland). Thin layer chromatography (TLC) and high-performance thin layer chromatography (HPTLC) were performed on analytical TLC or HPTLC silica gel 60 F_{254} (10x10 or 10x20 cm) glass plates, layer thickness 0,2 mm (Merck, Darmstadt, Germany). Cd and Pb content were determined using a Varian SpectrAA 220 atomic absorption spectrophotometer, equipped with a GTA 110 furnace, with constant temperature zone. Hg levels were determined on the same system as above, coupled to a VGA 77 hydride system.

Chemicals

A reference standard of aescin was purchased from Fluka (Buchs, Switzerland). All reagents and solvents were of analytical grade. Ferric chloride hexahydrate was purchased from LaChema (Neratovice, Czech Republic), while anisaldehyde, methanol, ethanol, glacial acetic acid, hydrochloric acid, concentrated sulfuric acid, ethyl acetate, chloroform, diethyl ether, n-propanol and n-butanol were from Merck (Darmstadt, Germany). Nitric acid was purchased from J.T. Baker (Center Valley, USA), and hydrogen peroxide from Kemika (Zagreb, Croatia).

Plant material

Plant material (flowers and seeds of *A. hippocastanum* and *A. x carnea*) was collected in May and September 2011 in Vršac urban area, and dried naturally in a shaded and well-ventilated place, at the room temperature. The seeds of both species were peeled off and the hulls were considered as separate samples.

Extraction procedures

The plant material (1 g of each sample) was reduced to a coarse powder, mixed with 10 ml of 60% aqueous ethanol (V/V) and extracted for 30 min on a hot water bath, under a reflux. The extracts were filtered and left overnight in a stream

of air to evaporate. Dry residues were accurately weighed and kept refrigerated until analysed.

Identification of aescin

The presence of aescin in the plant material was confirmed by TLC according to German Pharmacopoeia (DAB 10) [9]. In brief, test solutions were obtained by boiling 1 g of powedered plant material with 10 ml of ethanol (70% V/V) under a reflux for 15 min. Reference solution was prepared by dissolving 10 mg of aescin standard in 1 ml of ethanol (70% V/V). After the chromatogram has been developed in suitable mobile phase (glacial acetic acid – water – n-butanol, 10:40:50, V/V/V; upper layer), zones resembling to aescin were visualised by spraying with anisaldehyde reagent (10% solution of anisaldehyde in concentrated sulfuric acid) and subsequent heating at 100-105 °C.

Spectrophotometric determination of aescin

The content of aescin in all samples was determined according to DAB 10 [9]. Briefly, samples were heated with 65% aqueous methanol (V/V) under reflux condenser for 30 min. Dry residues obtained after evaporating the solvent were dissolved in 0,1 M HCl and extracted twice with a mixture of n-propanol and chloroform. After the organic layer was evaporated to dryness, obtained dry residue was washed with diethyl ether, dissolved in glacial acetic acid, transferred to 50 ml volumetric flask and filled up to the volume with the same solvent. A 5 ml aliquot of resulting solution was transferred to 25 ml volumetric flask and filled up to the volume with $FeCl_3$ reagent ($FeCl_3$ dissolved in a mixture of acetic acid and sulfuric acid). After incubation at 60 °C for 25 min and cooling, the absorbance was measured at 540 nm. The results are calculated as % (aescin) = 13.89 x A/m, where A stands for absorbance, and m for sample weight (g).

HPTLC densitometric determination of aescin

Preparation of test solutions was performed essentially in the same manner as described in the procedure for spectrophotometric determination, except for the final step, when the dry residue was dissolved in methanol, instead of in $FeCl_3$ reagent. The procedure was performed as described by Constantini, with slight modifications [10].

For the purpose of quantitative determination, both test and reference solutions were applied spotwise (2 µl, in duplicate) on analytical HPTLC silicagel plates, 10 mm from the edge of the plate. Reference solutions were prepared by dissolving aescin standard in methanol, covering the concentration range between 1 and 5 mg/ml.

Chromatographic separation was performed using the mixture of n-propanol, ethyl acetate and water (35:45:20, V/V/V) as a mobile phase, over a path

of 10 cm, in a saturated vertical chromatographic chamber. After complete removal of solvents by a stream of lukewarm air, aescin contents was determined by linear scanning in reflectance mode at 212 nm, by means of a computer-assisted Camag TLC-scanner. The spots were analysed using the following setup: single-beam reflectance mode; monochromator bandwidth 10 nm (micro position); slit dimension 0.3 x 0.4 mm; scanning speed 0.5 mm/s; automatic zeroing before each track; automatic sensitivity adjustment. The net chromatograms of each analyte (standards and test solutions) were obtained by substracting via computer, in the same plate, the registered signal of a blank track from the signal of the analyte.

Determination of heavy metals

After homogenisation, the samples (1 g) were digested with 8 ml of HNO_3 (65%, V/V) and 2 ml of H_2O_2 (30%, V/V), using the method of acid microwave digestion (ETHOS Milestone).

Cd and Pb contents were determined using graphite furnace atomic absorbtion spectrometry (GFAAS) technique. Cd was measured at 228.8 nm and Pb at 283.3 nm. The limits of quantification (LOQ) for Cd and Pb were 0.005 and 0.05 mg/kg, respectively. Analyses of Hg were carried out by cold vapor technique. The LOQ for Hg was 0.005 mg/kg. Recoveries for all examined elements ranged from 95% to 102% and variation coefficient between 4% and 9%. Quantification was performed by using standards with different concentrations and instrument contamination was monitored by analyzing blank samples in the sample series. The recoveries of Cd, Pb, and Hg were determined by adding a known amount of a particular standard solution into the samples. All determinations were done in compliance with ISO standard 17025.

RESULTS AND DISCUSSION

The plant material (cotyledons, hulls and flowers) of both chestnut species yielded between 4.99% and 25.30% (w/w) of matter extractable with 60% aqueous ethanol (V/V), based on the dry plant material weight. The lowest quantity of extractable matter has been recovered from the hulls of *Aesculus x carnea*, while the highest yield was recorded for the cotyledons of the same plant species. All the plant material could be characterized as rich in extractable matter, except the seed hulls of both plant species.

The results of spectrophotometric and HPTLC densitometric determination of aescin in investigated plant material and the extracts obtained with 60% aqueous ethanol (V/V) are presented in Table 2. Spectrophotometric assay of aescin revealed that this constituent was present in investigated material in a wide concentration range, between 0,17% (*A. hippocastanum* hulls) and 2,95% (*A.*

hippocastanum cotyledons). Similar, but slightly lower results were obtained by HPTLC densitometric procedure: again, aescin content was lower in hulls than in flowers and, especially, cotyledons of both investigated plant species. Observed difference between spectrophotometric and densitometric assay can be attributed to lower specificity of spectrophotometric determination, because the interference arising from the presence of the other, non-saponin or related constituents might also be accounted. Finally, the results of aescin determination in obtained extracts showed a remarkable share of aescin in all investigated extracts (5.12-13.23%).

Table 1. Plant material
Tabela 1. Biljni materijal

Sample No.	Description	Hull to cotyledon ratio	Extractable matter (%)
1	*Aesculus x carnea* cotyledons	1:5,17	25.30
2	*Aesculus x carnea* hulls		4.99
3	*Aesculus hippocastanum* cotyledons	1:4,82	25.02
4	*Aesculus hippocastanum* hulls		8.96
5	*Aesculus hippocastanum* flowers	-	25.90
6	*Aesculus x carnea* flowers	-	20.96

Qualitative analysis by TLC revealed that aescin could be detected in both the plant material and investigated extracts. After chromatographic separation and chemical derivatization of obtained chromatograms with anisaldehyde/sulfuric acid reagent, aescin was detected in upper half, as a deep red zone.

Tabela 2. Određivanje escina u ispitivanom materijalu
Table 2. Determination of aescin in investigated material

Sample	Aescin content in plant material (%)		Aescin content in extracts (%)
	Spectrophotometry	HPTLC densitometry	
A. x carnea cotyledons	2.79	2.39	13.23
A. x carnea hulls	0.18	0.10	4.58
A. hippocastanum cotyledons	2.95	1.92	11.29
A. hippocastanum hulls	0.17	0.10	9.41
A. hippocastanum flowers	1.31	0.27	5.12
A. x carnea flowers	1.24	0.30	6.02

Aescin levels in the plant material were found to be lower than minimal pharmacopoeial requirements [9]. However, from technological point of view, the plant material (removed from the streets and green areas as a solid municipal organic waste) still contains a significant quantity of valuable constituents, which can be produced and used in a sustainable and cost-effective manner.

Environmental pollution and constant exposure to heavy metals are considered to be among the most important threats to human health today. The increased and still growing levels of heavy metals in the environment are a consequence of their utilization in various industrial activities. Due to their non-biodegradable nature, heavy metals tend to accumulate in biological compartments and move through the food chains. Although some of them are micronutrients, in high concentrations they are toxic to various life forms [11]. Therefore, the next step in our study was to investigate the health safety of the plant material and obtained extracts, taking into the consideration the levels of certain heavy metals.

Metals such as mercury, cadmium and lead were chosen as the indicators of health safety, as they enter the environment primarily as a consequence of industrial emissions or via disposal of products containing them. Because of their widespread use, there is a general background level in the environment and they are consequently present in many foodstuffs and medicinal plants in low levels.

Tabela 3. Određivanje sadržaja teških metala u ispitivanom materijalu
Table 3. Determination of heavy metals in investigated material

Sample	**Cd (mg/kg)**		**Pb (mg/kg)**		**Hg (mg/kg)**	
	Plant material	Extract	Plant material	Extract	Plant material	Extract
A. x carnea cotyledons	0.005	0.005	< 0.05	0.71	< 0.005	< 0.005
A. x carnea hulls	0.014	0.008	< 0.05	1.73	< 0.005	< 0.005
A. hippocastanum cotyledons	0.01	0.007	< 0.05	0.35	< 0.005	< 0.005
A. hippocastanum hulls	0.015	0.014	< 0.05	0.59	< 0.005	< 0.005
A. hippocastanum flowers	0.037	0.007	0.62	0.62	< 0.005	< 0.005
A. x carnea flowers	0.043	0.009	0.92	0.45	< 0.005	< 0.005

As heavy metals pose a hazard to human and animal health, their content in plants used for consumption or medicinal purposes must be limited. For this reason limits for heavy metals have been set for foodstuffs and medicinal products by health authorities. However, the limits for toxic elements in herbal products are yet to be set at the global level. Table 3 compiles limits for lead, cadmium and mercury set or proposed so far in different regulatory frameworks [12-14].

Comparing our results to the limits for lead, cadmium and mercury set or proposed so far in different regulatory frameworks, it is obvious that both the investigated plant material and prepared extracts can be generally considered as safe with regards to the content of heavy metals as a measure of environmental pollution. In other terms, although the plant material was collected in urban area, the concentration of toxic metals was found to be low or at least acceptable, indicating that it could be considered as a potential raw material for industrial or semi-industrial production of aescin-rich concentrate, or derived products.

Tabela 4. Primeri nacionalnih i regionalnih limita za sadržaj olova, kadmijuma i žive u biljnim proizvodima

Table 4. Examples of national and regional limits for lead, cadmium and mercury content in herbal products

State/Authority	Pb	Cd	Hg	Comments
	(mg/kg)			
Canada	10	0,3	0,2	In crude herbal drugs
	0,02	0,006	0,02	In finished herbal products (mg/day)
China	10	1	0,5	In crude herbal drugs
Malaysia	10	-	0,5	In finished herbal products
Singapore	20	-	0,5	In finished herbal products
Thailand	10	0,3	-	In both crude herbal drugs and finished herbal products
WHO	10	0,3	-	In crude herbal drugs
Ph. Eur. monograph Kelp (2007)	5	4	0,1	
Ph. Eur. draft monograph Herbal drugs (2008)	5	0,5	0,1	
Regulation (EC) 396/2005 (2008)	-	-	0,02	For herbal infusions and spices
Regulation (EC) 629/2008	3	1 (3 for seaweed products)	0,1	For food supplements

In dendroflora of Vršac, both *Aesculus hippocastanum* and *Aesculus x carnea* (horse chestnut and red horse chestnut) are predominant plant species, making as much as almost two-thirds of all trees planted across the urban area. Being introduced as back as in 19th century, by Danube Swabian settlers of southern Banat, chestnut trees became popular for their ornamental qualities and, by time, a characteristic floral symbol of Vršac. Some of the specimens have reached imposing proportions and age, particularly in the Municipal Garden, where most of the chestnut trees are located, and where the plants enjoyed proper care and protection for more than 150 years of its two-

century long history. Similar considerations can be made for almost every town or city in Serbia, since both species of chestnut are widely present in urban areas, being among the most popular landscape elements in public and private parks, gardens and alleys [15].

However, although decorative, chestnut is also considered as a „messy“ tree, which produces a lot of solid waste material, particularly during the flowering and fruiting. In urban areas, this material is more or less regularly removed from streets but, in most cases, dumped indiscriminately, or subjected to some kind of mechanical treatment (for example, composting, incineration or industrial co-combustion, at best).

Waste generation is closely linked to population, urbanization and affluence. It arises from human activities – domestic, commercial, industrial, agricultural, etc. If the waste is not properly handled and treated, it has a negative impact on the hygienic conditions in urban areas. In most developed and developing countries with increasing population, prosperity and urbanization, waste remains a major challenge for municipalities to collect, recycle, treat and dispose it. A cornerstone of sustainable development is the establishment of affordable, effective and truly sustainable waste management practices [16].

By the National Waste Management Strategy 2010-2019, several methods of waste management practices have been proposed, such as decrease of waste at sources, re-use, recycling, composting, anaerobic digestion, incineration and other waste treatment procedures. This framework seems to be flexible enough to include some other innovative approaches to the problem of organic solid waste, such as considering it as a suitable and inexpensive source of various raw materials for pharmaceutical and chemical industry [17].

In the case of the organic waste produced by chestnut trees after the flowering and fruiting stage, the advantages of proposed approach are as follows: final product (aescin or aescin-rich extract) has certain and relatively high value on the market of pharmaceutical and chemical products, which should result not only in partial cost return, but a chance for local self-governments to achieve a substantial supplementary income. The space for the location of processing equipment is relatively small, as well as transportation costs, with relatively low environmental influence of the production process itself. On the other hand, such equipment may call for bigger capital investments. Finally, the market for pharmaceutical and chemical products is large and relatively stable, providing a good opportunity for further development of local communities.

CONCLUSIONS

Our results strongly suggest that the seeds and flowers of chestnut trees in urban areas could be considered as a raw material for chemical and pharmaceutical industry. The levels of aescin, although lower than minimal pharmacopoeial

requirements, were found to be high enough for sustainable and cost-effective industrial or semi-industrial production of aescin-rich concentrate and/or derived products.

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SEME I CVET KESTENA U GRADSKIM PODRUČJIMA: KOMUNALNI OTPAD ILI SIROVINA?

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IZVOD

Osnovni cilj ovog rada je bio da se utvrdi ukupni sadržaj escina u cvetu i plodu *Aesculus hippocastanum* L. i *Aesculus x carnea* Hayne (Hippocastanaceae), sakupljenim u gradskom području, kao i sadržaj Pb, Cd i Hg kao indikatora potencijalnog gradskog aerozagađenja. Identifikacija escina u ispitivanom materijalu je izvršena primenom TLC. Ukupni sadržaj escina je određen primenom UV-VIS spektrofotometrije i HPTLC denzitometrije, kako u biljnom materijalu, tako i u ekstraktima dobijenim ekstrakcijom pomoću 60% etanola. Sadržaj Pb, Cd i Hg je određen primenom AAS. Rezultati spektrofotometrijskog određivanja pokazuju da se koncentracija escina nalazi u širokom rasponu između 0,17% (semenjača ploda *A. hippocastanum*) i 2,95% (kotiledoni semena *A. hippocastanum*). Nešto niži rezultati su dobijeni primenom HPTLC denzitometrije, između 0,10% (semenjača ploda obe biljne vrste) i 2,39% (kotiledoni semena *A. x carnea*). Biljni materijal sadrži između 5% i 26% ekstraktibilnih materija, sa visokim udelom ukupnog escina (5% - 13%, u zavisnosti od izvora). Koncentracija Pb, Cd i Hg, kako u biljnom materijalu, tako i u ekstraktima, bila je niska, što implicira da zdravstvena ispravnost uzoraka biljnog materijala nije ugrožena činjenicom da je biljni materijal sakupljen u gradskoj sredini. Naši rezultati ukazuju na to da seme i cvet obe vrste kestena, koje se gaje u urbanim sredinama radi svojih dekorativnih svojstava, ali proizvode značajnu količinu komunalnog otpada, mogu da se koriste kao sirovina za proizvodnju vrednih proizvoda za upotrebu u hemijskoj i farmaceutskoj industriji.

Ključne reči: *Aesculus hippocastanum* L., *Aesculus x carnea* Hayne, escin, zdravstvena ispravnost, spektrofotometrija, HPTLC denzitometrija, AAS.