Highly invasive alien plant *Reynoutria japonica* Houtt. represents a novel source for pharmaceutical industry – Evidence from phenolic profile and biological activity

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Abstract: *Reynoutria japonica* is on the IUCN list of the Worlds' 100 worst invasive species, but it is also, especially its rhizome, an integral part of traditional chinese medicine. The objective of this study was to determine the amount of selected phenolic compounds in rhizome, stems, leaves and inflorescence methanol extracts of this plant, their antioxidant and anticholinesterase activity. The chemical profile of the examined extracts was obtained by a high-performance liquid chromatography. *In vitro* assays on DPPH, OH and NO radicals were used to estimate antioxidant potential and Ellman's method was applied for the determination of anticholinesterase activity. Leaves and rhizome extracts were found to be rich in rosmarinic and chlorogenic acid, and selected flavonoids. Resveratrol was exclusively present in rhizome and stems extracts. All the investigated extracts expressed certain antioxidant activity, where leaves extract was the most active. However, rhizome extract was the strongest inhibitor of acetylcholinesterase. These findings indicate that there is a possibility of *R. japonica* exploitation for the isolation of biologically active phenolic compounds used in pharmaceutical and food industry.

Keywords: *Polygonum cuspidatum*; *trans*-resveratrol; antioxidant effect; anticholinesterase activity; phenolic acids; flavonoids.

INTRODUCTION

Reynoutria japonica Houtt. – Japanese knotweed (syn. *Fallopia japonica* (Houtt.) Rouse Decreane var. *japonica*, *Polygnum cuspidatum* Sieb. *et* Zucc.) is a member of the family Polygonaceae, whose native range covers East Asia region, but it is a worldwide ornamental plant species. First records in Europe date back to XVIII century, when it was introduced to botanical gardens of Western Eur-

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ope, in Netherland and England. Nowadays, it is naturalized in Europe, North America, Asia, Australia and New Zealand.[1–5] Japanese knotweed is on the IUCN list of the 100 worst invasive species,[5] and it is one of the invasive alien species with the highest negative impact on the environment in Germany, UK and France.[6] It propagates from rhizomes vegetatively and endangers other plants. Once it appears at the site *R. japonica* spreads very fast and forms dense mono-cultures.[7] Many European countries develop control measures and suppression programs regarding this invasive species.

Interestingly, its usage in traditional systems of medicine dates back over 1800 years. Currently, it is listed in the Pharmacopoeia of the People's Republic of China as *Rhizoma Polygoni Cuspidati*. The rhizome of this plant is used as a main constituent in various pharmaceutical formulations intended for treatment of different ailments of liver, bile and urinary tract followed by inflammation such are acute hepatitis and cirrhosis, hepatitis B infection, cholecystitis, append-icitis, prostatitis, chronic pelvic pain syndrome and urinary tract infections.[8] Furthermore, *R. japonica* rhizome is used for curing burns, inflammation, various bacterial infections, rheumatic arthritis, acute gouty arthritis, arthralgia, myalgia, atherosclerosis, hyperlipidemia and diabetes.[9] Besides its medicinal use, the ten-der stems of *R. japonica* are consumed as a delicious foodstuff in the Eastern world.[8] Numerous studies have been conducted concerning phytochemistry, pharmacodynamics and potential application of *R. japonica*. It is important to emphasize that the majority of the available scientific data are focused at the rhizome and that the other parts, such are the aerial parts of the plant, should also be investigated. *Reynoutria japonica* is the source of stilbenes (e.g. resveratrol), which are important regarding the fact that these secondary metabolites are found in only a few higher plants.[10,11] *R. japonica* also contains quinones (emodin and similar anthraquinones), coumarins and lignans, different volatile compounds, phenolic acids, flavonoids and, recently discovered, flavonol-fused stilbene gly-cosides.[12] So far, antioxidant, anti-inflammatory, antimicrobial, anticancer, heap-toprotective, lipid regulating, estrogenic, anti-shock and inhibition of melano-genesis effects have been investigated with the *R. japonica* rhizome extracts or isolated compounds.[8] Since being an antioxidant agent and containing com-pounds which exhibit neuroprotective effects, *R. japonica* could be applied in prevention and co-therapy of Alzheimer's disease (AD).[13] Therefore, despite the negative impact on the environment and society, the invasive species, such as *R. japonica*, could be a potential natural resource of compounds that have signific-ant pharmacological effects.

The aims of the present study were to evaluate the amounts of the selected phenolic compounds (*i.e.* resveratrol, phenolic acids and flavonoids) in *R. japo-nica* rhizome, stems, leaves and inflorescence methanol extracts, as well as to estimate the *in vitro* antioxidant (neutralization of $DPPH^\bullet$, $^\bullet OH$ and $NO^\bullet$) and

anticholinesterase potential in the aspect of potential use in the treatment of AD and in pharmaceutical and food industry in general.

EXPERIMENTAL

Plant material and preparation of extracts

Rhizome, stems, leaves and flowers of the wild-growing *R. japonica* in full blossom were collected at location Kamenjar in Novi Sad, Serbia, in September 2014. Voucher specimen (no. 2-2119) was confirmed and deposited at the Herbarium of Department of Biology and Ecology (BUNS), Faculty of Sciences, University of Novi Sad. Plant material was air dried and kept in paper bags prior to analysis.

Maceration with 70 % methanol (herbal drug:solvent, 1:1) at room temperature was used for obtaining the extracts of *R. japonica* rhizome (RJ-1), stems (RJ-2), leaves (RJ-3) and flowers (RJ-4). After the maceration, the extracts were filtered and evaporated under vacuum to dryness, while the mass of dry extract (d. e.) was measured by analytical balance (accuracy 0.1 mg).

Evaluation of chemical composition

Total phenolic compounds and flavonoid content. The amount of total phenolic compounds (TP) in the investigated extracts was determined spectrophotometrically with the Folin-Ciocalteu (FC) reagent[14] and was expressed in mg of gallic acid equivalents (GAE) per g of dry extract (d. e.), using a standard calibration curve of gallic acid. All measurements were done in triplicate. Total flavonoid content (TF) in the extracts was also determined spectrophotometrically, using a method based on the formation of a flavonoid–aluminum complex with an absorptivity maximum at 430 nm.[14] Flavonoid content was expressed in mg of quercetin equivalents (QE) per g of d. e., using a standard curve of quercetin. All measurements were replicated three times.

HPLC-DAD analysis of selected phenolic compounds. Qualitative and quantitative characterization of phenolic compounds present in the examined extracts was performed by high performance liquid chromatography (HPLC). An Agilent HP 1100 HPLC – diode array (DAD) and fluorescence (FLD) detection system equipped with an autosampler (Agilent, Waldbronn, Germany) was used. The separation was done on Zorbax CB-C18 column (4.6 mm×150 mm, i. d., 5 μm particle size) at 25 °C with solvent A being 0.1 % aqueous solution of acetic acid and B 0.1 % solution of acetic acid in acetonitrile. Gradient mode of elution was used (3.25 min – 10 % B; 8 min – 12 % B, 15 min – 25% B, 15.8 min – 30 % B, 25 min – 90 % B, 25.4 min – 100 % B) with the flow rate of 1 mL min^{-1}. The injection volume was 15 μL. Analytical standards of ferulic, chlorogenic, *p*-hydroxybenzoic, rosmarinic, coumaric and *trans*-cinnamic acid, quercetin, naringenin, rutin and epicatechin were dissolved in methanol and injected to system under the same analytical conditions for the needs of calibration and quantification of compounds in the examined plant extracts. The detection was carried out at wavelength of 280 nm.[15]

HPLC-DAD/FLD analysis of trans-resveratrol. The HPLC analysis was performed using a reverse phase Zorbax, SB-C18, 4.6 mm×150 mm (5 μm) column, at a flow-rate of 1 mL min^{-1}, as previously described.[16]

Biological potential

In vitro *antioxidant activity*. Free radical scavenging capacity (*RSC*) of the investigated extracts was evaluated by measuring the scavenging activity on DPPH$^\bullet$, $^\bullet$OH and NO$^\bullet$. All the

measurements were done in triplicate and the percentage of all RSC processes was calculated by the following equation:

$$RSC = 100(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}$$

where A_{control} is an absorbance of reaction mixture without the examined extract, and A_{sample} is the absorbance of reaction mixtures containing increasing volumes of plant extract solution, whose antioxidant potential was estimated.

DPPH assay was performed as previously described.[17] The disappearance of DPPH$^\bullet$ was detected spectrophotometrically at 515 nm. The scavenging capacity of the examined *R. japonica* extracts for hydroxyl radicals was evaluated by measuring the degradation of 2-deoxy-D-ribose by $^\bullet$OH generated in a Fenton reaction.[17] The neutralization of NO$^\bullet$ was evaluated by measuring the accumulation of nitrite (formed in the reaction of NO$^\bullet$ with oxygen), according to the Griess reaction.[18] The results describing antioxidant potential of the examined extracts were compared with antioxidant potential of propyl gallate (PG) and *trans*-resveratrol, estimated under the same experimental conditions.

Anticholinesterase activity. The inhibition of acetylcholinesterase (AChE) was determined spectrophotometrically using *S*-acetylthiocholine iodide as a substrate in a modified Ellman's method.[19,20] Final concentrations of the extracts in the reaction mixture were 630 and 1260 μg mL^{-1}. The tests were carried out in triplicate, while the percentage of inhibition of AChE was calculated by the following equation:

$$I = 100(1 - (A_{\text{sample}}/A_{\text{control}}))$$

Statistical analysis

The data were reported as mean values ± standard deviations (*SD*). Values representing the concentrations of investigated extracts that cause 50 % of neutralization or inhibition (IC_{50}) were determined by regression analysis of *RSC*.

RESULTS

Chemical characterization

The results of total phenolic and flavonoid content, as well as dry extracts (d. e.) yield are given in Table I. Leaves yielded the highest amount of dry extract followed by inflorescence, rhizome and stems. Similar results were obtained also for the total phenolic content and the highest amount was recorded in the aerial parts of the plant, specifically in the stem extract (203.03 mg GAE/g d. e.). The extracts obtained from inflorescence and leaves contained higher amounts of flavonoids (18.21 and 16.31 mg QE/g d. e., respectively) when compared to stem and rhizome extracts (2.4 and 2.84 mg QE/g d. e., respectively).

TABLE I. The content of d. e., *TP* and *TF* in the investigated extracts of *Reynoutria japonica*

Sample	d. e. yield, mg/g of dry plant material	*TP* / mg GAE/g d. e.	*TF* / mg QE/g d. e.
RJ-1	89.14±0.01	94.97±3.35	2.84±0.03
RJ-2	73.26±0.09	203.03±6.07	2.4±0.08
RJ-3	158.86±0.13	186.45±1.32	16.31±0.09
RJ-4	117.8±0.08	117.59±3.42	18.21±0.41

Detailed chemical analysis of the obtained extracts by liquid chromatography resulted in quantification of the range of phenolic compounds (Table II, Fig. 1). The leaves extract was rich in rosmarinic and chlorogenic acid. Furthermore, a considerable amount of rutin was recorded in the leaves, whereas aglycones (epicatechin and quercetin) were not detected or were present in low amount (naringenin). The rhizome extract was also rich in rosmarinic and chlorogenic acid, but these amounts were lower when compared to the leaves extract. However, considerable amounts of epicatechin and quercetin were quantified. Although all of the investigated phenolic compounds were detected in the stem extract, their concentration was lower than in leaves and rhizome extracts. None of the selected phenolic compounds were detected in *R. japonica* inflorescence extracts.

TABLE II. Quantification of selected phenolic compounds in *R. japonica* extracts; n. d. – not determined

| Compound	Concentration of selected phenolic compounds, mg / g of d. e.			
	RJ-1	RJ-2	RJ-3	RJ-4
Phenolic acids				
p-Coumaric acid	1.67±0.05	0.41±0.02	2.57±0.09	n. d.
Ferulic acid	n. d.[a]	0.40±0.03	4.40±0.18	n. d.
p-Hydroxybenzoic acid	2.88±0.14	0.33±0.01	2.36±0.15	n. d.
trans-Cinnamic acid	0.27±0.02	0.62±0.03	0.32±0.05	n. d.
Rosmarinic acid	3.30±0.13	0.40±0.03	13.42±0.32	n. d.
Chlorogenic acid	4.89±0.12	0.48±0.04	4.21±0.17	n. d.
Flavonoids				
Epicatechin	19.69±0.15	2.08±0.11	n. d.	n. d.
Quercetin	2.26±0.03	1.83±0.12	n. d.	n. d.
Naringenin	0.77±0.02	0.73±0.04	0.87±0.10	n. d.
Rutin	6.87±0.21	1.27±0.07	7.69±0.42	n. d.
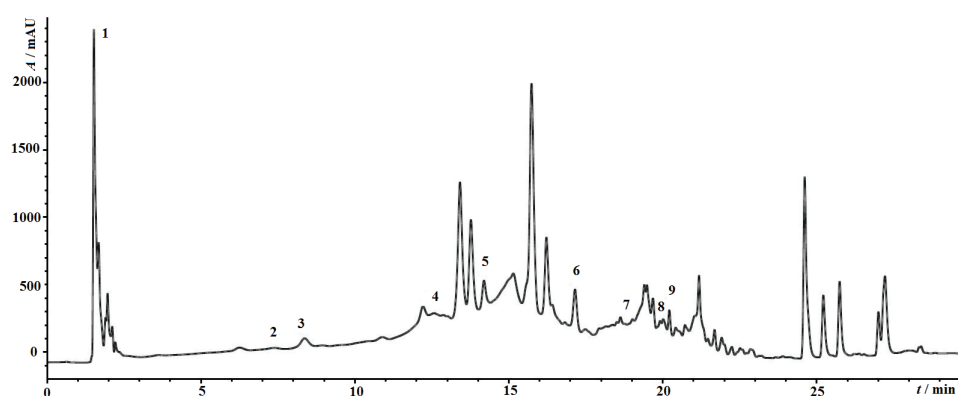
Fig. 1. Chromatogram of the examined *R. japonica* rhizome extract (RJ-1) at 280 nm: 1 – rutin, 2 – chlorogenic acid, 3 – *p*-hydroxybenzoic acid, 4 – epicatechin, 5 – *p*-coumaric acid, 6 – rosmarinic acid, 7 – quercetin, 8 – *trans*-cinnamic acid, 9 – naringenin.

The quantification of *trans*-resveratrol showed its presence in stem (0.087 µg/mg d. e.) and rhizome (0.068 µg/mg d. e.) extracts, while it was not determined in leaves and inflorescence extracts (Fig. 2).
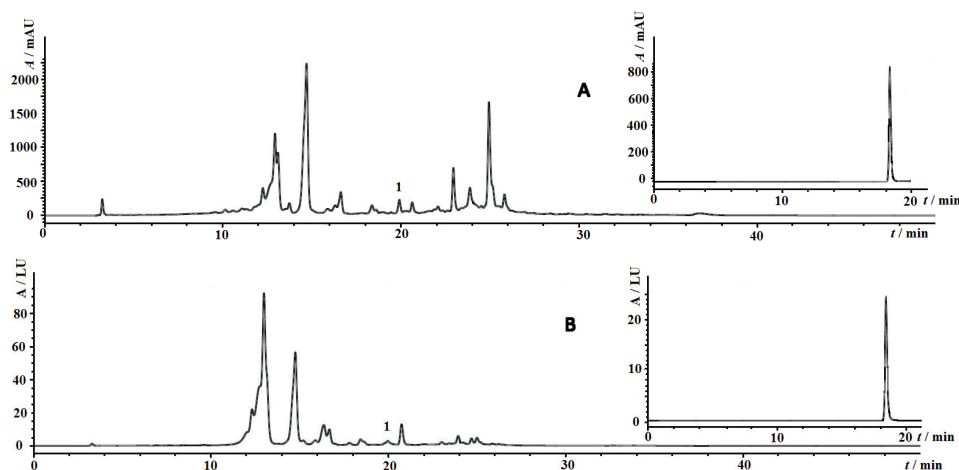

Fig. 2. Chromatograms of: A – the examined *R. japonica* rhizome extract and standard *trans*-resveratrol with detection at 280 nm; B – the examined *R. japonica* rhizome extract and standard *trans*-resveratrol with detection at $Ex = 330$ nm, $Em = 374$ nm (B); 1 - *trans*-resveratrol.

Biological potential

The second part of the study was focused on the antioxidant potential of different *R. japonica* extracts examined through neutralization of DPPH$^\bullet$, $^\bullet$OH and NO$^\bullet$, as well as on their anticholinesterase activity (Table III). The highest potential to neutralize DPPH$^\bullet$ was recorded in the leaves extract (RJ-3, $IC_{50} = 0.76$ µg mL^{-1}), while the lowest antioxidant potential was exhibited by the rhizome extract (RJ-1, $IC_{50} = 5.13$ µg mL^{-1}). The results obtained for *RSC* of the examined extracts in DPPH-test system were comparable with the antioxidant potential of synthetic antioxidant – PG ($IC_{50} = 0.58$ µg mL^{-1}, Table III). Similar results were obtained when the ability of *R. japonica* extracts to neutralize $^\bullet$OH was analyzed. Leaves extract (RJ-3) expressed the highest potential ($IC_{50} = 21.4$ µg mL^{-1}) whilst the highest IC_{50} value was recorded for the rhizome extract (RJ-1, $IC_{50} = 138.28$ µg mL^{-1}). However, in the NO test-system, the stem extract (RJ-2) expressed the highest activity ($IC_{50} = 13.65$ µg mL^{-1}), followed by the leaves extract (RJ-3, $IC_{50} = 19.19$ µg mL^{-1}), rhizome extract (RJ-1) and inflorescence extract (RJ-4). Similar as in the DPPH-test system, the lowest IC_{50} values obtained for leaves and stems extracts in OH- and NO-test systems, respectively, were comparable with the antioxidant potential of PG measured under the same experimental conditions. The rhizome extract (RJ-1) was found to inhibit AChE

considerably; 62.41 % and 79.05 % in applied concentrations (630 and 1260 µg mL^{-1}, respectively).

TABLE III. Antioxidant and anticholinesterase potential of the examined extracts

Sample	Antioxidant potential			Inhibition of AChE	
	IC$_{50}$, µg / mL			Concentration, µg/mL	
				Inhibition, %	
	DPPH$^\bullet$	$^\bullet$OH	NO$^\bullet$	630	1260
RJ-1	5.13±0.11	138.28±3.15	29.6±1.39	62.41±2.15	79.05±3.68
RJ-2	2.23±0.05	90.48±4.78	13.65±0.78	31.55±1.32	48.69±4.97
RJ-3	0.76±0.03	21.40±1.58	19.19±1.54	18.32±1.12	38.21±2.14
RJ-4	1.94±0.11	127.66±6.41	40.00±0.47	39.46±2.14	51.19±3.54
PG	0.58±0.03	8.76±0.18	9.04±0.23	/	/
trans-Resveratrol	3.57±0.08	/	/	/	/

DISCUSSION

Since the first investigations of *R. japonica* rhizome, traditional herbal medicine in China, a lot of different chemical constituents were characterized.[8] However, very few studies are dealing with the chemical analysis of the aerial parts. Japanese knotweed represents one of the most invasive species in Europe. Therefore, it is important to elucidate the efficient way of its eradication, and what is more, regarding its numerous pharmacological effects, to find the efficient way of its exploitation for the needs of pharmaceutical industry. A detailed chemical analysis of the selected phenolic compounds showed that the most abundant source of phenolic acids (especially chlorogenic and rosmarinic acid) is the *R. japonica* leaves extract. So far, chlorogenic acid has been detected only in rhizome extracts and found to be one of the major compounds responsible for free radical scavenging, antiproliferative, hepatoprotective, neuroprotective and anti--HIV potential of *R. japonica*.[21,22] Also the majority of studies determine only 5--caffeoylquinic acid, which is the most abundant chlorogenic acid in nature.[23] Yet, beside caffeoylquinic acids, there are dicaffeoylquinic acids and feruloylquinic acids found in plants, each group with at least three isomers. Presumably, they all play an important role in exhibiting beneficial effects to human health.[24] However, there are no data on rosmarinic acid content in *Reynoutria* species. This may be of importance since rosmarinic acid possesses notable biological activities (antioxidant, antimicrobial, antiviral, anti-inflammatory and neuroprotective).[25] Moreover, chlorogenic and rosmarinic acid were present in considerable amounts in the studied rhizome extracts. Along with phenolic acids, several flavonoids were quantified in the examined *R. japonica* extracts. They were detected in relatively high concentrations in rhizome extract (RJ-1), especially flavonoid aglycones epicatechin and quercetin. These findings concur with the results of Kuznetsova[26] and confirm the hypothesis that invasive species from

ruderal flora, such is *R. japonica*, could be a potential natural resource of biologically active compounds. Interestingly, flavonoid aglycones were either not detected or present in low amount in the leaves extract (RJ-3), whereas rutin, as flavonoid glycoside, was present in a relatively high concentration. The absence of investigated phenolic acids and flavonoids in the inflorescences may be explained by the white color of flowers. Namely, the presence of leucoanthocyanins is characteristic for this type of flowers where different types of phenolic compounds are present in colored flowers (yellow, red, purple, *etc.*).[27]

The content of *trans*-resveratrol was determined in both, rhizome and aerial parts. Resveratrol is a phenolic compound characteristic for red wine and leaves of eucalyptus. Several health benefits are related to its presence, especially in wine, such are decrease in cardiovascular disease incidence, often addressed to as French Paradox, cancer prevention and ability to prevent neurodegenerative ailments such is AD.[28] It is also considered to be responsible for numerous pharmacological effects of *R. japonica* rhizome. The concentrations of resveratrol and its derivatives in rhizome are well studied.[11] Nevertheless, a slightly lower concentration was determined in the rhizome extract (RJ-1) in our study, as compared to some other investigations. Taking into consideration that stilbene compounds have a role in the plant organism for the resistance against certain pathogens,[29] basic assumption is that *R. japonica* in the investigated area is a newcomer without, or with a few native enemies. Furthermore, it is determined that resveratrol, when present in plant material, is followed by a series of derivatives and oligomers (predominantly glycosides as piceid).[8] There are very few data on resveratrol content in the aerial parts of the plant. In comparison to a study of Vrchotova, Sera and Triska[30] similar results were obtained for resveratrol content in the studied stem extract (RJ-2).

The second part of the study was dealing with free radical scavenging and potential in the prevention and possible treatment of AD (inhibition of acetylcholinesterase) of examined *R. japonica* extracts. Similar activities were obtained when the ability of extracts to neutralize DPPH$^\bullet$ and $^\bullet$OH was analyzed. Namely, the leaves extract (RJ-3), which is found to be the richest source of selected phenolic compounds, possesses the highest antioxidant potential. However, in the NO$^\bullet$ neutralization test, the stem extract was the most active, followed by the leaves extract (RJ-3). The results describing antioxidant potential of the examined extracts are particularly emphasized when being compared to the antioxidant potential of PG evaluated under the same experimental conditions. These findings confirm hypothesis that phenolic compounds play an important role in prevention of oxidative processes, which are involved in pathogenesis of different ailments.[31] Previous *in vitro* studies reported significant potential of *R. japonica* extracts to neutralize DPPH radicals.[32] Also, conducted *in vivo* studies, suggested that the addition of *R. japonica* extract to diet of Jian carp suffering from liver

injury induced by CCl$_4$, increases the levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and decreases the levels of malondialdehyde (MDA).[33] Namely, AD belongs to the diseases of modern age. There are several current hypotheses about its ethiopathology. Oxidative stress in brain tissue can lead to oxidation of glycated proteins and formation of advanced glycation end-products, which are present in amyloid plaques.[34] It is also well known that the lowering of levels of ACh is considered responsible for the majority of symptoms of AD. Therefore, inhibition of AChE is already an approved approach to the treatment of AD. Galantamin, which was isolated from *Galanthus woronowii*, Amaryllidaceae, is one of the most potent AChE inhibitors administered to patients suffering from AD.[35] However, new biological sources of potentially strong AChE inhibitors are more than welcome. In our study, rhizome extract expressed the strongest inhibitory effect on AChE. Since stem extract (RJ-2) was the richest source of resveratrol, it is possible that other compounds found in the rhizome (especially phenolic compounds and quinones) contribute to the inhibition of the examined enzyme.

CONCLUSIONS

It can be concluded that, beside the *R. japonica* rhizome, that is well studied and known for notable antioxidant and anticholinesterase activity, the aerial parts (especially leaves and stems) could be a significant source of biologically active phenolic secondary metabolites (resveratrol, rosmarinic and chlorogenic acid, as well as rutin). This may indicate that there is an interest for the exploitation of this highly invasive species in pharmaceutical and food industry for the isolation of dominant compounds or for the preparation of extracts with several potential health beneficial effects.

И З В О Д
ВИСОКОИНВАЗИВНА АЛОХТОНА ВРСТА *Reynoutria japonica* HOUTT. ПРЕДСТАВЉА НОВУ СИРОВИНУ ЗА ФАРМАЦЕУТСКУ ИНДУСТРИЈУ – ДОКАЗИ НА ОСНОВУ ХЕМИЈСКОГ САСТАВА И БИОЛОШКЕ АКТИВНОСТИ

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Reynoutria japonica се налази на IUCN листи 100 најгорих светских инвазивних врста, али такође је, нарочито њен ризом, саставни део традиционалне кинеске медицине. Циљ истраживања била је квантификација одабраних фенолних компоненти у

ризому, гранама, листовима и цвастима метанолних екстраката ове биљке, као и одређивање антиоксидантог и антихолинестеразног потенцијала добијених екстраката. Хемијски састав екстраката одређен је високоефикасном течном хроматографијом. Антиоксидантни потенцијал је одређен помоћу *in vitro* тестова неутализације DPPH, OH и NO радикала, док је Елманова (Ellman) метода употребљена за испитавање антихолинестеразне активности. У екстрактима листова и ризома је уочен висок садржај розмаринске и хлорогенске киселине, као и одређених флавоноида. Резвератрол је био присутан искључиво у екстрактима ризома и гранчица. Сви испитани екстракти су исказали одређени антиоксидантни потенцијал, нарочито екстракт листова. Међутим, као најјачи инхибитор активности ацетилхолинестеразе показао се екстракт ризома. Добијени резултати сугеришу могућност експлоатације *R. japonica* за изолацију биолошки активних једињења за потребе фармацеутске и прехрамбене индустрије.

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