December 2024 | Volume 44 | ISSN 0455-6224

Natural Medicinal Materials
Lekovite sirovine

The journal that covers all areas of medicinal plants research

Institute for Medicinal Plants Research "Dr. Josif Pančić"

Lavandula angustifolia Mill.

LEKOVITE SIROVINE

PUBLISHER: Institute for Medicinal Plants Research "Dr. Josif Pančić", Tadeuša Košćuška 1, Belgrade, Serbia
PERSON RESPONSIBLE FOR PUBLISHING: Milan Lukić, Director of the Institute

Vol. 44 (2024)

Aims and scope: The journal Lekovite Sirovine (transl. Natural Medicinal Materials) is dedicated to all areas of Medicinal Plants research, Cultivation, Ethnopharmacology, Phytotherapy, Phytomedicine, Medicinal fungi, etc. It is a scientific journal with a long tradition, since 1951. The journal Lekovite Sirovine publishes original papers that have not been published previously. The journal Lekovite Sirovine publishes full-length research papers, reviews, and professional papers. Lekovite Sirovine is a peer-reviewed open access journal with no publication charges, costs or fees for publication. Contributions to the journal may be submitted in the English language. The Journal is issued once a year, but online publication ahead of print will be available as soon as the article is accepted. The journal is indexed in SCIndex. The journal Lekovite Sirovine is academic journal deeply committed to the dissemination of scientific information and in this regard, it strives to meet the highest possible standards of publication ethics, whereas the publication malpractice is strictly prohibited by all possible measures. Ethics and malpractice statements of the journal are based on COPE's Best Practice Guidelines for Journal Editors and pertain to all parties involved in the publishing: the peer reviewer, the editor, and the author.

SOURCES OF SUPPORT: Ministry of Science, Technological Development and Innovation of the Republic of Serbia supported hard-copy printing cost of this journal.

PRODUCT INFORMATION
Printed at JP Službeni glasnik, Belgrade
Serbia
Printed in 80 copies
ISSN 0455-6224
December, 2024

Table of Content

GC-MS, FTIR, Phytochemical Profiling and Antibiogram Pattern of *Ipomoea asarifolia* on Bacterial Strains from Wound

S.K.S. Ojo[1,3*], A.M. Ojo[2], A. Alaba[3], O.E. Adelugba[3], S. Oriowo[3], O.O. Ademola[3], A.A. Yusuf[3], I.A. Ojo[1], K.T. Aliu[1], M.A. Adelabu[1] and O.P. Odeleye[1]

[1]*Drug Discovery & Infectious Diseases Research Group, Department of Microbiology, Federal University Oye-Ekiti. P.M.B. 373, Oye-Ekiti, Ekiti State, Nigeria.*
[2]*Department of Chemistry, Federal University Oye-Ekiti, Ekiti State, Nigeria.*
[3]*Department of Biological Sciences, Joseph Ayo Babalola University, Ikeji-Arakeji, Osun State, Nigeria*
**Corresponding author: stephen.ojo@fuoye.edu.ng*

Received: November 4, 2023
Accepted: November 8, 2023
Published on-line: December 23, 2024
Published: December 25, 2024

This study aims to assess the GC-MS and FTIR profiles and secondary metabolites of *Ipomoea asarifolia* vis-a-vis its antibacterial action against bacteria, associated with wound infections. We conducted phytochemical screenings, GC-MS, and FTIR analyses on *I. asarifolia*. The antibacterial effects of the extracts were tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Reports indicated the presence of saponins (57.9, 13.81, 46 mg/g), phenolics (18.4, 4.14, 21.05 mg/g), and alkaloids (0.49, 0.62, 1.12 mg/g) in the leaf, stem, and whole plant, respectively. *I. asarifolia* contained eighteen (18 of 20) essential amino acids, including glycine, alanine, serine, and proline. We also found phytosterol, fatty acids, phospholipids, and vitamins (B, C, D, E, and K) in the samples. We detected distinctive absorption bands in the leaf and stem samples, corresponding to the stretching of O-H bonds. The absorption band at 3419.95 cm^{-1} was discovered in the leaf, while the absorption band at 3416 cm^{-1} was observed in the stem. A prominent adsorption peak at 1637.83 cm^{-1}, corresponding to the stretching of the alkene C=C bond in lignin, was detected in the leaf sample. The zone size inhibition for leaf and stem extracts ranges from 5 mm to 8 mm, depending on the extract concentration (0.625–5.0 mg/mL). *S. aureus* exhibited susceptibility to ciprofloxacin and norfloxacin but showed resistance to 15 other antibiotics. In contrast, *P. aeruginosa* displayed resistance to all tested antibiotics. The study provided confirmation and clarification of the traditional applications of *I. asarifolia*, a herbal plant that necessitates further investigation.

Keywords: wound bacteria; GC-MS; FTIR; phytochemicals; *Ipomoea asarifolia*; ethnomedicine

https://doi.org/10.61652/leksir2444001O

1. INTRODUCTION

Plants in Africa, Asia, and Latin America have been used for medicinal purposes since ancient times (Aliyu et al., 2011; Karou et al., 2006). Researchers have repeatedly validated the effectiveness of these medicinal plants in the laboratory. Aliyu et al. (2011) and Fatope (2001) reported that approximately 80% of plants selected based on ethnomedicinal information demonstrate significant pharmacological activity. Medicinal plants are a cheap and renewable source of pharmacologically active substances. Plants remain a vital source of medicines for a large proportion of the world's population, particularly in developing countries (Gurib-Fakim, 2006). Falode et al. (2016) reported that researchers have isolated a number of modern drugs from plants that have been used for many years to treat various diseases worldwide. Currently, herbal medicine is widely accepted globally as a legal, alternative system of therapy for treating and curing various diseases and physiological conditions in traditional treatments in the form of pharmaceuticals (Falode et al., 2016; Gutteridge, 1984). *Ipomoea asarifolia* (Desr.) Roem. and Schult. (Convolvulaceae) is a glabrous succulent perennial plant trailing on the ground. It reproduces from seeds and stem shoots. It is a perennial, creeping or trailing, growing on sandy soil or wastelands. It is native to tropical America but is now pantropical. It occurs throughout West Africa, spanning from Cameroun to Senegal, Mali, the

Cape Verde Islands, and tropical Asia. It is a common weed in hydromorphic soils, low-lying and inland valleys, streams, and riverbanks (Jegede et al., 2009). In Nigeria, the traditional names include "Dumankada" in Hausa and "Gboroayaba" in Yoruba (Jegede et al., 2009). In Senegal, the plant is used for various gynecological purposes, including urinary problems during pregnancy, hemorrhage, as an ecbolic and abortifacient, and also in general for wound dressing and the treatment of ophthalmia, neuralgia, headaches, arthritic pains, and stomach aches. People in Ivory Coast mix the pulped-up leafy stem with citron and water and take it as an ecbolic. In some cases, people internally take leaf decoctions as a wash to alleviate feverish chills and rheumatic pains. In the middle belt region of Nigeria, people use the leaves to treat dysmenorrhea (painful menstruation). In northern Nigeria, people in the region apply a leaf poultice to guinea worm sores, steam their faces over a hot decoction of the plant along with husks of bulrush millet, and boil the flowers with beans to eat as a remedy for syphilis (Aliyu et al., 2011; Burkill, 1985; Jegede et al., 2009). The focus of this research was on GCMS and FTIR profiling and the quantification of phytonutrients in *I. asarifolia* and their effects on bacterial strains from wounds (*in vitro*).

2. MATERIALS AND METHODS

2.1. Study area

The study was carried out at the Microbiology laboratory of the Department of Microbiology, Federal University Oye-Ekiti, Nigeria and the Department of Biological Sciences, Joseph Ayo Babalola University, Nigeria.

2.2. Study site

Fresh leaf, stem and whole plants of *I. asarifolia* were harvested from Oye-Ekiti and identified at the herbarium section of Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria with UHAE2017/051.

2.3. Collection and identification of clinical strains

Clinical strains of *S. aureus* and *P. aeruginosa* of wound origin were obtained from the Drug Discovery & Infectious Diseases Research Group into freshly prepared nutrient broth and incubated overnight at 37 °C. The overnight broth culture was standardized with 0.5 McFarland turbidity standards before streaking on selective media (mannitol salt and cetrimide agar - oxoid) for confirmation and incubated at 37 °C for 24 h. We also performed other biochemical tests.

2.4. Processing of plants

Fresh leaf, stem and whole plants of *I. asarifolia* were properly washed in tap water, rinsed in sterile distilled water and left to air-dry for several weeks. An electric blender (Magic Blender, Nakai Japan; model number SG-KIPN) micronized the plants into powdered form. The pulverized plants were stored in air-tight containers until required.

2.4.1. Cold Extraction Method

A pulverized plant sample of 75 g was soaked in 500 mL of methanol as the extracting solvent and agitated manually. After allowing it to extract for 48 hours, we filtered each extract using Whatmann No. 1 filter paper. We evaporated the solvent using a rotary evaporator (Senco Technology Co. Ltd., model no. R205, SN 13605) under pressure for 15 min at 39–40 rpm. The extracts were stored until needed in amber vial tubes away from light (Gujjeti and Mamidala, 2013; Wang, 2020).

2.4.2. Microbe-free proof of the extracts

The extracts were tested for presence or absence of turbidity using the Millipore filtration technique by introducing 2 mL of these extracts into 10 mL of sterile Mueller-Hinton broth and incubated at 37 °C for 24 h. The absence of turbidity or clearness of the broth after the incubation period indicated a microbe-free extract (Ojo et al., 2017).

2.5. Phytochemical analysis

2.5.1. Qualitative Phytochemical screening

The qualitative phytochemical tests on the leaf, stem, and whole plant extracts were performed, and the different secondary metabolites were characterized with reference to the technical procedures as described by various authors. Test for flavonoid (Gul et al., 2017), test for tannins (Usman et al., 2010), test for saponins (Iqbal et al., 2015), test for alkaloids (Usman et al., 2010; Wadood, 2013), test for sterols and triterpenoids (Gupta et al., 2013), test for anthraquinones glycoside (Borntrager's test) (Iqbal et al., 2015), tests for glycosides (Gul et al., 2017), cardiac glycoside (Keller-Killiani test) (Iqbal et al., 2015), test for terpenoids (Salkowski test) (Iqbal et al., 2015), test for steroids and triterpenoids (Liebermann-Burchard test) (Iqbal et al., 2015), test for phlobatannins (Wadood, 2013), test for phenolics (Ferric Chloride test) (Banu and Cathrine, 2015), and cardenolides (cardiac glycosides and aglycones-The Kedee's test) (Jagessar and Allen, 2012) were performed.

2.5.2. Qualitative Phytochemical screening

The quantitative analyses were conducted as described by various authors: alkaloids (Gupta et al., 2013; Senguttuvan et al., 2014), flavonoids, phenols, tannins, saponins (Gupta et al., 2013), anthraquinone contents (Soladoye and Chukwuma, 2012).

2.6. Gas Chromatography Mass Spectrometry (GC-MS) analysis on Ipomoea asarifolia

2.6.1. Phytosterol extraction and analysis

This was carried out by following the modified official methods of AOAC (AOAC, 2005). We weighed 5 g of powdered sample, transferred it to a Stoppard flask, and soaked it with petroleum ether until the powder was fully saturated. The flask was shaken every hour for the first 6 h and then it was kept aside and shook after 24 h. This process was repeated for three days, and then the extract was filtered. The extract was collected and evaporated to a constant mass using a nitrogen stream. The extract of 0.5 g from the sample was added to the screw-capped test tube. The samples were saponified at 95 °C for 30 min by using 3 mL of 10% KOH in ethanol, to which 0.20 mL of benzene had been added to ensure miscibility. 3 mL of deionized water was added, and 2 mL of hexane was used in extracting the non-saponifiable materials, e.g., sterols. The extractions, each with 2 mL of hexane, were carried out for 1 h, 30 min and 39 min, respectively, to achieve complete extraction of the sterols. For gas chromatography analysis, we concentrated the hexane to 2 mL in an Agilent vial.

2.6.2. The GC–MS analysis

GC-MS analysis remains one of the most accurate techniques to identify different volatile and semi-volatile bioactive constituents in organic and inorganic materials (Al-Huqail et al., 2018; Deshpande and Kadam, 2013; Payum, 2016). The GC–MS analysis of bioactive compounds from *I. asarifolia* was done using Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 mm in diameter × 0.25 mm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with flow rate

of 1mL/min. The initial temperature was set at 50 °C –150 °C with increasing rate of 3 °C /min and holding time of about 10 min. Finally, the temperature was increased to 300 °C at 10 °C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in a splitless mode. Relative quantity of the chemical compounds present in each of the extracts of *I. asarifolia* was expressed as percentage based on peak area produced in the chromatogram(Casuga et al., 2016). The content of the extract was analyzed by GC-MS to identify various compounds using NIST library (Sheik Uduman et al., 2016).

2.7. Fourier transform infrared (FTIR) spectroscopy of leaves and stem of Ipomoea asarifolia

The pulverized form of the leaves and stem were separately subjected to Fourier transform infrared (FTIR) spectroscopy (mid-IR spectra) on a Perkin-Elmer FTIR. Dried powder sample (10 mg) was encapsulated in 100 mg of KBr pellet as so to prepare translucent sample discs. The measurements were carried out at 25–27 °C within the spectra range of 4000 to 400 cm^{-1}. The peak frequencies were compared to the reference literature to determine the functional groups present.

2.8. Antibacterial Susceptibility Test

2.8.1. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MICs for the *S. aureus* and *P. aeruginosa* strains were determined by the macro-dilution method recommended by CLSI (CLSI, 2008). Macro-dilution test tubes were dispensed with 5mL of Mueller-Hinton (MH) broth containing 1 mL of the appropriate plant extracts concentrations (0.0625, 1.25, 2.5 and 5.0 mg/mL) and a final concentration of 10^5 CFU/mL of the test strains. After an incubation of 24 h at 35 °C, test tubes were examined for turbidity, indicating growth or absence of turbidity. The MBCs were determined by plating out 0.1mL of the test tubes indicated to have MIC on MH agar incubated at 35 °C for 24 h. Reference type *S. aureus* strain (ATCC 29523) was included.

2.8.2. Disk diffusion method

The *S. aureus* strain obtained were cultured on cation adjusted Mueller–Hinton agar and incubated at 35 °C while *P. aeruginosa* was cultured on Mueller–Hinton agar and incubated at 37 °C. The antibacterial disk diffusion susceptibility profiles of the isolates were determined and used as positive control (CLSI, 2008). Reference-type strains of *Staphylococcus aureus* ATCC 25923 were also included. Paper disks of 5 mm in size were impregnated for 30 min in different concentrations of methanol extract of *I. asarifolia* before being aseptically placed on the cultured agar plate.

2.9. Statistical Analysis

SPSS version 20 software was used for the statistical analysis of the data using one-way ANOVA. A p-value of less than or equal to 0.05 was considered to be statistically significant ($p \leq 0.05$).

3. RESULTS

3.1. Phytochemicals in Ipomoea asarifolia

The findings on the qualitative phytochemicals revealed the presence of secondary metabolites among the different parts of the plant under study (Table 1). Table 1 shows that the leaf contains certain phytochemicals (anthraquinones and flavonoids) that are absent in the stem.

Table1. Qualitative phytochemical constituents of *Ipomoea asarifolia*

Phytochemical constituents	Leaf	Stem	Whole plant
Anthraquinones	+	-	+
Saponins	+	+	+
Phenolics	+	+	+
Triterpenes	-	-	-
Flavonoids	-	+	+
Tanins	+	+	+
Alkaloids	+	+	+
Glycosides	-	-	-
Phlabotaninns	-	-	-
Terpenoids	-	-	-
Steroids	-	-	-
Cardiac Glycoside	-	-	-
Cardenolides and Dienolides	-	-	-

Key: + = present; - = not present

Table 2 revealed that the phenolic content in the whole plant was 48.3% (21.05 mg/g) higher than the leaf content of 18.40 mg/g (42.2%), while the stem part had a significantly lower content of 4.14 mg/g (9.5%). The stem part of the plant contained 4.34 mg/g (24.5%) of flavonoid content, while the whole plant was rich in flavonoid with 13.39 mg/g (75.5%). The leaf was in abundance of saponin with 57.90 mg/g (49.2%) when compared with the whole plant at 46 mg/g (39.1%), but very low in the stem part at 13.82 mg/g (11.7%). Overall, the whole plant contains the highest concentrations of phytochemicals, followed by the leaf and the stem, respectively (Table 2).

Table 2. Quantitative phytochemical constituents present in *Ipomoea asarifolia*

Phytochemical constituent	Leaf (mg/g)	Stem (mg/g)	Whole Plant (mg/g)
Alkaloid	0.49	0.62	1.12
Flavonoid	ND	4.34	13.39
Phenolics	18.40	4.14	21.05
Tannin	8.56	3.94	9.45
Saponin	57.90	13.82	46.00
Anthraquinones	2.08	ND	1.49

Key: ND = Not Determined since absent in qualitative phytochemical

3.2. Antibacterial Susceptibility Pattern

The zone size inhibition of methanol extract of *I. asarifolia* showed that for the leaf and stem, no antibacterial activity was detected, whereas the whole plant, for all the concentrations tested, had an 8-mm zone size diameter (Table 3).

Table 3. Diameter of zones of inhibition (mm) of *Ipomoea asarifolia* methanolic extract against test isolates.

Test organism	Zones of inhibiton (mm)/concentration(mg/mL)												
	Leaf				Stem				Whole Plant				NCTR
	0.625	1.25	2.5	5.0	0.625	1.25	2.5	5.0	0.625	1.25	2.5	5.0	
Staphylococcus aureus	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	8.0	8.0	8.0	8.0	5.0
Pseudomonas aeruginosa	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	8.0	8.0	8.0	8.0	5.0

Key: NCTR = Negative control (5% DMSO); - = No zone of inhibition

The MIC value obtained from Table 4 (0.625 mg/mL) indicated no bactericidal activity (MBC) at all tested concentrations.
Only ciprofloxacin and norfloxacin effectively inhibited *S. aureus* in the antibiotic sensitivity (positive control) test, whereas *P. aeruginosa* was resistant to all the antibiotics (Table 5).

3.3. Phytonutrients present in the extract

The GC-MS findings of *I. asarifolia* revealed that it is very rich in phytoconstituents. *I.asarifolia* has 18 essential amino acids, which can be seen in Figure 1 and Table 6. These are glycine, alanine, serine, proline, valine, threonine, isoleucine, leucine, and cystine.
Figure 2 also showed the presence of some bioactive phytosterols, including cholesterol, cholestanol, ergosterol, campesterol, stigmasterol, savenasterol, and sitosterol.

Table 4. Minimum inhibitory and bactericidal concentration of *Ipomoea asarifolia* against test isolates

Test isolates	Leaf (mg/mL)		Stem (mg/mL)		Whole Plant (mg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus	0.625	G	0.625	G	0.625	G
Pseudomonas aeruginosa	0.625	G	0.625	G	0.625	G

Key: NCTR = Negative control (5% DMSO); - = No zone of inhibition

Table 5. Antibiotic susceptibility of test isolates against commercially available antibiotics (Positive control)

	Antibiotics (mm)																
Test Isolates	CPX	NB	CN	AML	SY	RD	E	CH	APX	LEV	OFX	PEF	AU	CEP	NA	SXT	PN
Staphylococcus aureus	24S	22S	5R	5R	5R	8R	5R	5R	5R	5R	5R	5R	5R	5R	5R	5R	5R
Pseudomonas aeruginosa	5R	5R	5R	5R	7R	5R	5R	5R	5R	5R	5R	5R	5R	5R	5R	5R	5R

Key: OFX- Ofloxacin, PEF- Pefloxacin, CPX- Ciprofloxacin, AU- Augmentin, CN- Gentamicin, SY- Streptomycin, CEP-Ceporex, NA-Nalixidic acid, SXT-Septrin,PN-Ampicillin, NB-Norfloxacin, AML-Amoxil, RD-Rifampicin, E-Erythromycin,CH-Chloramphenicol, APX-Ampiclox, LEV-Levofloxacin. S – Sensitive; R - Resistant

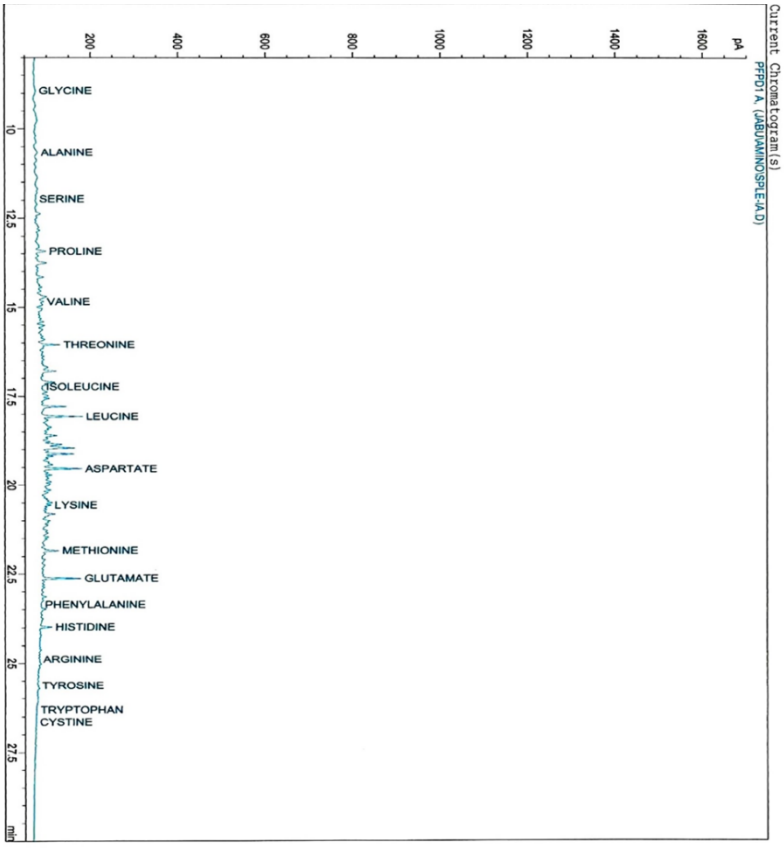

Fig 1. Chromatogram of bioactive amino-acids present in *Ipomoea asarifolia*

Table 6: Amino acid composition in *Ipomoea asarifolia*

Ret. Time (min)	Area (pA*s)	Amount (g/100 g of P)	Name
8.927	71.93903	5.83846	Glycine
10.657	99.26823	6.16317	Alanine
11.954	25.44103	6.59447	Serine
13.430	117.92834	6.89387	Proline
14.846	106.18715	5.20593	Valine
16.049	200.50868	4.81622	Threonine
17.237	63.92682	5.21033	Isoleucine
18.425	115.44450	7.37385	Leucine
19.532	384.54706	11.10560	Aspartate
20.548	124.42369	2.05546	Lysine
21.831	282.02722	$8.31015e^{-1}$	Methionine
22.614	342.46295	13.72507	Glutamate
23.357	60.85301	4.00780	Phenylalanine
23.975	151.49039	2.93474	Histidine
24.884	52.81997	5.80350	Arginine
25.625	54.71838	3.64856	Tyrosine
26.307	23.11975	1.54162	Tryptophan
26.646	30.86706	3.49211	Cystine
Total		**97.24176**	

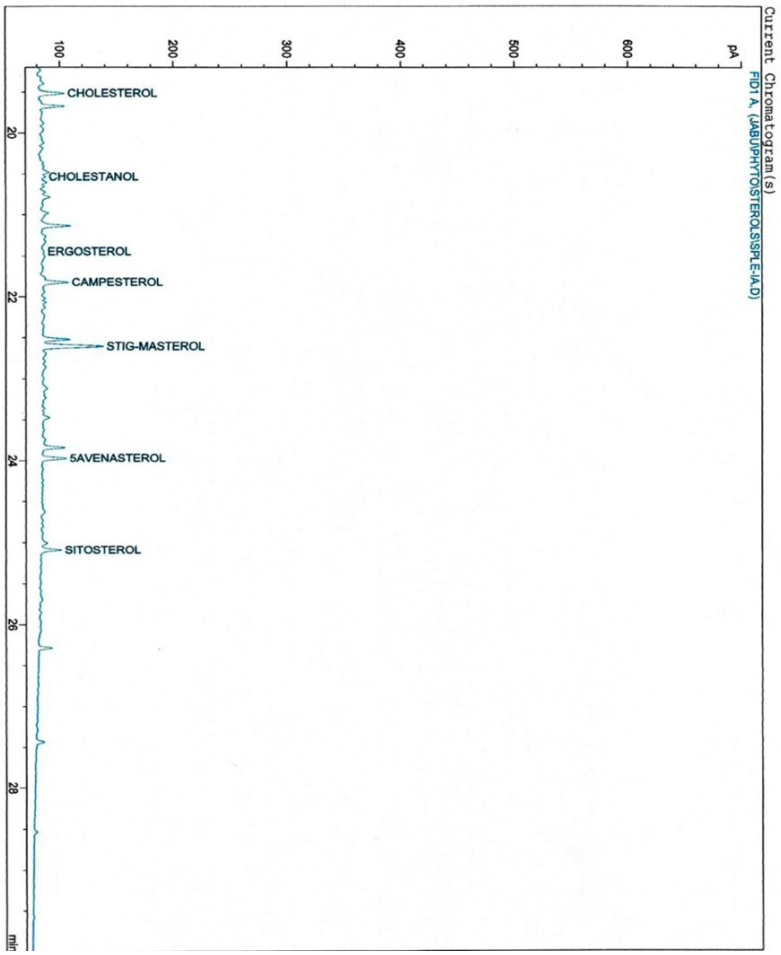

Fig 2. Chromatogram of bioactive phytosterol in *Ipomoea asarifolia*

The fatty acid compositions shown in Figure 3 are caproic acid, carpylic acid, decanoic/carpic acid, lauric acid, myristic acid, tetradecanoic acid, palmitic acid, and stearic acid.
Figure 4 shows that phospholipids are made up of phosphatidylethanolamine, phophatidylcholine, phophatidylserine, lysophophatidylcholine, and phophatidylinositol.
Figure 5 displays the vitamins that are present, which are vitamins A, B1, B2, B3, B4, B6, B9, B12, C, D, E, and K.

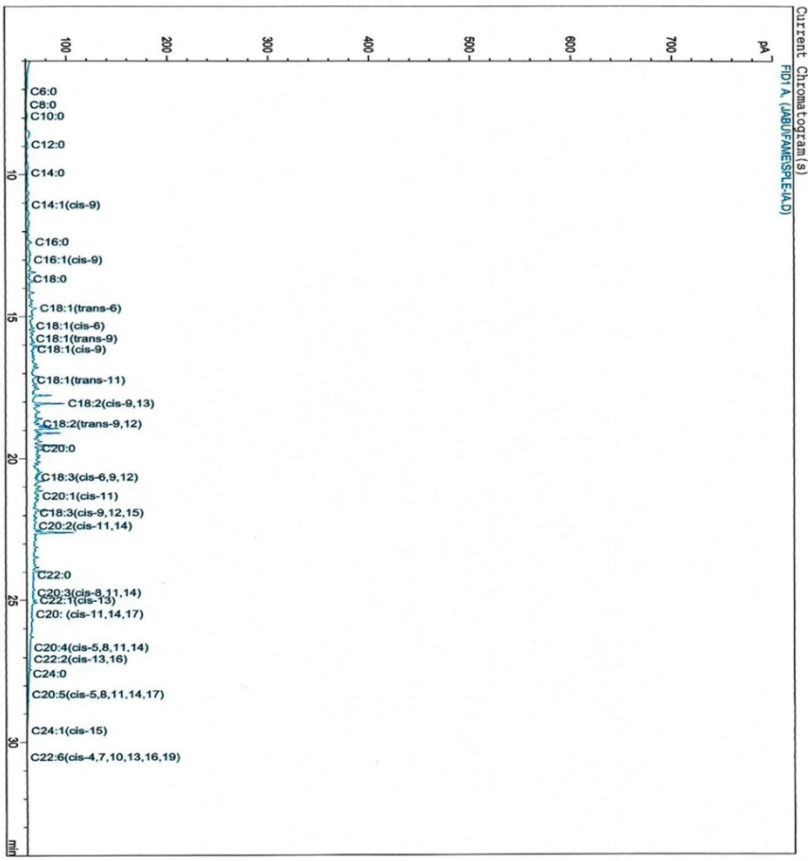

Fig 3. Chromatogram for bioactive fatty acids present in *Ipomoea asarifolia*

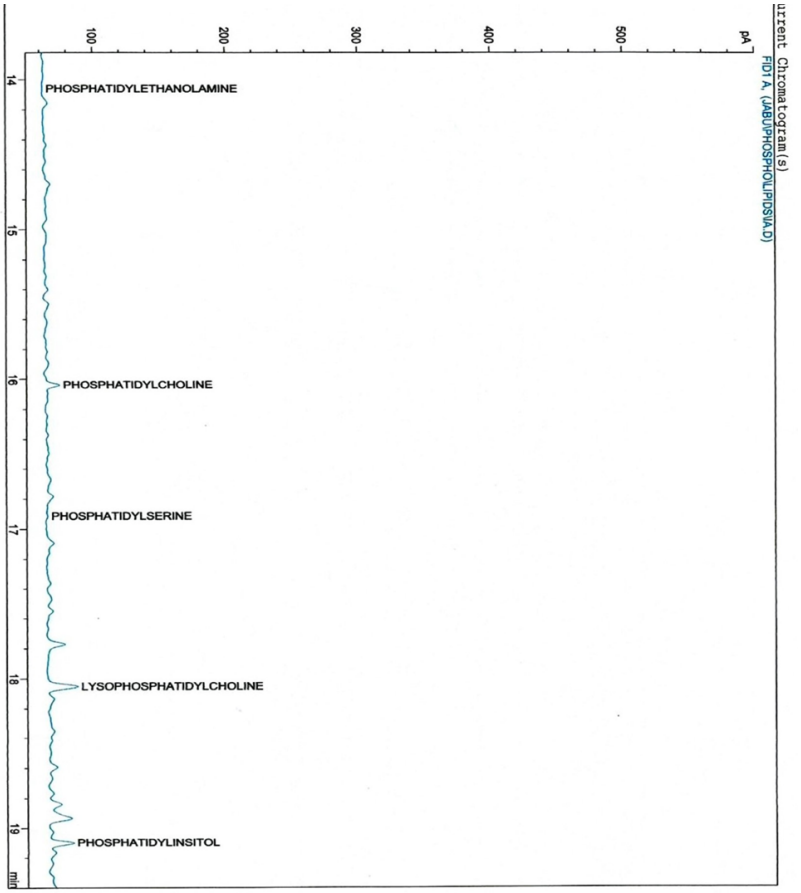

Fig 4. Chromatogram for phospholipid present in *Ipomoea asarifolia*

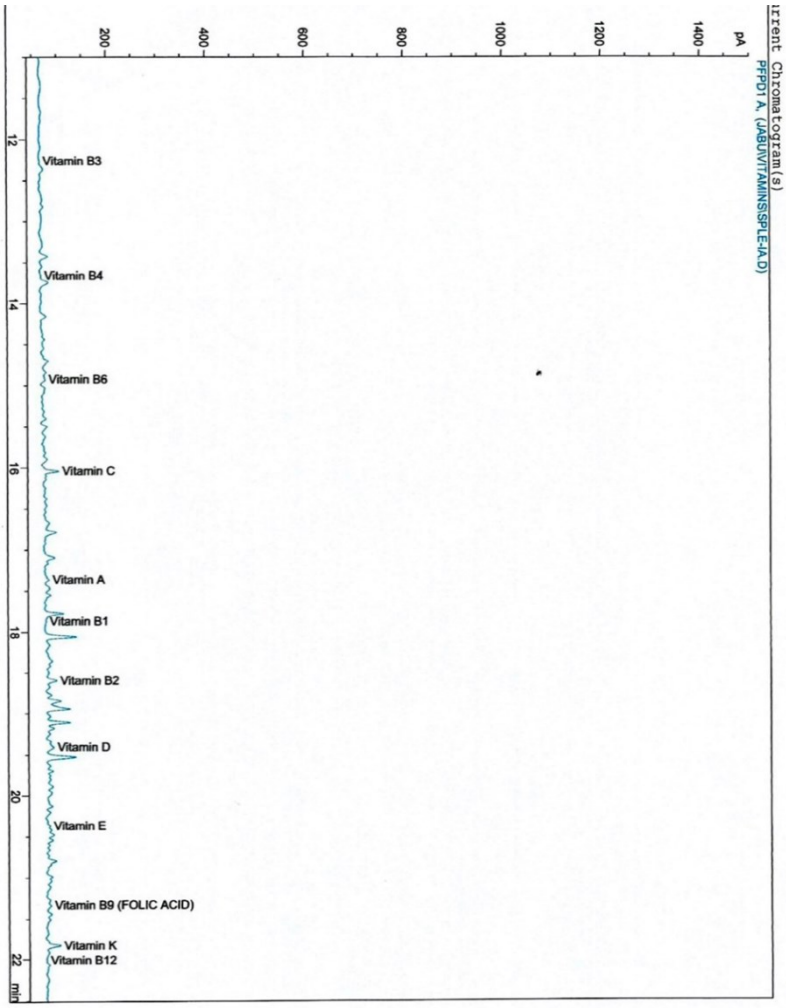

Fig 5. Chromatogram for bioactive vitamins present in *Ipomoea asarifolia*

4. DISCUSSION

The results obtained from this research work have clearly shown that the methanol extract of *I. asarifolia* (leaf, stem and whole plants) contain certain secondary metabolites such as saponin, tannin, flavonoids, phenol and alkaloids, which was earlier corroborated (Jegede et al., 2009). However, the presence of anthraquinones in the leaf of *I. asarifolia* from our study does not agree with Jegede et al. (2009). Phytochemical qualitative reports from Aliyu et al. (2011), on *I. asarifolia* leaf had similar results with our study except phenols, which were not reported in their study. In contrast to previous authors, this study reported the quantitative values of the phytochemicals. Leaf contains 0.49 mg/g, stem contains 0.62 mg/g, and whole plants contain 1.12 mg/g of alkaloids, whereas phenolics are 18.40 mg/g in leaf, 4.14 mg/g in stem, and 21.05 mg/g in whole plants.

Secondary metabolites in plants are responsible for their therapeutic activity, as reported by Cowan (1999) and Sibanda and Okoh (2007). Thus, the lower antibacterial activity of *I. asarifolia* observed can be attributed to the metabolites present. It is worth noting that the low antibacterial activity in this study could be due to the lower concentrations assayed as compared to works on medicinal plants using high concentrations.

The antibacterial activity of *I. asarifolia* methanol leaf extract as reported by Aliyu et al. (2011) using varying concentrations (12.5, 25, 50, 100, and 200 mg/mL) against *E. coli*, *S. aureus* and *P. aeruginosa* revealed the zone size inhibition ranging between 6.0 and 17.5mm against *S. aureus* (at 12.5, 25, 50, 100 mg/mL), zone size inhibition against *E. coli* ranges between 6.0 mm and 14.0 mm (at 200 mg/mL) while the zone size inhibition against *P. aeruginosa* was 6.0 mm on all the concentrations. This study had a similar trend using lower concentrations of the methanol extract of the leaf, and stem. However, in the present study, we observed that the whole plant had a zone-size antibacterial activity of 8 mm against *S. aureus* and *P. aeruginosa* for all the concentrations used which were not reported elsewhere.

As shown in Figure 6, the spectra of the leaves and stem are similar, with few variations. Characteristic absorption bands in the leaves and stem identified various functional groups. Strong absorption bands are seen in the leaves at 3419.95 cm^{-1} O–H stretch (Stuart, 2005), medium absorption bands are seen at 2923.53 cm^{-1} C–H stretch. These are asymmetrical stretching vibrations of methylene groups in lipids (Stuart, 2005; Zeier and Schreiber, 1999).

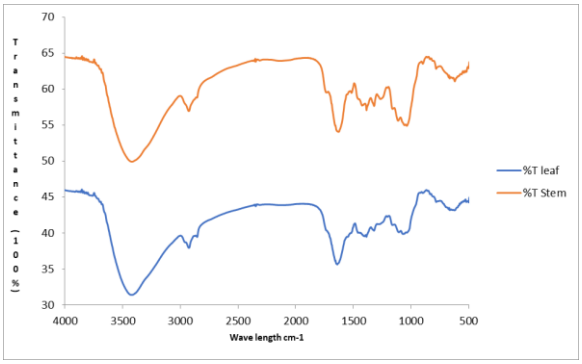

Fig. 6. FTIR spectra of leaf and stem of *Ipomoea asarifolia.*

The strong absorption band at 1637.83 cm^{-1} alkene C=C stretch indicates lignin (Morán et al., 2008), at 1384.64 cm^{-1} of methyl CH_3 group representing saturated aliphatic alkyl molecules. One type of OH in the bend plane is either a primary or secondary alcohol. Another type is linked to the C-O stretching mode of the C-OH groups of serine, threosine, and tyrosine in proteins (Fujioka et al., 2004). Finally, the C-F stretch is of aliphatic organohalogen compounds at 1064.03 cm^{-1}. The 892.65 cm^{-1} C-H is an out-of-plane bend for aromatics, while the 781.26 cm^{-1} and 620.60 cm^{-1} of C-Cl and C-Br stretch correspond to aliphatic organohalogen compounds (Nandiyanto et al., 2019). The spectrum of the stem shows absorption bands at 3416 cm^{-1} O-H stretch (Stuart, 2005), 2924.58 cm^{-1} C-H asymmetric and symmetric stretch of methylene groups in lipids (Stuart, 2005; Zeier and Schreiber, 1999), and 1627.33 cm^{-1} is indicative of oleifinic unsaturation in C=C bond stretching vibration (Morán et al., 2008). At 1384.30 $cm^{-1}$the methyl (CH_3) corresponds to a saturated alkyl group, while at 1318.47 cm^{-1}, the OH in the bend plane is a hydroxyl group of primary or secondary alcohol (Coates, 2000). At 1156 cm^{-1} is the C-N stretching in secondary amino acids of protein molecules (Coates, 2000). The fingerprint regions showed characteristic absorption bands at 1156.5 cm^{-1} C-N stretching of secondary amino acids, while 1053.9 cm^{-1} is indicative of C-O stretching coupled with C-O bending of the C-OH of carbohydrates (Wang et al., 1997). The weak absorption band at 895.58 cm^{-1} signifies C-H out of the plane bend of vinylidene compounds. Other absorption bands at 778.32 $cm^{-1}$and 618.89 cm^{-1} are C-Cl stretch and C-Br stretch, respectively, signifying the presence of aliphatic organohalogen compounds (Nandiyanto et al., 2019). The absorption band at 1731 cm^{-1} in the stem implies the presence of a carbonyl compound associated with the ester bond, while aldehyde is absent in the leaf (Coates, 2000). The presence of C-N stretch at 1200 cm^{-1} which is peculiar to tertiary amine in the leaf is however absent in the stem. Non-detection of absorption in the region 2300–1990 cm^{-1} of both samples implies the absence of nitrogen compounds such as cyanides (nitriles), cyanates, isocyanates, thiocyanates, and diazo compounds in the stem and leaf. The appearance of moderate to intense absorption bands within the range of 1600–1300, 1200–1000, and 800–600 cm^{-1} indicates a high amount of hydroxyl group compounds in the stem and leaf of *I. asarifolia* (Coates, 2000). The identified compounds revealed and confirmed that *I. asarifolia* is a very rich medicinal plant, and many of the compounds present have been verified in the literature to have great biological activities like anti-inflammatory, antidiabetic, anticancer, antimicrobial, antifungal, and antioxidant activities, among others (Asha et al., 2017). Owing to the paucity of information on this plant, further research into the use of purified bioactive compounds, their toxicological effects, and mechanisms of action on the host immune system should be considered.

5. CONCLUSION

The biological activities of compounds present in *Ipomoea asarifolia* leaf, stem and whole plant extracts form the basis for application of this plant in treating wound infection. The study revealed major bioactive compounds, vitamins and other phytonutrients present in the different parts of the plant extracts. Identification of these essential compounds, form the basis in determining possible health benefits of the plant, leading to further biologic and pharmacologic studies.

Significance Statement

This study discovered essential amino acids and phytonutrients that are necessary for human growth and development, can be beneficial for wound therapy, and serve as a good alternative to orthodox medicine. This study will help the researchers uncover the critical areas of mechanism of action, therapeutic dose, and toxic dose of *I. asarifolia*. Thus, the researchers can inevitably discover a new drug for animal and clinical trials.

ETHICAL APPROVAL

The authors wish to appreciate the University ethical committee for approving this study under the guidance of the Department of Plant Science and Biotechnology, Federal University Oye-Ekiti.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ACKNOWLEDGMENTS

The authors wish to the technical staff of the department of Biological Sciences, Joseph Ayo Babalola University, Ikeji-Arakeji, Osun State, Nigeria and department of Microbiology, Federal University Oye-Ekiti, Ekiti State, Nigeria for their technical support.

CONFLICT OF INTEREST

The authors wish to declare that there is no conflict of interest on this study.

REFERENCES

Al-Huqail, A. A., Elgaaly, G. A., and Ibrahim, M. M. (2018): Identification of bioactive phytochemical from two Punica species using GC–MS and estimation of antioxidant activity of seed extracts, *Saudi Journal of Biological Sciences*, **25**(7), 1420–1428. https://doi.org/10.1016/j.sjbs.2015.11.009

Aliyu, M. S., Lawal, U., Tijjani, M. B., Doko, M. H. I., and Garba, I. (2011): Phytochemical and antibacterial properties of leaf extracts of *Lpomoea asarifolia*, *Nigerian Journal of Basic and Applied Science*, **19**(2), 236–240.

Asha, K. R., Priyanga, S., Hemmalakshmi, S., and Devaki, K. (2017): GC-MS Analysis of the Ethanolic Extract of the whole Plant *Drosera indica* L, *International Journal of Pharmacognosy and Phytochemical Research*, **9**(5), 685–688.

Banu, K. S., and Cathrine, L. (2015): General techniques involved in phytochemical analysis, *International Journal of Advanced Research in Chemical Science (IJARCS)*, **2**(4), 25–32.

Burkill, H. M. (1985): *The useful plants of west tropical africa* (2nd ed.), Royal Botanic Gardens, Kew, Richmond, United Kingdom, 960.

Casuga, F. P., Castillo, A. L., and Corpuz, M. J.-A. T. (2016): GC–MS analysis of bioactive compounds present in different extracts of an endemic plant *Broussonetia luzonica* (Blanco) (Moraceae) leaves, *Asian Pacific Journal of Tropical Biomedicine*, **6**(11), 957–961. https://doi.org/10.1016/j.apjtb.2016.08.015

Coates, J. (2000): Interpretation of infrared spectra, a practical approach, 10815–10837 *in* R. A. Meyers, ed., *Encyclopedia of analytical chemistry*, John Wiley & Sons Ltd, Chichester.

Cowan, M. M. (1999): Plant Products as Antimicrobial Agents, *Clinical Microbiology Reviews*, **12**(4), 564–582. https://doi.org/10.1128/CMR.12.4.564

Deshpande, S. N., and Kadam, D. G. (2013): GCMS analysis and antibacterial activity of *Piper betle* (Linn) leaves against *Streptococcus mutans*, *Asian J Pharm Clin Res.*, **6**(Suppl 5), 99–101.

Falode, J. A., Obafemi, T. O., Akinmoladun, A. C., Olaleye, M. T., Boligon, A. A., and Athayde, M. L. (2016): High-Performance Liquid Chromatography (HPLC) fingerprinting and comparative antioxi-

dant properties of fruit and leaf extracts of Kigelia Africana, *IJPPR*, **8**(10), 1645–1656.

Fatope, M. O. (2001): Natural Products: Looking backward and looking forward.

Fujioka, N., Morimoto, Y., Arai, T., and Kikuchi, M. (2004): Discrimination between normal and malignant human gastric tissues by Fourier transform infrared spectroscopy, *Cancer Detection and Prevention*, **28**(1), 32–36. https://doi.org/10.1016/j.cdp.2003.11.004

Gujjeti, R. P., and Mamidala, E. (2013): Phytochemical screening and thin layer chromatographic studies of *Aervalanata* root extract, *International Journal of Innovative Research in Science, Engineering and Technology*, **2**(10), 5725–5730.

Gul, R., Jan, S. U., Faridullah, S., Sherani, S., and Jahan, N. (2017): Preliminary Phytochemical Screening, Quantitative Analysis of Alkaloids, and Antioxidant Activity of Crude Plant Extracts from *Ephedra intermedia* Indigenous to Balochistan, *The Scientific World Journal*, **2017**, 1–7. https://doi.org/10.1155/2017/5873648

Gupta, M., Thakur, S., Sharma, A., and Gupta, S. (2013): Qualitative and quantitative analysis of phytochemicals and pharmacological value of some dye yielding medicinal plants, *Oriental Journal of Chemistry*, **29**(2), 475–481.

Gurib-Fakim, A. (2006): Medicinal plants: Traditions of yesterday and drugs of tomorrow, *Molecular Aspects of Medicine*, **27**(1), 1–93. https://doi.org/10.1016/j.mam.2005.07.008

Gutteridge, J. M. C. (1984): Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate, *Biochemical Journal*, **224**(3), 761–767. https://doi.org/10.1042/bj2240761

Iqbal, E., Salim, K. A., and Lim, L. B. L. (2015): Phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of *Goniothalamus velutinus* (Airy Shaw) from Brunei Darussalam, *Journal of King Saud University - Science*, **27**(3), 224–232. https://doi.org/10.1016/j.jksus.2015.02.003

Jagessar, R. C., and Allen, R. (2012): Phytochemical screening and atomic absorption spectroscopic studies of solvent type extract from leaves of *Terminalia catappa* (Almond), *Natural and Applied Sciences*, **3**(3), 16–26.

Jegede, I. A., Nwinyi, F. C., Ibrahim, J., Ughabe, G., Zarma, S. D., and Kunle, O. F. (2009): Investigation of phytochemical, anti-inflammatory and anti-nociceptive properties of *Lpomoea asarifolia* leaves, *Journal of Medicinal Plant Research*, **3**(3), 160–164.

Karou, D., Savadogo, A., Canini, A., Yameogo, S., Montesano, J. S., Colizzi, V., and Traore, A. S. (2006): Antibacterial activity of alkaloids from *Sida acuta*, *African Journal of Biotechnology*, **5**(2), 195–200.

Morán, J. I., Alvarez, V. A., Cyras, V. P., and Vázquez, A. (2008): Extraction of cellulose and preparation of nanocellulose from sisal fibers, *Cellulose*, **15**(1), 149–159. https://doi.org/10.1007/s10570-007-9145-9

Nandiyanto, A. B. D., Oktiani, R., and Ragadhita, R. (2019): How to Read and Interpret FTIR Spectroscope of Organic Material, *Indonesian Journal of Science and Technology*, **4**(1), 97. https://doi.org/10.17509/ijost.v4i1.15806

Ojo, S. K. S., Esumeh, F. I., Osanyinlusi, S. A., and Jeje, T. O. (2017): Phytochemical and antibacterial properties of *Diodia scandens* and *Phyllanthus amarus* on staphylococci isolated from patients in tertiary hospitals in Nigeria, *Journal of Medicinal Plants for Economic Development*, **1**(1). https://doi.org/10.4102/jomped.v1i1.7

Payum, T. (2016): GC- MS Analysis of Mussaenda roxburghii Hk.f.: A Folk Food Plant Used Among Tribes Of Arunachal Pradesh, India., *Pharmacognosy Journal*, **8**(4), 395–398. https://doi.org/10.5530/pj.2016.4.14

Senguttuvan, J., Paulsamy, S., and Karthika, K. (2014): Phytochemical analysis and evaluation of leaf and root parts of the medicinal herb, *Hypochaeris radicata* L. for *in vitro* antioxidant activities, *Asian Pacific Journal of Tropical Biomedicine*, **4**, S359–S367. https://doi.org/10.12980/APJTB.4.2014C1030

Sheik Uduman, M. S. T., Rathinam, P., Karuru, Y., Obili, G., Chakka, G., and Janakiraman, A. K. (2016): GC-MS Analysis of Ethyl Acetate Extract of Whole Plant of *Rostellularia diffusa*, *Pharmacognosy Journal*, **9**(1), 70–72. https://doi.org/10.5530/pj.2017.1.13

Sibanda, T., and Okoh, A. (2007): The challenges of overcoming antibiotic resistance: Plant extracts as potential sources of antimicrobial and resistance modifying agents, *African Journal of Biotechnology*, **6**(25).

Soladoye, M. O., and Chukwuma, E. C. (2012): Quantitative phytochemical profile of the leaves of Cissus populnea Guill. and Perr. (Vitaceae) – An important medicinal plant in central Nigeria, *Arch. of Appl. Sci. Res.*, **4**(1), 200–206.

Stuart, B. (2005): *Infrared spectroscopy*, Wiley Online Library.

Usman, H., Abdulrahman, F. I., and Usman, A. (2010): Qualitative phytochemical screening and *in vitro* antimicrobial effects of methanol stem bark extract of *Ficus thonningii* (Moraceae), *African Journal of Traditional, Complementary and Alternative Medicines*, **6**(3). https://doi.org/10.4314/ajtcam.v6i3.57178

Wadood, A. (2013): Phytochemical Analysis of Medicinal Plants Occurring in Local Area of Mardan, *Biochemistry & Analytical Biochemistry*, **02**(04). https://doi.org/10.4172/2161-1009.1000144

Wang, H. P., Wang, H.-C., and Huang, Y.-J. (1997): Microscopic FTIR studies of lung cancer cells in pleural fluid, *Science of The Total Environment*, **204**(3), 283–287. https://doi.org/10.1016/S0048-9697(97)00180-0

Wang, X. (2020): Groundless Research Published on the International Journal of Plant Sciences, *Voice of the Publisher*, **06**(04), 167–169. https://doi.org/10.4236/vp.2020.64020

Zeier, J., and Schreiber, L. (1999): Fourier transform infrared-spectroscopic characterisation of isolated endodermal cell walls from plant roots: chemical nature in relation to anatomical development, *Planta*, **209**(4), 537–542. https://doi.org/10.1007/s004250050758

Sonication of smoke tree extract-loaded liposomes: the antioxidant potential of particles

ALEKSANDRA JOVANOVIĆ[1*], MILENA MILOŠEVIĆ[2], MIROSLAV NOVAKOVIĆ[2] AND ALEKSANDAR MARINKOVIĆ[3]

[1]*Institute for the Application of Nuclear Energy INEP, University of Belgrade, Banatska 31b, 11080 Belgrade, Serbia*
[2]*Institute of Chemistry, Technology, and Metallurgy, National Institute of the Republic of Serbia, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia*
[3]*Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia*
**Corresponding author: ajovanovic@inep.co.rs*

Received: August 13, 2024
Accepted: October 24, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

With the aim to examine the radical scavenging activity of smoke tree extract-loaded liposomal particles before and after ultrasound exposure (45 or 70% amplitude and 15 or 30 min), ABTS and DPPH tests were employed. The antioxidant activity of the pure extract was 11.37 ± 0.52 μmol Trolox equivalent (TE)/mL and 79.7 ± 0.5%. Multilamellar liposomes with extract showed significantly higher antioxidant activity in both assays (12.02 ± 0.54 μmol TE/mL and 81.9 ± 0.4%) compared to sonicated liposomes (10.75-11.00 μmol TE/mL and 79.3-80.9%) and pure extract. There was no significant difference between the ABTS radical scavenging activity of the liposomes treated by different amplitudes and times, while prolonged sonication and a higher amplitude caused a significant drop in the anti-DPPH capacity of extract-loaded liposomal vesicles. The presented results and the differences between the obtained data provide a good insight into the overall antioxidant capacity of smoke tree extract-loaded multilamellar and sonicated unilamellar liposomal vesicles.

Keywords: antioxidants; liposomes; smoke tree; sonication

https://doi.org/10.61652/leksir2444003J

1. INTRODUCTION

Smoke tree (*Cotinus coggygria* Scop.) from the family Anacardiaceae is an ornamental tree or large bush that has medicinal properties and multiple biological activities (Matić et al., 2011, 2016; Teixeira Da Silva et al., 2018). The plant has a wide distribution, including southern Europe, the Mediterranean, Moldova, the Caucasus, the Himalayas, and central China (Matić et al., 2016). The species is an important source of essential oil and extracts with a wide range of health-promoting effects (Matić et al., 2016). Various properties, including antioxidant, antibacterial, antifungal, antiviral, anticancer, antigenotoxic, hepatoprotective, and anti-inflammatory have been demonstrated for all parts of the plant by in vivo and in vitro studies (Matić et al., 2011, 2016; Teixeira Da Silva et al., 2018). The antioxidant potential of smoke tree extracts and essential oil is a biological property of great interest because the mentioned formulations can preserve food, pharmaceutical, and cosmetic products from the toxic and degrading effects of oxidants and/or free radicals (Maestri et al., 2006). In traditional medicine, its syrup showed the potential to protect the liver from chemical damage, reduce the tension of the choledochal sphincter, enhance bile flow, and raise immunity (Shen et al., 1991). Ethanol infusions of the wooden parts of the smoke tree were used in the treatment of gastric ulcers, diarrhea, cancer, and eye ailments, and as a cholagogue and antipyretic agent (Matić et al., 2016).

The encapsulation of the extract in various carriers, such as liposomes, can provide a longer and controlled release of its bioactives, i.e., antioxidants, as well as their protection. Additionally, disruption of multilamellar liposomal vesicles (MLVs) using sonic energy (sonication) by ultrasound bath or probe can provide small unilamellar vesicles (SUVs) with improved characteristics (Rieth and Lozano, 2020). Therefore, in the present study, the antioxidant property of smoke tree extract-loaded liposomes (MLVs and SUVs) was examined.

2. MATERIALS AND METHODS

2.1. Extract preparation

Smoke tree (collected in Belgrade, Serbia) extract was prepared using 5 g of the wooden part (dried material was grinded in the laboratory mill) and 200 mL of 80% ethanol (Fisher Science, United Kingdom) in an ultrasound bath (Sonorex Super RK, Bandelin, Germany) for 30 min. Erlenmeyer flask (250 mL) was covered with aluminum foil to avoid light exposure and ethanol evaporation. The obtained extract was filtered through a cellulose filter (fine pore, 0.45 μm). The extract was stored at 4 °C in a dark place until further experiments.

2.2. Liposomal preparation and sonication

Smoke tree extract-loaded MLVs were prepared using a previously published proliposome procedure (Jovanović et al., 2022). Ethanol extract (20 mL) was mixed with 2 g of phospholipids (soy L-α-phosphatidylcholine, Avanti Polar Lipids, USA), and heated to 60 °C for 30 min. After cooling, ultra-pure water (Simplicity UV® water purification system, Merck Millipore, Germany) was added in small portions to a total volume of 20 mL, and the dispersion was stirred for 2 h at 800 rpm. Plain liposomes (without active compounds) were prepared as a control.

With the aim to produce SUVs, an ultrasound probe, Sonopuls (Bandelin, Berlin, Germany), at 45% amplitude for 15 min (40 s on-10 s off) or at 70% amplitude for 30 min (40 s on-10 s off) was employed. The sample temperature was 25 °C; a flask with the liposomes was continuously cooled using ice coating during the sonication and the temperature was measured and controlled.

2.3. Determination of size and zeta potential of liposomes

The obtained liposomes' particle size and zeta potential were measured in Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., Malvern, UK). Each sample was diluted 200 times and measured three times at 25 °C. The measurement was repeated after three months for the extract-loaded liposomes.

2.4. Determination of antioxidant potential (ABTS and DPPH assays)

The ABTS and DPPH radical scavenging capacity of pure smoke tree extract and obtained MLVs and SUVs with extract were examined using spectrophotometric methods. The absorbance was measured using the UV Spectrophotometer UV-1800, Shimadzu, Japan. The measurements were performed on the 1st day and after three months of storage at 4 °C. In the ABTS assay, $ABTS^{\bullet+}$ solution (2 mL) was mixed with liposomes or extract (20 μL of the solution diluted with water in a ratio 1:10) (Li et al., 2013). After 6 min of incubation in the dark, the absorbance was measured at 734 nm. The results are expressed as μmol Trolox equivalent (TE)/mL. In the DPPH assay, non-diluted liposomes or extract (20 μL) were mixed with 1.8 mL of ethanol $DPPH^{\bullet}$ radical solution (Xi and Yan, 2017). After 20 min of incubation in the dark, the absorbance was measured at 517 nm. The results are expressed as the percentage of neutralization of free DPPH radicals. All reagents used in the antioxidant assays were from Sigma Aldrich (Germany).

3. RESULTS AND DISCUSSION

In the present study, smoke tree ethanol extract was encapsulated in liposomal particles that were further exposed to sonication. The antioxidant potential of prepared liposomal systems with extract (MLVs and two types of SUVs) was examined using two antioxidant assays. The particle size of MLVs with extract was 3131 ± 17 nm, while the size of SUVs with extract was from 272.9 to 512.6 nm. The size of empty MLVs was 2125 ± 48 nm, whereas the vesicle size of empty SUVs was from 141.7 to 217.9 nm. The zeta potential of the extract-loaded liposomes amounted to –27.7 ± 0.5 mV (for MLVs) and ~ –12.8 mV (for SUVs), while the mentioned parameter was significantly lower for empty parallels (< 10 mV). The mentioned parameters did not significantly change after three months of storage at 4°C in all liposomes with extract. Namely, the size and zeta potential of MLVs with extract were 3085 ± 60 nm and –25.8 ± 1.5 mV, respectively, while the diameter and zeta potential of both types of SUVs with extract were 154.1-261.0 nm and ~ –13 mV. The antioxidant activity of pure extract (diluted to achieve the same concentration as in liposomes) was also determined. The data of the measurements are presented in Table 1.

As can be seen from Table 1, MLVs showed significantly higher antioxidant capacity in both assays (12.02 ± 0.54 μmol TE/mL and 81.9 ± 0.4%, respectively) in comparison to sonicated vesicles (10.75-11.00 μmol TE/mL and 79.3-80.9%, respectively) and pure extract (11.37 ± 0.52 μmol TE/mL and 79.7 ± 0.5%, respectively). Namely, the higher antioxidant potential of the extract encapsulated in liposomes was expected due to the presence of antioxidants (added to the phospholipid mixture used by the producer) and phosphatidylcholine. The obtained results are in agreement with the literature data where was shown a slight antioxidant effect of plain liposomal particles that originated from synthetic antioxidant compounds already presented in phospholipids, as well as phosphatidylcholine (De Luca et al., 2022). At the same time, sonication has caused a decrease in the antioxidant potential of the liposomal samples (Table 1). The ultrasound probe can cause changes in the antioxidant capacity of the sample because of its potential to generate free radicals. Hence, sonication can damage natural antioxidants, particularly during extended exposure (Horžić et al., 2012). However, there was no statistically significant difference between the anti-ABTS effect of the samples treated by different amplitudes of ultrasound waves (45 and 75%) and times (15 and 30 min).

Table 1. Antioxidant potential of multilamellar and small unilamellar (sonicated) smoke tree extract-loaded liposomes (MLVs and SUVs, respectively) and pure extract.

Sample	ABTS (μmol TE*/mL)		neutralization of DPPH radicals (%)	
	1st day	After 3 months	1st day	After 3 months
MLVs	12.02 ± 0.54^{a}	13.01 ± 0.18^{a}	81.9 ± 0.4^{a}	82.9 ± 1.0^{a}
SUVs (15 min, 45% amplitude)	10.75 ± 0.53^{b}	10.01 ± 0.29^{b}	80.9 ± 0.4^{b}	79.8 ± 0.9^{b}
SUVs (30 min, 70% amplitude)	11.00 ± 0.24^{b}	10.52 ± 0.48^{b}	79.3 ± 0.6^{c}	78.7 ± 1.1^{b}
Extract	11.37 ± 0.52^{ab}	12.03 ± 0.32^{ab}	79.7 ± 0.5^{c}	79.0 ± 0.7^{b}

*TE, Trolox equivalent; different letters in each column showed statistically significant difference ($p<0.05$, n=3, one-way ANOVA followed by Duncan's post hoc test)

Nevertheless, in the DPPH assay, a prolonged sonication period and a higher value of amplitude caused a significant drop in the antioxidant potential of smoke tree extract-loaded liposomes (Table 1). In addition, sonication of the liposomes with encapsulated compounds can result in a leakage of the entrapped components causing the reduction of liposome antioxidant activity. Considering that the two used antioxidant assays are based on various principles, reactions, and probes, and the measurements were performed at different pH values and wavelengths, the obtained data, as well as differences among them provide a good insight into the overall antioxidant potential of smoke tree extract-loaded multilamellar and small unilamellar liposomal particles. As can be seen from Table 1, the storage at 4 °C for three months did not cause significant changes in the antioxidant potential of the obtained liposomes confirming the protective role of liposomal particles on bioactive principles from smoke tree extract.

4. CONCLUSION

Smoke tree extract-loaded multilamellar and small (sonicated) unilamellar liposomal particles were prepared with the aim of providing better stability and bioavailability of the extract's bioactives, as well as their longer recovery. The multilamellar liposomal system with extract showed significantly higher antioxidant activity in both antioxidant tests compared to sonicated liposomes and pure extract. The application of prolonged sonication time and a higher amplitude resulted in significantly lower anti-DPPH activity of the liposomes, while the mentioned parameters did not significantly influence the ABTS radical scavenging potential of the samples. Future experiments should be focused on other biological properties of the obtained liposomes, including antimicrobial, anti-biofilm, anti-inflammatory, skin regeneration, and enzyme-inhibitory effects, as well as on monitoring the release of bioactive compounds in simulated skin conditions.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Science, Technological Development and Innovation of Serbia (451-03-66/2024-03/200019, 451-03-66/2024-03/200135, and 451-03-66/2024-03/200026).

CONFLICT OF INTEREST

The authors declare that they have no financial or commercial conflict of interest.

REFERENCES

De Luca, M., Lucchesi, D., Tuberoso, C. I. G., Fernàndez-Busquets, X., Vassallo, A., Martelli, G., Fadda, A. M., Pucci, L., and Caddeo, C. (2022): Liposomal Formulations to Improve Antioxidant Power of Myrtle Berry Extract for Potential Skin Application, *Pharmaceutics*, **14**(5), 910. https://doi.org/10.3390/pharmaceutics14050910

Horžić, D., Jambrak, A. R., Belščak-Cvitanović, A., Komes, D., and Lelas, V. (2012): Comparison of conventional and ultrasound assisted extraction techniques of yellow tea and bioactive composition of obtained extracts, *Food and Bioprocess Technology*, **5**(7), 2858–2870.

Jovanović, A. A., Ćujić, D., Stojadinović, B., Čutović, N., Živković, J., and Šavikin, K. (2022): Liposomal Bilayer as a Carrier of *Rosa canina* L. Seed Oil: Physicochemical Characterization, Stability, and Biological Potential, *Molecules*, **28**(1), 276. https://doi.org/10.3390/molecules28010276

Li, L., Yang, Y., Hou, X., Gu, D., Ba, H., Abdulla, R., Wu, G., Xin, X., and Aisa, H. A. (2013): Bioassay-guided separation and purification of water-soluble antioxidants from *Carthamus tinctorius* L. by combination of chromatographic techniques, *Separation and Purification Technology*, **104**, 200–207 https://doi.org/10.1016/j.seppur.2012.11.027

Maestri, D. M., Nepote, V., Lamarque, A. L., and Zygadlo, J. A. (2006): Natural products as antioxidants, 105–135 *in* I. F. Trivandrum, ed., *Phytochemistry: Advances in research*, Research Signpost, Córdoba.

Matić, S., Stanić, S., Bogojević, D., Vidaković, M., Grdović, N., Arambašić, J., Dinić, S., Uskoković, A., Poznanović, G., Solujić, S., Mladenović, M., Marković, J., and Mihailović, M. (2011): Extract of the plant *Cotinus coggygria* Scop. attenuates pyrogallol-induced hepatic oxidative stress in Wistar rats, *Canadian Journal of Physiology and Pharmacology*, **89**(6), 401–411. https://doi.org/10.1139/y11-043

Matić, S., Stanić, S., Mihailović, M., and Bogojević, D. (2016): *Cotinus coggygria* Scop.: An overview of its chemical constituents, pharmacological and toxicological potential, *Saudi Journal of Biological Sciences*, **23**(4), 452–461. https://doi.org/10.1016/j.sjbs.2015.05.012

Rieth, M. D., and Lozano, A. (2020): Preparation of DPPC liposomes using probe-tip sonication: Investigating intrinsic factors affecting temperature phase transitions, *Biochemistry and Biophysics Reports*, **22**, 100764. https://doi.org/10.1016/j.bbrep.2020.100764

Shen, Q., Shang, D., Ma, F., and Zhang, Z. (1991): Pharmacological study on anti-hepatitis effect of *Cotinus coggygria* Scop. syrup, *China Journal of Chinese Materia Medica*, **16**, 746–749.

Teixeira Da Silva, J. A., Pacholczak, A., and Ilczuk, A. (2018): Smoke tree (Cotinus coggygria Scop.) propagation and biotechnology: A mini-review, *South African Journal of Botany*, **114**, 232–240. https://doi.org/10.1016/j.sajb.2017.11.009

Xi, J., and Yan, L. (2017): Optimization of pressure-enhanced solid-liquid extraction of flavonoids from *Flos Sophorae* and evaluation of their antioxidant activity, *Separation and Purification Technology*, **175**, 170–176. https://doi.org/10.1016/j.seppur.2016.10.013

Total Phenolic Content and Radical Scavenging Potential of Celery Root and Celeriac Stalk and Leaf Extracts

ANA PLEĆIĆ[1], MARIJA GNJATOVIĆ[1] AND ALEKSANDRA JOVANOVIĆ[1*]

[1]*Institute for the Application of Nuclear Energy INEP, University of Belgrade, Banatska 31b, 11080 Belgrade, Serbia*
**Corresponding author: ajovanovic@inep.co.rs*

Received: August 16, 2024
Accepted: September 25, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

In the study, celery root and celeriac stalk and leaf extracts were prepared using heat-assisted extraction, and the total polyphenol concentration (TPC) and anti-DPPH radical potential of the obtained extracts were determined. The TPC values of the extracts were from 2.67 to 13.43 mg gallic acid equivalent/g, following the trend: ethanol celeriac leaf sample>water celeriac leaf sample>ethanol celeriac stalk sample>water celery root sample. Ethanol celery root and water celeriac stalk samples showed significantly lower TPC. The anti-DPPH activity follows the trend: ethanol celeriac leaf sample>water celeriac leaf sample>water celery root sample>water and ethanol celeriac stalk samples>ethanol celery root sample. The IC_{50} values (the concentration of the sample necessary to neutralize 50% of free radicals) varied in a range of 63.9 to 326.4 mg/mL. Both phenolic yield and antioxidant activity achieved the highest levels in the ethanol celeriac leaf sample.

Keywords: celeriac leaf, celeriac stalk, celery root, extraction, polyphenols, radical scavenging activity

https://doi.org/10.61652/leksir2444004P

1. INTRODUCTION

Celery (*Apium graveolens*), as an edible herb, was used in traditional therapy for cardiovascular diseases, high blood glucose levels, and hypertension, and as an antifungal, anti-inflammatory, anticoagulant, antioxidant, and antitumor agent (Al-Asmari et al., 2017; Tyagi et al., 2013)[1,2]. Various parts of celery are also employed in hepatic, spleen, and brain diseases, pain, and sleep problems (Al-Asmari et al., 2017). The constituents of celery include glycosides, steroids, polyphenols (furanocoumarins and flavones), as well as trace elements (sodium, potassium, calcium, and iron) (Hussain et al., 2013; Tyagi et al., 2013) Celeriac (*Apium graveolens* var. *rapaceum*) shows anticancer properties due to the presence of flavonoids, volatile oil, vitamins, and minerals in the root, stalk, and leaf(Turner et al., 2021). In addition, celeriac is a rich source of phthalides with health benefits on the central nervous system and cardiac performance, including anti-thrombotic modulation and protection against cerebral ischemia and high blood pressure (Lin et al., 2005; Turner et al., 2021).

Heat-assisted extraction represents a novel procedure for extracting different biologically active components from various herbal materials. Namely, the employment of thermal energy enhances the efficiency of the extraction by cell disruption, increasing membrane permeability, and breakdown interactions between polyphenols and other compounds, such as lipids or proteins (Jovanovic et al., 2017; Mustafa and Turner, 2011). Additionally, high temperature causes a decrease in the viscosity of the extraction solvent, allowing better penetration of the extraction medium into the plant material (Jovanovic et al., 2017; Miron et al., 2011). In comparison to traditional extraction protocols, heat-assisted extraction provides faster kinetics reducing time and energy costs (Jovanovic et al., 2017, 2017). In modern times, novel extraction procedures (ultrasound-, microwave, and enzyme-assisted extractions, sub- and supercritical extractions, extraction employing natural deep eutectic solvents, etc.) can be used for the extraction of phytoconstituents from celery root and celeriac stalk and leaf (Rahaman et al., 2023). However, the mentioned techniques require expensive devices and reagents or time-consuming pre-treatment. Since the celery root and celeriac stalk and leaf are dominantly edible plants with medicinal properties and are used in culinary, the focus of the present research was on the extraction method that can be performed in ordinary conditions without complex apparatus. Namely, heat-assisted extraction, although a traditional extraction technique, can provide a satisfactory extraction yield by using simple and one-step operational protocol. The presented manuscript also follows

world trends in the field of green chemistry, due to the employed extraction mediums (water and ethanol). Namely, according to the literature, ethanol, water or hydroethanolic mixtures are GRAS (Generally Recognized As Safe) solvents (Gil-Martín et al., 2022). In addition, celeriac stalks used for the extract preparation represent agro-food waste. Also, according to Rupérez and Toledano study (2003), both stalks alone and stalks with leaves represent two celery residues from the food industry.

Therefore, in the present study, heat-assisted extraction from *A. graveolens* root and *A. graveolens* var. *rapaceum* stalk and leaf was performed and the polyphenolic yield and DPPH radical neutralization potential of the prepared extracts were investigated.

2. MATERIALS AND METHODS

2.1. Extraction protocol

Celery root and celeriac stalks and leaves, purchased in the local market (Belgrade, Serbia), were washed and wiped off to remove excess water and subsequently cut and shredded using a mixer grinder. Fresh plant material was used for the extraction. Heat-assisted extraction from fresh and shredded celery root and celeriac stalks and leaves was performed at 60°C in the incubator shaker (IKA, Germany) and water or 30% ethanol (Fisher Science, United Kingdom), at a ratio of 1:20 g/mL (1 g of plant material and 20 mL of the extraction solvent), during 20 min. The samples were filtered and stored at 4°C before further analytical experiments.

2.2. Total polyphenol content

The total polyphenol concentration (TPC) was measured in a modified Folin-Ciocalteu method(Sari et al., 2023). The absorbance of the mixture (extract, water, Folin-Ciocalteu reagent, and sodium carbonate) was read at 765 nm against a blank (UV-VIS Spectrophotometer UV-1900i, Shimadzu, Japan) after incubation of 2 h. The data was expressed as mg of gallic acid equivalents per g of fresh herbal material (mg GAE/g). Sodium carbonate was from Fisher Science (United Kingdom), while Folin-Ciocalteu reagent and gallic acid were from Merck (Germany).

2.3. DPPH radical neutralization capacity

The antioxidant activity of the prepared extracts was examined in the DPPH test [13], and 2,2-diphenyl-1-picrylhydrazyl – DPPH was from Sigma-Aldrich (Germany). In the DPPH test, 200 μL of the extract and 1800 μL of ethanol DPPH$^{\bullet}$ radical solution was mixed. The absorbance was measured at 517 nm after the incubation of 20 min, and the data are presented as IC_{50}, the concentration of the sample necessary to neutralize 50% of DPPH radicals.

2.4. Statistical data processing

Statistical data processing was done in STATISTICA 7.0 software (one-way ANOVA and Duncan's *post hoc* test). The statistically significant differences were at $p<0.05$, n=3.

3. RESULTS AND DISCUSSION

The TPC and DPPH radical neutralization potential of celery root and celeriac stalk and leaf extracts were examined. The data are shown in Table 1. In the preliminary study, ultrasound-assisted extraction and different extraction buffers were employed but the polyphenol content and antioxidant capacity of the extracts were significantly lower in comparison to the extracts obtained using heat-assisted extraction and water-ethanol mixture (data not shown), probably because of the degradation potential of ultrasound waves, as well as production of free radicals by ultrasound.

As can be seen from Table 1, the polyphenol yield was from 2.67 to 13.43 mg GAE/g of fresh plant material, and the trend was as follows: ethanol celeriac leaf extract>water celeriac leaf extract>ethanol celeriac stalk extract>water celery root extract. Ethanol celery root and water celeriac stalk samples showed significantly lower phenolic concentrations. In the case of celery root, water extract showed significantly higher TPC in comparison to ethanol parallel. In contrast, ethanol celeriac extracts had significantly higher values of phenolics compared to aqueous extracts using both plant organs (stalks and leaves). The same trend can be observed for the antioxidant capacity (shown in Table 1). The anti-DPPH potential of the extracts follows the trend: ethanol celeriac leaf extract (73.9 ± 2.6 mg/mL)>water celeriac leaf extract (97.4 ± 2.5 mg/mL)>water celery root extract (161.9 ± 2.9 mg/mL)>water and ethanol celeriac stalk extracts (~210 mg/mL)>ethanol celery root extract (326.4 ± 9.7 mg/mL). Therefore, it can be concluded that antioxidant potential follows the trend of the phenolic content in most cases (higher TPC=higher antioxidant activity, *i.e.*, lower IC_{50} value) and both phenolic yield and antioxidant potential achieved the highest values in ethanol celeriac leaf extract. Septiana et al. (2023) have shown that the application of ethanol as an extraction solvent in celery extracts provided higher TPC and DPPH free radical scavenging potential in comparison to water. Since different extraction mediums gave the best results for different plant materials, every herbal matrix requires investigation of the appropriate extraction solvents for achieving the highest extraction efficiency in terms of TPC and antioxidant capacity (Batinić et al., 2022). Additionally, the differences in the TPC can be due to the use of various parts of the plant matrix in the process. Namely, the concentration of flavonoid compounds, as a large group of phenols with antiox-

Table 1. The total polyphenol concentration (TPC) and DPPH radical neutralization potential of celery and celeriac extracts.

Plant material	Extraction medium	TPC (mg GAE*/g)	IC_{50} DPPH (mg/mL)
Celery root	water	3.94±0.08^{d}	161.9±2.9^{c}
	30% ethanol	2.67±0.07^{e}	326.4±9.7^{e}
Celeriac stalk	water	2.75±0.13^{e}	209.3±2.6^{d}
	30% ethanol	4.66±0.17^{c}	211.5±3.3^{d}
Celeriac leaf	water	12.21±0.41^{b}	97.4±2.5^{b}
	30% ethanol	13.43±0.18^{a}	73.9±2.6^{a}

*GAE, gallic acid equivalent; IC_{50}, the concentration necessary to neutralize 50% of free DPPH radicals; analysis of variance (one-way ANOVA) and Duncan's *post hoc* test (the differences marked as different letters in each column were considered statistically significant at $p<0.05$, n=3).

idant potential, varies between different species, as well as the organ of the used herb (Anokwuru et al., 2011; Batinić et al., 2022). Golubkina et al. study (2020) reported that the TPC was higher in celery leaf and stalk samples in comparison to root parallels. Also, the study that dealt with industrial celery by-products showed that the stalk possessed a higher polyphenol yield and radical scavenging potential than the root (Beltrán Sanahuja et al., 2021). The neutralization of DPPH radicals is possible by the reducing potential of flavonoid compounds (Hirano et al., 2001) presented in the obtained extracts. According to the literature data and HPLC analysis, quercetin glucosides are the main compounds in celery root extracts, while the assay also quantified lower content of other flavonoids, including apigenin and luteolin glucosides, and naringenin (Nikolić et al., 2011). Various studies have shown that catechin, epicatechin, rutin, and quercetin are present in celery leaf extracts (Ashoush et al., 2017; Ingallina et al., 2020). Malonylated and acetylated derivatives of flavonoid compounds were also revealed by HPLC analysis in celeriac products (Kaiser et al., 2013). The fact that some extracts obtained in the present study showed better anti-radical activity despite lower TPC (the absence of a strict correlation between TPC and antioxidant activity) can be explained by the presence of some non-phenolic compounds which can enhance the reactions of neutralization of free radicals (Özyürek et al., 2011).

4. CONCLUSION

The aim of the present study was to compare different extraction mediums (water and water-ethanol mixture) and various plant parts in terms of polyphenol yield and antioxidant capacity. Therefore, celery root and celeriac stalk and leaf extracts were prepared using heat-assisted extraction, and the phenolic content and anti-DPPH radical capacity of the extracts were measured. Different extraction solvents gave the best results for different herbal sources; thus, it can be concluded that every material requires the investigation of the appropriate solvent to achieve the highest phenolic yield and antioxidant potential. The highest phenolic yield and antioxidant activity were obtained for ethanol celeriac leaf extract, therefore further analyses will be aimed to perform the chemical characterization of the above-mentioned sample and its components responsible for the radical scavenging, and other biological properties. Thus, the most prominent extract will be chosen for further experiments which should include a wider spectrum of *in vitro*, *in silico*, or *in vivo* tests related to the biological potential of the extract.

ACKNOWLEDGMENTS

The present research was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (451-03-66/2024-03/200019).

CONFLICT OF INTEREST

The authors declare that they have no financial or commercial conflict of interest.

REFERENCES

Al-Asmari, A., Athar, Md. T., and Kadasah, S. (2017): An updated phytopharmacological review on medicinal plant of arab region: *Apium graveolens* Linn, *Pharmacognosy Reviews*, **11**(21), 13. https://doi.org/10.4103/phrev.phrev_35_16

Anokwuru, C., Godswill Nduka, A., and Olusola, A. (2011): Effect of extraction solvents on phenolic, flavonoid and antioxidant activities of three Nigerian medicinal plants, *Nature and Science of Sleep*, **9**, 53–61.

Ashoush, Y. A. M., Ali, A. M. F., Abozid, M. M., and Salama, M. S. M. (2017): Comparative study between celery leaves and broccoli flowers for their chemical composition and amino acids as well as phenolic and flavonoid compounds, *Menoufia Journal of Agricultural Biotechnology*, **2**(1), 1–13. https://doi.org/10.21608/mjab.2017.175909

Batinić, P., Čutović, N., Mrđan, S., Jovanović, A., Čirić, K., Marinković, A., and Bugarski, B. (2022): The comparison of *Ocimum basilicum* and *Levisticum officinale* extracts obtained using different extraction solvents and techniques, *Lekovite Sirovine*, (42), 43–43. https://doi.org/10.5937/leksir2242043B

Beltrán Sanahuja, A., Ponce Landete, M., Domingo Martínez, M. I., Prats Moya, M. S., and Valdés García, A. (2021): Optimization of Volatile Compounds Extraction from Industrial Celery (*Apium graveolens*) By-Products by Using Response Surface Methodology and Study of Their Potential as Antioxidant Sources, *Foods*, **10**(11), 2664. https://doi.org/10.3390/foods10112664

Gil-Martín, E., Forbes-Hernández, T., Romero, A., Cianciosi, D., Giampieri, F., and Battino, M. (2022): Influence of the extraction method on the recovery of bioactive phenolic compounds from food industry by-products, *Food Chemistry*, **378**, 131918. https://doi.org/10.1016/j.foodchem.2021.131918

Golubkina, N. A., Kharchenko, V. A., Moldovan, A. I., Koshevarov, A. A., Zamana, S., Nadezhkin, S., Soldatenko, A., Sekara, A., Tallarita, A., and Caruso, G. (2020): Yield, Growth, Quality, Biochemical Characteristics and Elemental Composition of Plant Parts of Celery Leafy, Stalk and Root Types Grown in the Northern Hemisphere, *Plants*, **9**(4), 484. https://doi.org/10.3390/plants9040484

Hirano, R., Sasamoto, W., Matsumoto, A., Itakura, H., Igarashi, O., and Kondo, K. (2001): Antioxidant Ability of Various Flavonoids against DPPH Radicals and LDL Oxidation., *Journal of Nutritional Science and Vitaminology*, **47**(5), 357–362. https://doi.org/10.3177/jnsv.47.357

Hussain, M. T., Ahmed, G., Jahan, N., and Adiba, M. (2013): Unani description of Tukhme Karafs (seeds of *Apium graveolens* Linn) and its scientific reports, *International Research Journal of Biological Sciences*, **2**, 88–93.

Ingallina, C., Capitani, D., Mannina, L., Carradori, S., Locatelli, M., Di Sotto, A., Di Giacomo, S., Toniolo, C., Pasqua, G., Valletta, A., Simonetti, G., Parroni, A., Beccaccioli, M., Vinci, G., Rapa, M., Giusti, A. M., Fraschetti, C., Filippi, A., Maccelli, A., Crestoni, M. E., Fornarini, S., and Sobolev, A. P. (2020): Phytochemical and biological characterization of Italian "sedano bianco di Sperlonga" Protected Geographical Indication celery ecotype: A multi-methodological approach, *Food Chemistry*, **309**, 125649. https://doi.org/10.1016/j.foodchem.2019.125649

Jovanovic, A., Petrovic, P., Đordjevic, V., Zdunic, G., Savikin, K., and Bugarski, B. (2017): Polyphenols extraction from plant sources, *Lekovite Sirovine*, (37), 45–49. https://doi.org/10.5937/leksir1737045J

Kaiser, A., Hartmann, K. I., Kammerer, D. R., and Carle, R. (2013): Evaluation of the effects of thermal treatments on color, polyphenol stability, enzyme activities and antioxidant capacities of innovative pasty celeriac (*Apium graveolens* L. var. rapaceum (Mill.) DC.) products, *European Food Research and Technology*, **237**(3), 353–365. https://doi.org/10.1007/s00217-013-1998-6

Lin, G., Chan, S. S.-K., Chung, H.-S., and Li, S.-L. (2005): Chemistry and biological activities of naturally occurring phthalides, 611–669 *in Studies in Natural Products Chemistry*, Elsevier. https://doi.org/10.1016/S1572-5995(05)80065-1

Miron, T. L., Plaza, M., Bahrim, G., Ibáñez, E., and Herrero, M. (2011): Chemical composition of bioactive pressurized extracts of Romanian aromatic plants, *Journal of Chromatography A*, **1218**(30), 4918–4927. https://doi.org/10.1016/j.chroma.2010.11.055

Mustafa, A., and Turner, C. (2011): Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review, *Analytica Chimica Acta*, **703**(1), 8–18. https://doi.org/10.1016/j.aca.2011.07.018

Nikolić, N., Cvetković, D., and Todorović, Z. (2011): A characterization of content, composition and antioxidant capacity of phenolic compounds in celery roots, *Italian Journal of Food Science*, **23**, 214–219.

Özyürek, M., Güçlü, K., Tütem, E., Başkan, K. S., Erçağ, E., Esin Çelik, S., Baki, S., Yıldız, L., Karaman, Ş., and Apak, R. (2011): A comprehensive review of CUPRAC methodology, *Analytical Methods*, **3**(11), 2439. https://doi.org/10.1039/c1ay05320e

Rahaman, A., Kumari, A., Farooq, M. A., Zeng, X.-A., Hassan, S., Khalifa, I., Aadil, R. M., Jahangir Chughtai, M. F., Khaliq, A., Ahmad, N., and Wajid, M. A. (2023): Novel Extraction Techniques: An Effective Way to Retrieve the Bioactive Compounds from Saffron (*Crocus Sativus*), *Food Reviews International*, **39**(5), 2655–2683. https://doi.org/10.1080/87559129.2021.1967377

Rupérez, P., and Toledano, G. (2003): Celery by-products as a source of mannitol, *European Food Research and Technology*, **216**(3), 224–226. https://doi.org/10.1007/s00217-003-0663-x

Sari, K. R. P., Ikawati, Z., Danarti, R., and Hertiani, T. (2023): Microtiter plate assay for measurement of total phenolic and total flavonoid contents in medicinal plant extracts, *Arabian Journal of Chemistry*, **16**(9), 105003. https://doi.org/10.1016/j.arabjc.2023.105003

Septiana, E., Rahmawati, S. I., Izzati, F. N., Ahmadi, P., Wulandari, D. A., Bustanussalam, B., Warsito, M. F., and Putra, M. Y. (2023): Biological Activity of Celery Extract Using Different Extraction Methods, 312–326 *in* I. Nurlaila, Y. Ulfa, H. Anastasia, G. Putro, R. Rachmalina, R. Ika Agustiya, N. Sari Dewi Panjaitan, R. Sarassari, A. Lystia Poetranto, and S. Septima Mariya, eds., *Proceedings of the 1st International Conference for Health Research – BRIN (ICHR 2022)*, Atlantis Press International BV, Dordrecht. https://doi.org/10.2991/978-94-6463-112-8_30

Turner, L., Lignou, S., Gawthrop, F., and Wagstaff, C. (2021): Investigating the factors that influence the aroma profile of *Apium graveolens*: A review, *Food Chemistry*, **345**, 128673. https://doi.org/10.1016/j.foodchem.2020.128673

Tyagi, S., Chirag, P., Dhruv, M., Ishita, M., Gupta, A. K., Usman, M., Nimbiwal, B., and Maheshwari, R. (2013): Medical benefits of *Apium graveolens* (celery herb), *Journal of Drug Discovery and Therapeutics*, **1**(5), 36–38.

Total phenolic and flavonoid content in *Boswellia serrata* Roxb. resin extracts obtained with subcritical water

TANJA BREZO-BORJAN[1*] AND JAROSLAVA ŠVARC-GAJIĆ[1]

[1]*University of Novi Sad, Faculty of Technology Novi Sad, Bul. cara Lazara 1, 21000 Novi Sad, Serbia*
**Corresponding author: tanja.brezo.borjan@uns.ac.rs*

Received: October 14, 2024
Accepted: November 7, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

***Boswellia serrata* Roxb. is a tree that is mainly found in the dry regions of India. Its oleoresin, known internationally as Indian frankincense, is used in Ayurvedic, traditional Arabic and Chinese medicine. This gum resin contains 15-20% of boswellic, lupeolic and other pentacyclic triterpenic acids, of which the boswellic acids (beta-boswellic acid, keto-beta-boswellic acid and acetil-11-keto-beta-boswellic acid) have been shown to have anti-inflammatory, anticancer and antidiabetic properties and are used in the modern pharmaceutical industry. Besides its ability to prevent and treat various diseases (rheumatoid arthritis, osteoarthritis, Chron's disease, ulcerative colitis and asthma), other biological functions of *B. serrata* resin should not be neglected.**

The aim of this study was to analyze, for the first time, extracts of *B. serrata* resin obtained with subcritical water at different temperatures (110–190 °C) for their phenolic and flavonoid content. The total phenolic content (TPC) was determined by UV-spectrophotometry using the Folin-Ciocalteu method. The total flavonoid content (TFC) was also determined by UV-spectrophotometry using a simple method with $AlCl_3$.

With increasing extraction temperature, the TPC increased from 3.76 mg GAE/g DW at 110 °C to 13.78 mg GAE/g DW at 190 °C. The highest TFC was observed in the extract obtained at 170 °C (8.56 mg RE/g DW). The results of this study suggest that extracts of *B. serrata* resin obtained with subcritical water are a rich source of bioactive compounds that can be used in pharmaceuticals, dietary supplements and functional foods.

Keywords: *Boswellia serrata* Roxb. resin; subcritical water extraction; total phenolic content; total flavonoid content

https://doi.org/10.61652/leksir2444006B

1. INTRODUCTION

Frankincense is an aromatic resin obtained from trees of various species of the genus *Boswellia* by making incisions in the trunk of the trees. The resins obtained from the cuts of these trees play a role of a natural defense against wounds made in the bark, to repel herbivores and to attract animals to spread their pollen or seeds (Guta et al., 2024). The *Boswellia* genus is commonly cultivated in the dry regions throughout Africa, South Asia and the Arabian Peninsula (Camarda et al., 2007). It is used as a perfume, flavoring agent for cosmetics, in aromatherapy and as a traditional medicine to treat various diseases in Ayurvedic, traditional Arabic and Chinese medicine. Even the ancient Romans, Egyptians and Greeks recognized the therapeutic importance of frankincense (Al-Harrasi et al., 2019). More recently, frankincense has been used in European countries to treat various chronic inflammatory problems such as arthritis, chronic bowl diseases and asthma (Guta et al., 2024). The chemical profile of the oil and resin of the various *Boswellia* species is very different, which is reflected in the great chemical diversity of the various *Boswellia* species (Al-Harrasi et al., 2019). The resins of *Boswellia* species are rich in pentacyclic triterpenes (boswellic acids) and other triterpenoids (lupeol and lupeolic acids), which target pro-inflammatory signals and are responsible for its biological activity. Boswellic acids bind to IκB kinase (IKK) and inhibit its activity, leading to inhibition of the proinflammatory transcription factor NF-κB (Nuclear Factor Kapp B), while acetyl-

lupeolic acid (ALA) inhibits the kinase AKT, which also influences inflammation and oxidative stress. Inhibition of NF-κB by boswellic acids significantly reduces the expression of the important proinflammatory cytokine TNF-α in human monocytes. This provides a rational basis for the traditional use of *Boswellia* and suggests that the use of drugs containing *Boswellia* extracts may provide therapeutic benefit in inflammatory conditions (Schmiech et al., 2024).

The extremely valuable essential oil of *Boswellia serrata* Roxb., commonly known as the Indian frankincense tree, is used in the food, flavoring and perfume industries. This essential oil is rich in boswellic acid, lupeolic acid and other pentacyclic triterpenic acids (15-20%), of which beta-boswellic acid, keto-beta-boswellic acid and acetil-11-keto-beta-boswellic acid are the most important (Tironi de Castilho et al., 2023). They have been described to inhibit elastase in leukocytes, inhibit proliferation, induce apoptosis and inhibit topoisomerases (Baliga et al., 2013) and therefore they are considered to have biological activities such as anti-cancer, anti-inflammatory, anti-diabetic and antimicrobial. The pure natural substances of these acids are used in the modern pharmaceutical industry (Gupta et al., 2022). In addition to the therapeutic effects of *B. serrata* essential oil, the other biological functions of *B. serrata* resin as a whole should not be neglected, as this resin contains numerous bioactive compounds.

In general, *B. serrata* resin consists of 60–85% water insoluble pentacyclic triterpenes, 6-30% polysaccharides, and 5-9% essential oil, which is a mixture of monoterpenes and sesquiterpenes (Guta et al., 2024).

In order to fully utilize the biomedical potential of a plant material, it is very important to use a suitable extraction method (Radovanović et al., 2022). Subcritical water extraction (SWE) is suitable for the production of natural extracts due to its environmental friendliness and the safety of the final extracts by using water in the subcritical state (below the critical temperature of 374.15 °C and the critical pressure of 22.1 MPa) instead of synthetic organic solvents used in most conventional extraction processes. This technique can shorten the operating time and provides better yield and quality of the extract (Radovanović et al., 2022; Švarc-Gajić and Morais, 2022). Water is the safest and most cost-effective solvent that can change its properties by changing the temperature in SWE processes. Temperature plays a crucial role in determining extraction efficiency and selectivity (as the temperature changes, the dielectric constant, surface tension and viscosity of the water also change, altering the polarity and other properties of the solvent) (Plaza and Marina, 2023). Under subcritical conditions, it becomes an excellent solvent that can even dissolve compounds that are poorly-soluble in water at room temperature, making this technique particularly suitable for extracting compounds from the resin of *B. serrata*. On the other hand, the solubility of polar and moderately polar compounds does not change significantly.

In the scientific literature, extraction of bioactive compounds from *B. serrata* resin was performed with petroleum ether and ethyl alcohol (Alshafei et al., 2023; Sharma et al., 2016), and methanol (Gupta et al., 2021, 2022; Katragunta et al., 2019). Guta et al. (2024) performed selective extraction with subcritical and supercritical CO_2 methods from *Boswellia papayrifera* resin. To our knowledge, extraction with subcritical water has never been performed on any *Boswellia* species. With its exceptional properties of adjustable polarity, subcritical water could provide new insights into the chemical characterization of this complex material, which is insoluble in water at ambient conditions.

The aim of this study was to investigate, for the first time, the total phenolic content (TPC) and total flavonoid content (TFC) of *B. serrata* resin extracts obtained with SWE at different temperatures (110–190 °C). This study is the first step towards the characterization of *B. serrata* resin extracts obtained by SWE.

2. MATERIALS AND METHODS

2.1. Plant material and reagent

The plant material used in this study, *B. serrata* gum (Gond Kondru, Gond Kundru or Shallaki gum), was a commercial product imported from India (Sanchar Vihar Colony, Uttar Pradesh).

The sample was ground with a laboratory grinder and the obtained powder was stored in a plastic container until extraction. Gallic acid and rutin tryhydrate were purchased from Dr. Ehrenstorfer GmbH (Ausburg, Germany). Folin-Ciocalteu reagent was purchased from Lachner (Neratovice, Czech Republic). Sodium carbonate and aluminum chloride hexahydrate were purchased from Alpha Aesar GmbH & Co KG (Karlsruhe, Germany). Nitrogen under pressure (99.999%) was supplied by Messer (Bad Soden, Germany). All other chemicals were of analytical reagent grade.

2.2. Subcritical water extraction (SWE)

The SWE of the *B. serrata* resin was performed in a home-made subcritical water extractor/reactor with a high-pressure stainless steel process vessel of a total volume of 1.7 liters (Švarc-Gajić et al., 2017). The resin powder and distilled water were added to the process vessel in a ratio of 1:20. After closing, the extraction vessel was pressurized to constant pressure of 15 bar with nitrogen via the gas inlet valve installed in the lid of the vessel. The extraction vessel was placed on a heating/vibration platform. The heating rate was approximately 10 °C/min and the frequency of vibration was maintained at 3 Hz. After the operating temperature was reached (110–190 °C), the extraction time (30 min) was measured. After extraction, the process vessel was cooled to 20 ± 2 °C in a flow-through water bath and the pressure was released by opening the valve. The extracts obtained were filtered through a grade 1 Whatman filter paper, and stored in polyethylene bottles in a refrigerator (4 °C) for further analysis.

2.3. Spectrophotometric analyses

The content of polyphenols and flavonoids in the resin extracts of *B. serrata* obtained with subcritical water was determined spectrophotometrically.

The TPC was measured using the well-known Folin-Ciocalteu method (Li et al., 2007). The extract/standard solution (400 μL) was mixed with 2 mL of diluted Folin-Ciocalteu reagent (1:10, *v*/*v*). After 4 minutes, 1.6 mL sodium carbonate solution (7.5%, *w*/*w*) was added. The blank was prepared with distilled water instead of the extracts. The mixtures were incubated for 90 minutes at room temperature for color development (from yellow to indigo). The absorbance was measured at 765 nm in triplicate for each sample. Gallic acid dissolved in distilled water (0–200 mg/L) was used as a standard. The results were expressed as mg gallic acid equivalent per gramme of resin powder dry weight (mg GAE/g DW) and calculated as mean ± SD.

The TFC was measured using a simple spectrophotometric method with $AlCl_3$ (Benmerzoug et al., 2020). In brief, 2 mL of $AlCl_3$ solution (2%) was added to 2 mL of the extract/standard solution. After 10 minutes, the absorbance was measured at 430 nm. Measurements were performed in triplicate for each sample. Distilled water (2 mL) was used to prepare the blank by mixing it with $AlCl_3$. Rutin-trihydrate dissolved in distilled water (0–125 mg/L) was used as a standard. The results were expressed as mg rutin equivalent per gramme dry weight of resin powder (mg RE/g DW) and calculated as mean ± SD.

2.4. Statistical analysis

Three independent experiments were performed for each analysis. All data were expressed as means ± standard deviations (SD). A one-way analysis of variance (ANOVA: single factor test) was performed to compare the mean values and to determine significant differences ($P < 0.05$).

3. RESULTS AND DISCUSSION

Five samples of *B. serrata* resin extracts were prepared with subcritical water at five different temperatures (Table 1). The extraction time was 30 minutes, nitrogen pressure was 15 bar, rotation frequency was 3 Hz and the sample to solvent ratio was 1:20 (*w*/*w*) for all extractions. TPC and TFC were determined in the extracts obtained after appropriate dilution.

Table 1. Extracts of *B. serrata* obtained at different temperatures (110–190 °C) with subcritical water

B. serrata sample	Temperature of SW* (°C)
Extract 1	110
Extract 2	130
Extract 3	150
Extract 4	170
Extract 5	190

*SW – Subcritical Water.

As the most important parameter for extraction efficiency, the influence of temperature on the extraction of *B. serrata* resin, a complex matrix largely composed of water-insoluble compounds (Guta et al., 2024), was investigated over a wide range from 110 °C to 190 °C. As already mentioned, the polarity of water decreases with increasing temperature, as the dielectric constant, surface tension and viscosity decrease. The diffusion properties of water in the subcritical state are improved and the pressure keeps the water in its liquid state. This makes subcritical water an excellent extraction medium for less polar polyphenols and flavonoids.

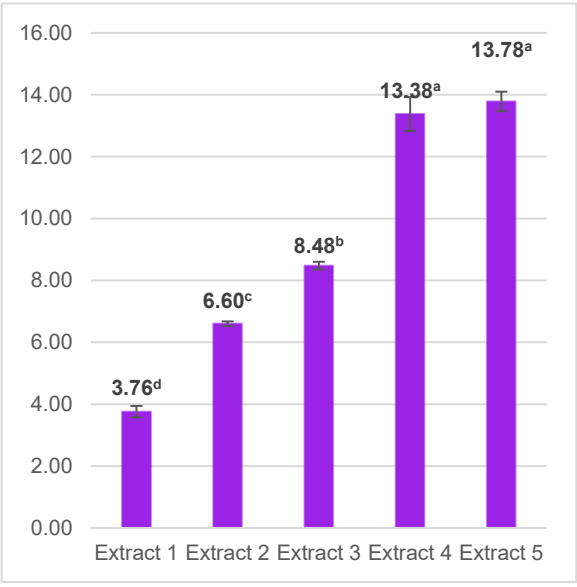

Fig. 1. The TPC (Total Phenolic Content) of *B. serrata* resin extracts obtained with subcritical water (mg GAE/g DW). The error bars indicate standard deviation (n = 3). Different letters (a, b, c, d) indicate a significant statistical difference in the observed data (P < 0.05).

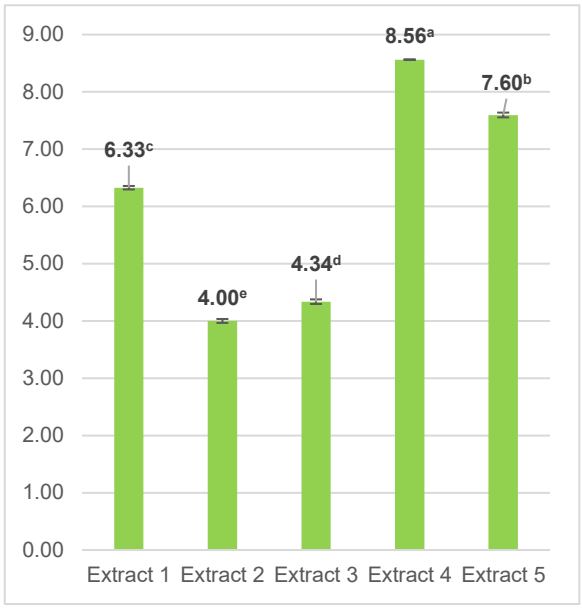

Fig. 2. The TFC (Total Flavonoid Content) of *B. serrata* resin extracts obtained with subcritical water (mg RE/g DW). The error bars indicate standard deviations (n = 3). Different letters (a, b, c, d, e) indicate a significant statistical difference in the observed data (P < 0.05)

The TPC of *B. Serrata* resin extracts is shown in Figure 1, while the TFC is shown in Figure 2.

According to the statistical analysis (ANOVA), the TPC values differed significantly ($p < 0.05$) for all extracts, except for extract 4 and extract 5. As can be seen from Figure 1, the highest TPC value (13.78 mg GAE/g DW) was obtained for Extract 5, which was close to that of extract 4 (13.38 mg GAE/g DW). These extracts were obtained at high temperatures, 190 °C and 170 °C, respectively. The sample obtained at the lowest temperature tested (Extract 1) had the lowest TPC value (3.76 mg GAE/g DW). In fact, the TPC value gradually increased with increasing temperature. This result contradicts the general knowledge that polyphenols are heat-sensitive compounds. However, in many studies oriented towards investigation of the optimal temperature for SWE, temperatures of up to 200 °C were reported (Antony and Farid, 2022; Correia et al., 2022; Palma et al., 2001; Vergara-Salinas et al., 2012; Vladić et al., 2020). A possible explanation for the results of this study could be the chemical composition of *B. serrata* resin, which consists mainly of polymers of pentacyclic triterpenes and gums consisting of polysaccharides. At elevated temperatures, these macromolecules may be degraded to simpler molecules that react with the Folin-Ciocalteu reagent and increase the TPC. The different extraction temperatures may also have affected the types of polyphenols extracted. At high temperatures, the formation of new compounds known as Maillard reaction and caramelization products can also affect the results of the spectrophotometric assay for polyphenols (Antony and Farid, 2022). These chemical reactions are not always desirable as they often lead to the formation of toxic compounds (Plaza and Marina, 2023).

As for the TFC of *B. serrata* resin, the ANOVA test showed significant differences between all extracts obtained at different temperatures. The highest value (8.56 mg RE/g DW) was observed for Extract 4 obtained at 170 °C. At a temperature of 190 °C (Extract 5), a slight decrease in the value was observed (7.06 mg RE/g DW), probably due to thermal degradation. These values were higher than the TFC values of the extracts obtained at lower temperatures (Extract 1, 2 and 3) which contradicts the fact that flavonoids as a group of polyphenols are

thermolabile compounds. Due to the chemical structure of the resin of *B. serrata*, a possible explanation for these results could be that the flavonoids are chemically and physically bound in the structure of the macromolecules of the resin. The temperatures of 170 °C and 190 °C are high enough to degrade the structure of the resin and release them during the extraction time of 30 minutes without degrading the flavonoids.

There is limited data in the scientific literature on the TPC and TFC as well as the polyphenolic profile of *B. serrata* resin. Alshafei et al. (2023) found that the TPC of *B. serrata* resin was 540.88 μg GAE/g of the aqueous extract, while the TFC was 0.118 μg QE/g of the aqueous extract of the resin. These results cannot be compared with the data in this paper as different units are used to express the content. The same authors provided a polyphenolic profile of *B. serrata* resin after HPLC analysis of the aqueous extracts and found catechin, rutin, naringenin, kaempferol, taxifolin, pyrocatechin, vanillic acid, caffeic acid, ellagic acid, cinnamic acid, and syringic acid. Gomma et al. (2019) found that the TPC in *B. serrata* resin extracts obtained with petroleum ether and ethanol was 25.84 μg GAE/mg DW, while the TFC of the same extracts was 15.09 μg RE/mg DW. These values are comparable to the results obtained in this study. The GC/MS analysis performed by the same authors confirmed the presence of phenolic compounds, mainly 2,7,8-trimethoxy-3-methyl-5,6-methylenedioxynaphtho-1,4-quinone (28.43%), terpenoid compounds including monoterpenes, diterpenes and triterpenes, the major terpenes being (E,E,10S)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadiene (18.54%) and boswellic acid (7.5%). The content of alkaloids was 5.02%. On the other hand, the chemical composition of the essential oil of the resin, which is the main carrier of bioactivity, is well studied (Ayub et al., 2018; Camarda et al., 2007). Some papers have investigated the TPC, TFC or antioxidant and anti-inflammatory activities of the leaf's extracts (Afsar et al., 2012; Subhashini Devi et al., 2014) and bark (Rao et al., 2024) of *B. serrata*.

However, to determine the individual phenolic composition of *B. serrata* resin extracts obtained by SWE and the formation of unwanted compounds due to Maillard and caramelization reactions, especially at high temperatures, further analyses with modern analytical instruments are required.

4. CONCLUSION

The present study is the first to address SWE of *B. serrata* resin, which is insoluble in water at ambient conditions, to obtain safe extracts rich in polyphenols and flavonoids. SWE is an environmentally friendly, powerful and safe technique that uses water at subcritical conditions and can extract polar and less polar compounds due to the adjustable polarity depending on the applied temperature. Samples were extracted at different subcritical water temperatures (110–190 °C). The extracts obtained were analyzed spectrophotometrically for TPC and TFC. According to the ANOVA statistical test, almost all TPC values and all TFC values differed significantly between all extracts ($p < 0.05$), indicating a strong influence of temperature on the analyzed parameters. The TPC increased from 3.76 mg GAE/g DW to 13.78 mg GAE/g DW with increasing temperature from 110 – 190° C, indicating a possible degradation of the resin macromolecule to compounds that react with the Folin-Ciocalteu reagent and increase the TPC. Since the highest TFC was observed for the extract obtained at 170 °C (8.56 mg RE/g DW), these results suggest that flavonoids may be incorporated into the structure of the resin macromolecule and released after its thermal degradation. However, these conclusions should be confirmed by further analyses of the polyphenolic profile and antioxidant activity of the extracts obtained.

The results of this study suggest that extracts of *B. serrata* resin obtained with subcritical water are a rich source of bioactive compounds such as polyphenols and flavonoids and can be considered as potential medicinal materials, dietary supplements and additives in functional foods after further chemical and biological characterization, once their efficacy and safety have been confirmed by standardized tests. In addition, further research could go in the direction of valorizing biowaste: The resin of *B. serrata*, which remains after the production of the essential oil, could be subjected to extraction with subcritical water to conduct further studies on health-promoting compounds.

ACKNOWLEDGMENTS

The Science Fund of the Republic of Serbia (project *In situ* pollutants removal from waters by sustainable green nanotechnologies – CleanNanoCatalyze, Grant No 7747845) and the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (project 451-03-66/2024-03/200134) are acknowledged.

CONFLICT OF INTEREST

The authors declare that they have no financial and commercial conflicts of interest.

REFERENCES

Afsar, V., Reddy Mohan, Y., and Saritha, K. V. (2012): In vitro antioxidant activity and anti-inflammatory activity of methanolic leaf extract of *Boswellia serrata*, *International Journal of Life Sciences Biotechnology and Pharma Research*, retrieved from internet: http://www.ijlbpr.com/currentissue.php, **1**(4).

Al-Harrasi, A., Hussain, H., Csuk, R., and Yar Khan, H. (2019): Chemistry and bioactivity of boswellic acids and other terpenoids of the genus boswellia.

Alshafei, M. M., Mabrouk, A. M., Hanafi, E. M., Ramadan, M. M., Korany, R. M. S., Kassem, S. S., and Mohammed, D. M. (2023): Prophylactic supplementation of microencapsulated *Boswellia serrata* and probiotic bacteria in metabolic syndrome rats, *Food Bioscience*, **51**, 102325. https://doi.org/10.1016/j.fbio.2022.102325

Antony, A., and Farid, M. (2022): Effect of Temperatures on Polyphenols during Extraction, *Applied Sciences*, **12**(4), 2107. https://doi.org/10.3390/app12042107

Ayub, M. A., Hanif, M. A., Sarfraz, R. A., and Shahid, M. (2018): Biological activity of *Boswellia serrata* Roxb. oleo gum resin essential oil: effects of extraction by supercritical carbon dioxide and traditional methods, *International Journal of Food Properties*, **21**(1), 808–820. https://doi.org/10.1080/10942912.2018.1439957

Baliga, M. S., Nandhini, J., Emma, F., Venkataranganna, M. V., Venkatesh, P., and Fayad, R. (2013): Indian medicinal plants and spices in the prevention and treatment of ulcerative colitis, *Bioactive Food as Dietary Interventions for Liver and Gastrointestinal Disease*, 173–185.

Benmerzoug, A., Svarc-Gajic, J., Nastic, N., Guettaf, S., and Harzallah, D. (2020): Subcritical water extraction of polyphenols from endemic Algerian plants with medicinal properties, *Acta Periodica Technologica*, (51), 191–206. https://doi.org/10.2298/APT2051191B

Camarda, L., Dayton, T., Di Stefano, V., Pitonzo, R., and Schillaci, D. (2007): Chemical Composition and Antimicrobial Activity of Some Oleogum Resin Essential Oils from *Boswellia* SPP. (Burseraceae), *Annali Di Chimica*, **97**(9), 837–844. https://doi.org/10.1002/adic.200790068

Correia, A., Silva, A. M., Moreira, M. M., Salazar, M., Švarc-Gajić, J., Brezo-Borjan, T., Cádiz-Gurrea, M. D. L. L., Carretero, A. S., Loschi, F., Dall'Acqua, S., Delerue-Matos, C., and Rodrigues, F. (2022): Salicornia ramosissima: A New Green Cosmetic Ingredient with Promising Skin Effects, *Antioxidants*, **11**(12), 2449. https://doi.org/10.3390/antiox11122449

Gomaa, A. A., Farghaly, H. S. M., El-Sers, D. A., Farrag, M. M., and Al-Zokeim, N. I. (2019): Inhibition of adiposity and related metabolic disturbances by polyphenol-rich extract of *Boswellia serrata* gum

through alteration of adipo/cytokine profiles, *Inflammopharmacology*, **27**(3), 549–559. https://doi.org/10.1007/s10787-018-0519-4

Gupta, M., Kumar, S., Kumar, R., Kumar, A., Verma, R., Darokar, M. P., Rout, P., and Pal, A. (2021): Inhibition of heme detoxification pathway in malaria parasite by 3-hydroxy-11-keto-β-boswellic acid isolated from *Boswellia serrata*, *Biomedicine & Pharmacotherapy*, **144**, 112302. https://doi.org/10.1016/j.biopha.2021.112302

Gupta, M., Singh, S., Kurmi, A., Luqman, S., Saikia, D., Thomas, M., and Rout, P. K. (2022): Correlation of boswellic acids with antiproliferative, antioxidant and antimicrobial activities of topographically collected *Boswellia serrata* oleo-gum-resin, *Phytomedicine Plus*, **2**(3), 100289. https://doi.org/10.1016/j.phyplu.2022.100289

Guta, M., Tan, H., and Zhao, Y. (2024): Selectively extracting oleoresin from *Boswellia papyrifera* frankincense resin by sub- and supercritical CO2 processes, *The Journal of Supercritical Fluids*, **205**, 106135. https://doi.org/10.1016/j.supflu.2023.106135

Katragunta, K., Siva, B., Kondepudi, N., Vadaparthi, P. R. R., Rama Rao, N., Tiwari, A. K., and Suresh Babu, K. (2019): Estimation of boswellic acids in herbal formulations containing *Boswellia serrata* extract and comprehensive characterization of secondary metabolites using UPLC-Q-Tof-MSe, *Journal of Pharmaceutical Analysis*, **9**(6), 414–422. https://doi.org/10.1016/j.jpha.2019.09.007

Li, H., Cheng, K., Wong, C., Fan, K., Chen, F., and Jiang, Y. (2007): Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae, *Food Chemistry*, **102**(3), 771–776. https://doi.org/10.1016/j.foodchem.2006.06.022

Palma, M., Piñeiro, Z., and Barroso, C. G. (2001): Stability of phenolic compounds during extraction with superheated solvents, *Journal of Chromatography A*, **921**(2), 169–174. https://doi.org/10.1016/S0021-9673(01)00882-2

Plaza, M., and Marina, M. L. (2023): Pressurized hot water extraction of bioactives, *TrAC Trends in Analytical Chemistry*, **166**, 117201. https://doi.org/10.1016/j.trac.2023.117201

Radovanović, K., Gavarić, N., Švarc-Gajić, J., Brezo-Borjan, T., Zlatković, B., Lončar, B., and Aćimović, M. (2022): Subcritical Water Extraction as an Effective Technique for the Isolation of Phenolic Compounds of *Achillea* Species, *Processes*, **11**(1), 86. https://doi.org/10.3390/pr11010086

Rao, H., Maurya, A., Kumar Raidas, H., Koram, B., Kumar Goswami, R., Singh Rajpoot, V., Khute, S., Subash, P., Chandra Mandal, S., Saha, S., and Rao Kareti, S. (2024): *In Silico* Exploration of Potential Phytoconstituents from the Bark Extract of *Boswellia serrata* for Hemorrhoidal Disease: Molecular Docking and Molecular Dynamics Analysis**, *Chemistry & Biodiversity*, **21**(2), e202301416. https://doi.org/10.1002/cbdv.202301416

Schmiech, M., Abdel-Kahaar, E., Ulrich, J., Pfeiffer, M., Duweb, A., Zolk, O., Syrovets, T., and Simmet, T. (2024): Single-dose comparative pharmacokinetic/pharmacodynamic study of a micellar formulation versus a native *Boswellia serrata* dry extract in healthy volunteers, *Phytomedicine*, **132**, 155863. https://doi.org/10.1016/j.phymed.2024.155863

Sharma, N., Bhardwaj, V., Singh, S., Ali, S. A., Gupta, D. K., Paul, S., Satti, N. K., Chandra, S., and Verma, M. K. (2016): Simultaneous quantification of triterpenoic acids by high performance liquid chromatography method in the extracts of gum resin of *Boswellia serrata* obtained by different extraction techniques, *Chemistry Central Journal*, **10**(1), 49. https://doi.org/10.1186/s13065-016-0194-8

Subhashini Devi, P., Satyanarayana, B., and Tarakeswara Naidu, M. (2014): Phytochemical Screening for Secondary Metabolites in *Boswellia serrata* Roxb. and Wrightia tinctoria (Roxb.) R.Br., *Notulae Scientia Biologicae*, **6**(4), 474–477.

Švarc-Gajić, J., Cvetanović, A., Segura-Carretero, A., Linares, I. B., and Mašković, P. (2017): Characterisation of ginger extracts obtained by subcritical water, *The Journal of Supercritical Fluids*, **123**, 92–100. https://doi.org/10.1016/j.supflu.2016.12.019

Švarc-Gajić, J., and Morais, S. (2022): Recent Advances in the Development and Application of Green Extraction Techniques, *Applied Sciences*, **12**(20), 10510. https://doi.org/10.3390/app122010510

Tironi de Castilho, A. L., de Liori Teixeira, L., and Leite Rozza, A. (2023): *Boswellia serrata*, 31–40 *in* A. Amalraj, S. Kuttappan, A. C. K. Varma, and A. Matharu, eds., *Herbs, spices and their roles in nutraceuticals and functional foods*, Elsevier Inc., London.

Vergara-Salinas, J. R., Pérez-Jiménez, J., Torres, J. L., Agosin, E., and Pérez-Correa, J. R. (2012): Effects of Temperature and Time on Polyphenolic Content and Antioxidant Activity in the Pressurized Hot Water Extraction of Deodorized Thyme (*Thymus vulgaris*), *Journal of Agricultural and Food Chemistry*, **60**(44), 10920–10929. https://doi.org/10.1021/jf3027759

Vladić, J., Jakovljević, M., Molnar, M., Vidović, S., Tomić, M., Drinić, Z., and Jokić, S. (2020): Valorization of Yarrow (*Achillea millefolium* L.) By-Product through Application of Subcritical Water Extraction, *Molecules*, **25**(8), 1878. https://doi.org/10.3390/molecules25081878

Anatomical study of Japanese Knotweed (*Reynoutria japonica* Houtt., Polygonaceae): key insights for identifying an invasive species with medicinal traits

Danilo Stojanović[1*], Miloš Zbiljić[1], Jelena Brezičanin[2] and Zoran Maksimović[2]

[1]*University of Belgrade, Faculty of Pharmacy, Department of Botany, Vojvode Stepe 450, 11000 Belgrade, Serbia*
[2]*University of Belgrade, Faculty of Pharmacy, Department of Pharmacognosy, Vojvode Stepe 450, 11000 Belgrade, Serbia*
**Corresponding author: danilo.stojanovic@pharmacy.bg.ac.rs*

Received: October 25, 2024
Accepted: November 13, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

The macroscopic and microscopic characteristics of the above- and underground organs, along with features of powdered plant material of the invasive species *Reynoutria japonica* Hout., Polygonaceae, as a potential raw material for the pharmaceutical industry, were examined. No specific characteristics of the stem, root, and rhizome were identified that could independently allow for species determination. However, identifying features include the leaf midrib with its protrusions, vascular bundle arrangement and trichomes. The leaf powder is characterized by presence of epidermal cells with wavy walls, anisocytic stomata, numerous crystal druses, and conical unicellular trichomes. The elements present in the rhizome and root powder are crystal druses, cork fragments, vessels, macrosclereids, and grouped starch grains. The use of *R. japonica* as medicinal raw material could help to reduce the negative impact of this species on habitats and biodiversity in Serbia.

Keywords: *Fallopia japonica*, invasive plant, microscopy, identification, rhizome, midrib

https://doi.org/10.61652/leksir2444008S

1. INTRODUCTION

Japanese knotweed, *Reynoutria japonica* Houtt., Polygonaceae, (synonyms: *Fallopia japonica* (Houtt.) Ronse Decr., *Polygonum cuspidatum* Siebold & Zucc.), is a perennial, semi-woody plant (hemicryptophyte) native to East Asia. It differs from other allochthonous species of this genus that occur in Serbia (*R. sahalinensis* (F. Schmidt) Nakai and *R. x bohemica* Chrtek & Chrtková) by the presence of red spots on the stem, a truncate leaf base and pointed leaf tips. In Serbia almost exclusively female individuals (with sterile stamens) are present in the distribution area - sexual reproduction does not occur (Stojanović et al., 2021).

R. japonica was introduced to Europe as an ornamental plant in the middle of the 19th century. At the beginning of the 20th century, the number of naturalized populations increased sharply, which was a consequence of its frequent planting as an exotic, fast-growing ornamental plant. In the second half of the 20th century, it became established in the Balkans, and towards the end of the century its presence was confirmed in ruderal habitats of Belgrade. Today, the species is widespread in Serbia. It is registered in 270 georeferenced locations. (Jovanović et al., 2022).

The International Union for Conservation of Nature (IUCN) has classified *R. japonica* as one of the 100 most invasive species in the world due to its ability to rapidly colonize large areas in non-native environments and severely disrupt natural ecosystems. By outcompeting native species for resources, invasive species generally pose a significant threat to indigenous biodiversity. They also cause economic damage, particularly in agriculture and forestry, by reducing yields and increasing management costs. Controlling and preventing their spread is essential for maintaining ecological balance and protecting habitats (Lowe et al., 2000). Once established, it is almost impossible to remove *R. japonica* from a habitat. Due to vegetative propagation, the species has a remarkable regenerative ability. Its root system is extraordinarily well developed, with rhizomes up to 20 m long and penetrating up to 3 m deep into the soil. Even very small rhizome fragments, no

larger than 1 cm, can generate new individuals, and if buried, they can even sprout from a depth of 2 m. Seedlings can even penetrate asphalt (Alberternst and Böhmer, 2011; Barney et al., 2006; Stojanović et al., 2021).

However, *R. japonica* is used in traditional Chinese and Japanese medicine and is also a common food plant in Japan (Khalil et al., 2020; Stojanović et al., 2021). The rhizomes and roots of *R. japonica* have been reported to exhibit a spectrum of beneficial properties, such as antitumor, anti-inflammatory and antibacterial activities (Liu et al., 2022).

In China, the use of *R. japonica* dates back to ancient times. It was traditionally prepared as a decoction, powder or infusion. In a monograph on pediatric treatment from 1119, its use for treating fever and night sweats in children is described. Over the centuries, *R. japonica* was included in 77 prescriptions, either alone or as a part of herbal mixtures (Dong et al., 2016). Since 1977, Japanese knotweed has been included in the Pharmacopoeia of the People's Republic of China (under the name *Polygoni cuspidati Rhizoma et Radix*), and its root is used as an active ingredient in more than 100 formulations (Peng et al., 2013). It is also official according to the Taiwanese pharmacopoeia. The traditional use of this plant is widespread and includes the treatment of diseases of the liver, the cardiovascular system and the endocrine system (Liu et al., 2022).

In the cosmetic industry, this plant is used as a source of yellow pigment, which serve not only as a colorant but also exhibits antioxidant properties helping to protect the skin from oxidative stress and premature aging (Liu et al., 2022).

One hundred and ten biologically active compounds have been isolated and identified from the rhizome and roots of *R. japonica*, the most important of which are anthraquinones, flavonoids and stilbenes (Liu et al., 2022). Anthraquinone emodin has been shown to exhibit antiviral, antibacterial, antiallergic, immunosuppressive and hepatoprotective effects (Dong et al., 2016). The flavonoids in the root of Japanese knotweed include flavonols such as quercetin, quercitrin, isoquercitrin and hyperoside, while the leaves contain reynoutrin (quercetin-3-D-xyloside). The methanol extract of the flowers is rich in rutin, kaempferol and quercetin. These compounds contribute significantly to the antibacterial and antiviral effects, particularly rutin and quercetin, which also protect the cardiovascular system. In addition, the procyanidins isolated from the root bark enhance the overall antioxidant effect. The gallic and protocatechuic acids contained in the plant demonstrate the ability to reduce blood sugar levels and protect against cardiovascular diseases such as atherosclerosis and coronary artery disease (Liu et al., 2022).

The underground organs of *R. japonica* contain relatively high concentrations of stilbenes, including resveratrol and polydatin, both of which have important biological activity. Resveratrol is a natural plant polyphenol found in significant concentrations in grapes, berries and peanuts (Kovarova et al., 2010). Both resveratrol and its glycoside derivative polydatin exhibit beneficial effects on cancer prevention, inhibition of platelet aggregation and antioxidant protection (Liu et al., 2022).

In addition, extracts from this species have been shown to protect crops from fungal infections. The species also has the ability to accumulate significant amounts of zinc, copper and cadmium, making it a potential candidate for the decontamination of heavy metals in soil (Stojanović et al., 2021).

Considering that currently, the rhizome and root of *R. japonica* are a major source of natural resveratrol used in dietary supplements worldwide (Chen et al., 2013) this species with its widespread distribution and high utilization potential represents a remarkable opportunity as a medicinal raw material, and its harvesting could simultaneously mitigate its impact on native ecosystems. It may be more effective to control the spread of this invasive plant species by intensifying its use rather than relying on eradication measures, which can involve significant costs.

The trichome morphology in the section *Reynoutria* in Korea was studied by Moon et al. (2011), who identified three main types of trichomes: conical unicellular trichomes, uniseriate filiform trichomes, and peltate glandular trichomes. Recently, a comparative anatomical and chemical study of Korean native *Reynoutria* species was conducted by Khalil et al. (2020), however, there are certain inconsistencies concerning the anatomy of underground organs in this paper. On the other hand, Vinogradova et al. (2021) conducted a study of the taxonomic characteristics of the vegetative organs of invasive *Reynoutria* species in Central and Northern Europe, focusing on the micromorphological features of the leaf epidermis; however, they did not address internal anatomy, particularly that of underground organs.

In this paper, to characterize the organs of *R. japonica* both macroscopically and microscopically as potential medicinal raw material, we provide detailed information on the anatomy of the aboveground and underground vegetative organs (stem, leaf, rhizome and root) of this species as well as the noteworthy characteristics of the powdered plant material (pulvis).

2. MATERIALS AND METHODS

2.1. Plant material

The underground and above-ground parts of several specimens of *R. japonica* were collected on 09.11.2023. from a ruderal habitat in Serbia, near Kumodraž (on the outskirts of Belgrade) (44°45'02"N 20°29'59"E). The collected material was identified by Assistant Professor Dr. Miloš Zbiljić from the Department of Botany, Faculty of Pharmacy, University of Belgrade. The voucher specimen has been deposited in the Herbarium of the Department of Botany, Faculty of Pharmacy, University of Belgrade (HFF), under the number 4293. The collected plant material was separated into plant organs. The rhizome and root were cleaned of soil and impurities, washed and cut into slices about 1 cm long. One part of the fresh material was left to dry in a dark, well-ventilated place at room temperature, while the other, smaller part, was fixed in 50% ethanol.

2.2. Macroscopic and microscopic analysis

The anatomical features of the plant organs of *R. japonica* were studied in the laboratory of the Department of Botany, Faculty of Pharmacy, University of Belgrade. Temporary and permanent slides of the rhizome, root, stem and leaves were prepared from plant material previously fixed in 50% ethanol. For each organ, six consecutive cross-sections, approximately 15 μm thick, were obtained using the sliding microtome (Reichert, Vienna). For temporary slides, sections were stained with a general reagent according to Tucakov (Kundaković et al., 2017). This complex reagent differentially stains various plant structures according to their chemical nature. Aniline sulfate from reagent stains lignified structures yellow, while Sudan III stains suberized structures reddish-brown. It was also used to determine the characteristic features of powdered plant material. The dried plant organs were ground into a powder and then sampled using the tip of a needle and immersed in the reagent. For permanent slides, sections were decolorized with sodium hypochlorite (NaClO), rinsed, and then stained with safranin (Merck, Darmstadt, Germany) (1% w/v in 50% ethanol) for lignified and alcian blue (Acros Organics, New Jersey, USA) (1% w/v in water) for unlignified structures. Excess stain was removed by passing the sections through a series of ethanol solutions of increasing concentrations (50%, 70%, 96%, and absolute ethanol) (Sani-hem, Novi Sad, Serbia). Finally, the

sections were mounted in D.P.X. mountant (HiMedia Laboratories Pvt. Ltd. Mumbai, India) and analyzed.
Representative samples of the plant material were examined using an Olympus SZ61 stereomicroscope, and the anatomical sections, along with the features of the powdered material, were studied with a trinocular microscope Olympus BX31 and photographed with an Olympus SC30 color camera for light microscopy (Olympus Corporation, Tokyo, Japan).

3. RESULTS

3.1. Stem anatomy

Macroscopically, the annual stem of *R. japonica* is glabrous, green with red spots, cylindrical, jointed and hollow (Figure 1A).
Microscopically in a cross-section, sparse unicellular trichomes can be observed at the stem surface. The epidermal cells have a slightly convex outer wall covered with a cuticle. Few layers of collenchyma are located under the epidermis with the lowest transitioning into chlorenchyma. Clusters of calcium oxalate – crystal *druses* (about 50 μm in diameter) are scattered in the cells of the primary cortex parenchyma as well. The pericycle consists of well-developed continuous sclerenchyma, with closed collateral vascular bundles attached to its inner side. The bundles are arranged in a circular pattern, divided by sclerenchymatic parenchyma, leaving practically no medullary rays of parenchyma. Inner parenchyma of the central cylinder is consisting of large round cells with a significant number of crystal druses. In the very center, there is a cavity/canal formed by the rupture of large parenchymatous cells (Figure 2A, 2B).
In the powder obtained from the stem, fragments of sclerenchyma fiber bundles (Figure 2C), clusters of calcium oxalate in the form of crystal druses (Figure 2D), as well as elements of the xylem vessels with annular and pitted thickenings of the cell walls, can be identified (Figure 2E).

3.2. Leaf anatomy

Macroscopically, the leaf is heart-shaped, with entire margins, a truncate leaf base and pinnate-reticulate semicraspedodromous venation (Figure 3A). Leaf lamina is dark green on the upper surface, and grayish-green underside (Figure 1B). Leaf veins are yellowish, prominent on both the axial and adaxial sides. The leaf is glabrous, but non-glandular trichomes can be observed on the main and secondary veins, more frequently on the upper surface under a stereomicroscope (Figure 3B). Longitudinally along the midrib at the abaxial side two protrusion lines are observable. Additionally, small brown spots representing glands are sporadically visible on the lower surface of the leaf (Figure 3C).
Microscopically the cross-section reveals a typical dorsiventral structure of the leaf. The cells of the upper epidermis are twice as large as those of the lower epidermis. The palisade tissue is single-layered and is approximately equally represented as the spongy parenchyma. Between these layers, large cells containing crystal druses are present, spaced apart from one another (Figure 4A). The midrib rises in a triangular shape on the upper side, while it is semicircular on the lower side. Two distinct central protrusions and two faint lateral ones are visible on the lower side. On the upper triangular protrusion, there are conical unicellular trichomes (around 50 μm long) and rare multicellular, uniseriate filiform trichomes (around

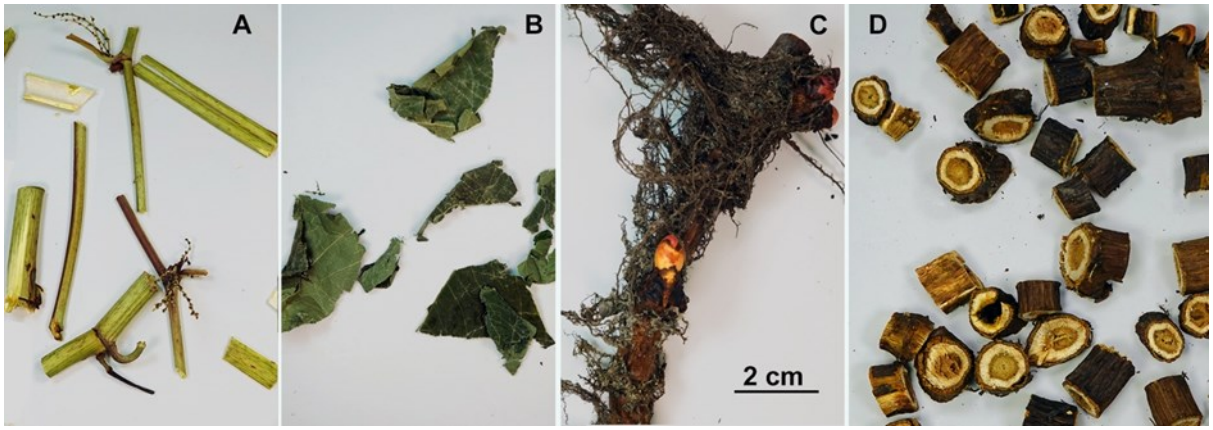

Fig. 1. Desicated vegetative organs of *R. Japonica;* A - annual stem; B – leaf; C - rhizome with roots; D - pieces of rhizome.

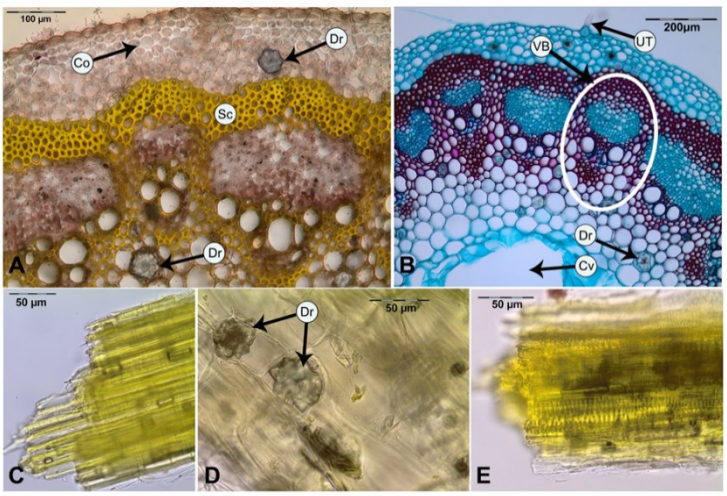

Fig. 2. Anatomical features of the stem of *R. Japonica;* A – cross section of the stem stained with general reagent by Tucakov; B - cross section of the stem stained with safranin and alcian blue; C –sclerenchyma fibers in powdered stem; D - details of the powdered stem with crystal druses. E – fragment of xylem vessels with pitted and annular thickenings. *Abbreviations*: Co – collenchyma; Dr – druse; Sc – sclerenchyma/pericycle; VB - vascular bundle; Ut – unicelluler trichome; CV – cavity/canal in the center of the stem.

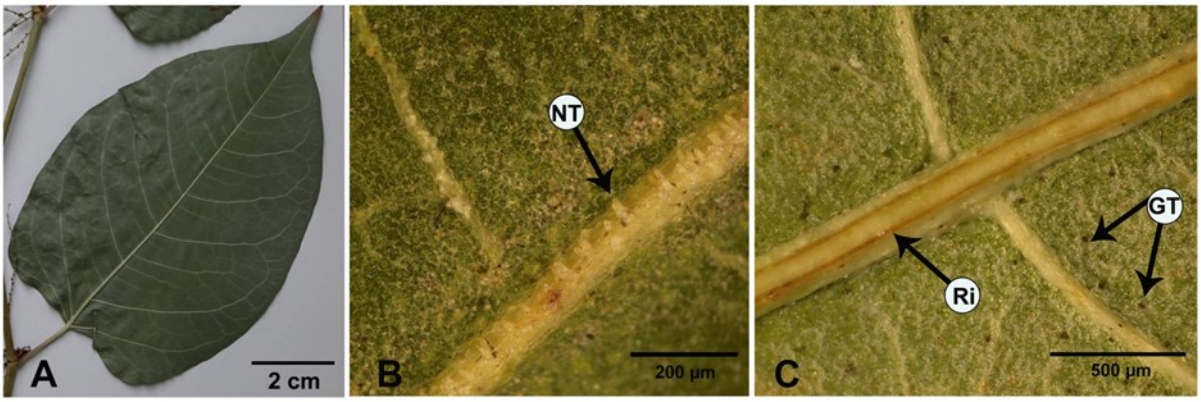

Fig. 3. Leaf of *R. Japonica;* A – shape and venation of the leaf; B – detail of the upper surface with trichomatous prominent midrib; C – midrib at the lower side with scattered glands on the surface. *Abbreviations*: Nt – nonglandular trichomes; Ri – midrib protrusion at lower side; GT – glandular trichomes.

150 µm). At the midrib, the epidermis on both the upper and lower sides is single-layered, composed of rectangular cells with a convex outer wall covered by a thin layer of cuticle. Beneath the epidermis, there are two to three layers of collenchymatous cells, and the majority of the cross-section is occupied by parenchyma with numerous scattered idioblasts containing calcium oxalate druses (app. 50 µm in diameter). Four collateral vascular bundles are arranged in a circular pattern, with the xylem oriented towards the center of the section. The largest bundle, crescent-shaped, is located on the lower side, with a smaller one opposite it on the upper side, and two small bundles on the sides. Two layers of lignified fibers are located next to the phloem (Figure 4B). Glandular trichomes of the peltate type with consisting of four-celled head and about 50 µm in diameter, are sporadically observed in the lower epidermis (Figure 4A, 4C).

In the powder obtained from the leaf, the cells of the lower epidermis with wavy anticlinal walls and anisocytic stomata (Figure 4D), druses and tracheids (Figure 4E), and striated conical unicellular trichomes (Figure 4F) can be identified.

3.3. Rhizome and root anatomy

Macroscopically, the rhizome is hard, woody, and predominantly cylindrical. The surface is slightly longitudinally grooved, dark brown in color, covered with numerous adventitious roots (Figure 1C). When sliced into short pieces, at a transversely cut surface, the bark is relatively thin, the wood is broad, white-yellowish, with radial rays. The pith is yellowish-brown, entire or cracked, rarely hollow (Figure 1D).

Microscopically, in cross-section, the outer part of the secondary cortex of the rhizome consists of the periderm with cork, four to five layers thick, which peels off at the periphery upon contact with the soil. Secondary cortex parenchyma contains some intercellular spaces resembling aerenchyma to some extent (Figure 5A). A large number of starch grains (and elaioplasts) are observed in the parenchyma of the secondary cortex at a fresh section stained with a general reagent (Figure 5B), while round bundles of sclerenchyma fibers are visible at different levels adjacent to the secondary phloem (Figure 5A, C). Crystal druses are scattered throughout the cortical parenchyma, but in the medullary rays of the phloem, they are often arranged in radial rows (Figure 5A). In the xylem, tracheids dominate the cross-section, with scattered individual vessels

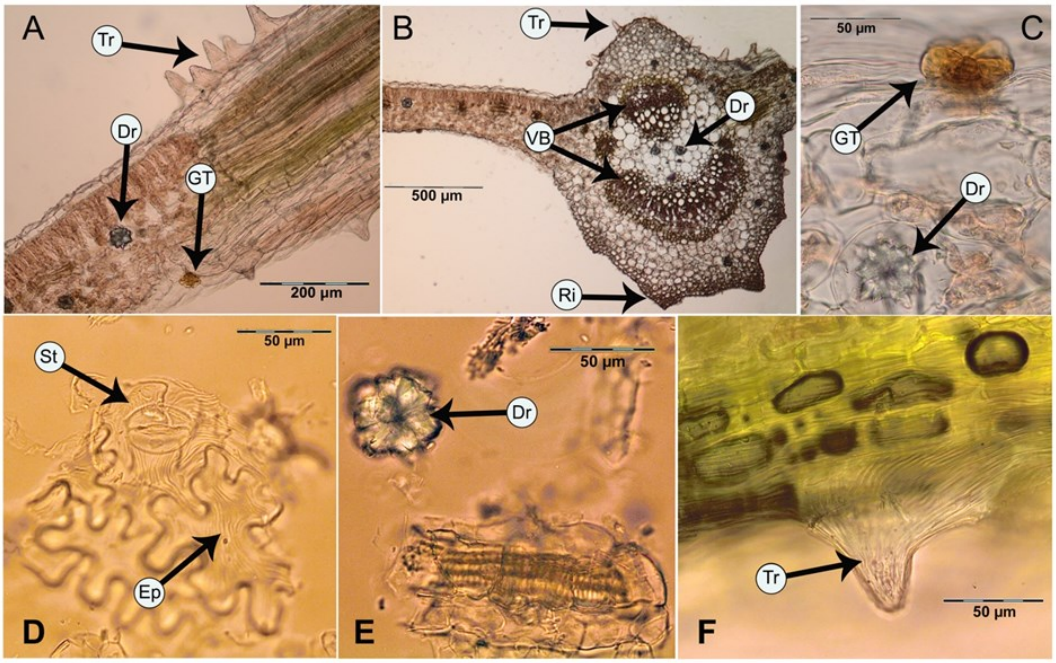

Fig. 4. Anatomical features of the leaf of *R. japonica;* A - cross section of the leaf lamina stained with general reagent by Tucakov; B - cross section of the midrib; C – lower epidermis with glandular trichome; D, E, F – details of the powdered leaf. Abbreviations: Tr – trichome; Dr – crystal druse; GT –glandular trichome/gland; VB – vascular bundles; Ri – midrib protrusion; St – anisocytic stoma; Ep – epidermal cell.

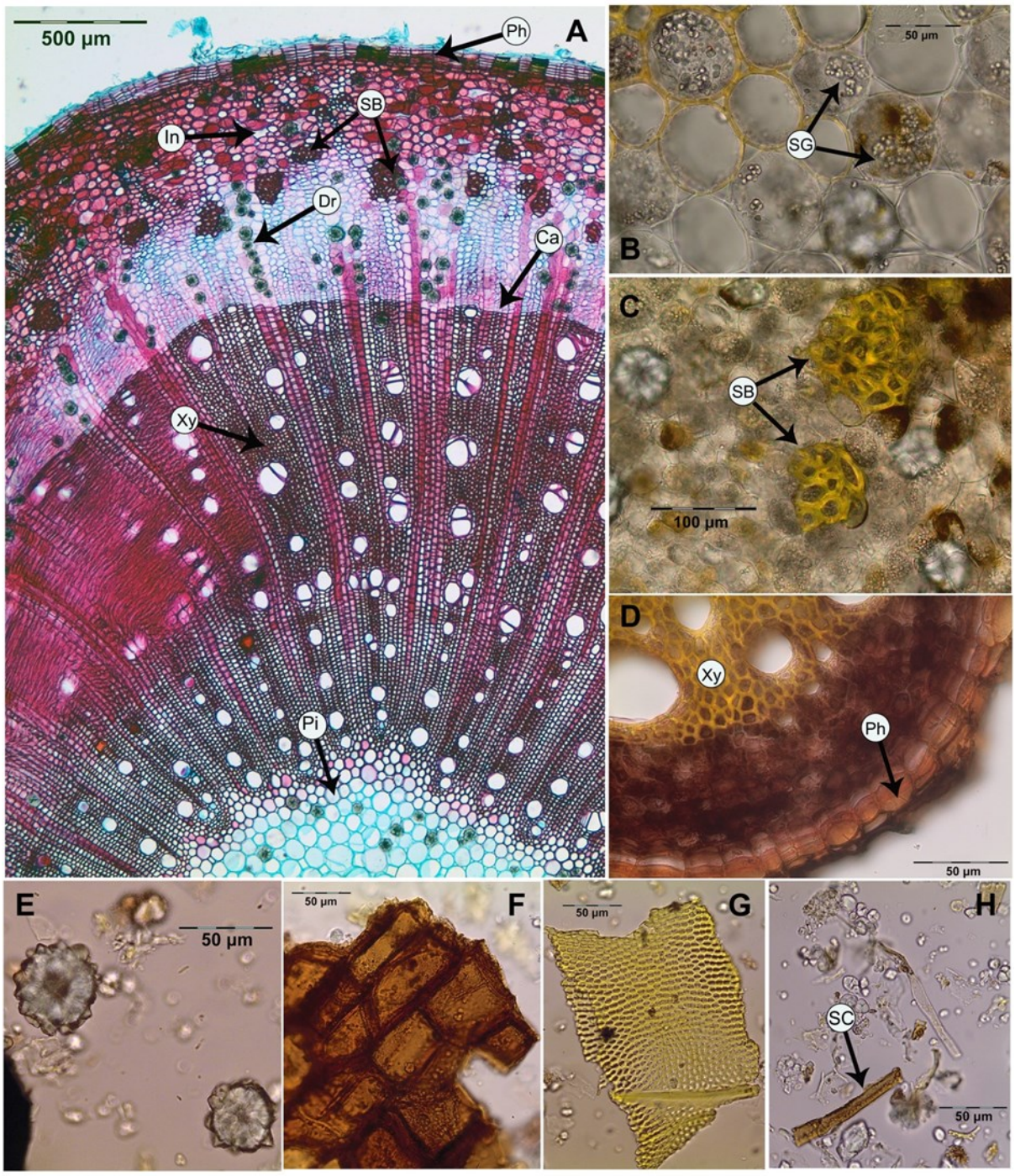

Fig. 5. Anatomical features of underground organs of *R. japonica;* A - cross section of the rhizome; B – secondary cortex parenchyma with starch grains; C – scelerenchyma fiber bundles; D - cross section of the adventitous root; E – crystal druses; F – fragments of cork tissue; G - pitted vessels; H - cylindrical stone cells. Abbreviations: Ph – cork (phelem); In – intercellular space; SG – starch grains; SB – sclerenchyma bundles; Dr – crystal druses; Ca – cambium; Xy – xylem; Pi – pith; St – starch grains in parenchyma; SC - stone cell (macrosclereid).

or, occasionally, groups of two to three. The diameter of vessels is 60–100 µm. Growth rings are hardly visible. The pith parenchyma is intact and rich in idioblasts containing large crystal druses (Figure 5A).

Unlike the rhizome, in cross-section, the adventitious root with secondary growth lacks the pith in the center, with the xylem derived from a radial vascular bundle in the core. The cortical parenchyma is rich in starch grains, with numerous scattered idioblasts containing crystal druses, and the surface is covered by periderm (Figure 5D).

In the powder obtained from the rhizome and roots, clusters of calcium oxalate in form of druses are frequent (Figure 5E), along with large fragments of cork tissue (phelem) with subpolygonal to subsquare cells (Figure 5F), as well as segments of pitted vessels from the xylem (Figure 5G). Cylindrical stone cells, approximately 50 µm in length with simple pits, are present, along with numerous individual round or elliptical starch grains often grouped into compound granules composed of 2–4 units (Figure 5H).

4. DISCUSION

The anatomical features of the above- and underground vegetative organs of *Reynoutria japonica* reflect on the one hand the life form and adaptation to moist (ruderal) habitats, and on the other hand they represent a number of characteristics that are typical for the genus *Reynoutria* and for this particular species. As a hemicryptophyte, *R. japonica* has an annual, above-

ground primary stem that is hollow in the center, while its overwintering organ, the perennial rhizome, has features of the secondary stem, as well as adaptations to habitats with moist, soft soils.

The parenchyma of the secondary cortex of the rhizome contains intercellular spaces and numerous starch grains. "Enveloped" starch grains, often grouped into compound granules of 2 to 4 units, are a characteristic feature of the starch of species from the Polygonaceae family (Czaja, 1978). Vessels with a diameter of more than 100 μm are found in the xylem, which is common in the genera *Reynoutria* and *Fallopia* within the family Polygonaceae. The relatively small number of vessels in the xylem is also a characteristic of the genus *Reynoutria* (Schweingruber et al., 2011). In addition, the scattered bundles of lignified fibers in the secondary phloem represent a synapomorphy for the genus *Reynoutria* (Khalil et al., 2020). It is interesting that Khalil et al. (2020) mistakenly described the structure of the rhizome as root anatomy, even though pith parenchyma with numerous crystal druses is present in the center of the cross-section.

However, as features of the rhizome can vary depending on age and environmental conditions of the habitat, they cannot be reliably used as diagnostic features for species identification. One of the most important anatomical diagnostic features of *R. japonica* is the shape of the leaf midrib in cross-section (Khalil et al., 2020). In particular, the prominent triangular protrusion on the upper side of the midrib, together with three to four protrusions on the lower side (two of which are even visible to the naked eye), in combination with the number and position of vascular bundles, serves as a reliable identification feature for the leaves of *R. japonica*. As suggested by Moon et al. (2011) the conical unicellular trichomes and uniseriate filiform trichomes on the midrib as well as peltate glandular trichomes with a four-celled head on the abaxial leaf surface are additional anatomical characteristics that can aid in the identification of *R. japonica*.

Since the rhizome and roots of this species are widely used in traditional medicine in East Asia and serve as a source for the extraction of active ingredients, the combined presence of various structures observed in the powdered drug may be important for quality control. For the identification of rhizome and root powder, it is necessary to microscopically identify the specific pitted vessels with a diameter of about 100 μm, crystal druses, sclereids, granules with 2–4 starch grains and segments of cork tissue.

5. CONCLUSION

Reynoutria japonica Houtt. (Polygonaceae), the highly invasive species in Serbia was collected in a typical ruderal habitat in Kumodraž, a southern suburb of Belgrade. Given the importance of the species as a source of resveratrol and other biologically active compounds, the plant organs were dried and examined macroscopically and microscopically using stereo and light microscopy to determine characteristics important for the identification of the plant as a potential medicinal raw material.

Macroscopically, the green, hollow annual stem lacks specific identifying features. However, the leaf, even if fragmented, can be identified by the yellow midrib, which is raised on the upper side with conical, non-glandular trichomes. On the underside, the midrib has two raised distinctive lines, while the flat area of the leaf contains numerous small, dark, glandular trichomes, while non-glandular trichomes are absent. The most reliable microscopic features for leaf and species identification are the shape of the midrib in cross-section and the arrangement of the vascular bundles.

Characteristic fragments in the stem powder are sclerenchyma fibers, xylem elements with annular and pitted thickenings of the cell walls as well as groups of calcium oxalate crystals in the form of large druses. In the leaf powder, the epidermal cells of the abaxial surface with waved anticlinal walls and anisocytic stomata, numerous crystal druses and conical unicellular trichomes with striated cuticular thickenings are common features.

When cleaned from adventitious roots and sliced, rhizome with its thin bark and broad white-yellowish wood macroscopically resembles many similar drugs. Microscopically, noticeable features include pith rays filled with rows of crystal druses, the wood consisting mainly of tracheids and numerous bundles of sclerenchyma fibers. Both the rhizome and the roots contain starch and numerous calcium oxalate druses. Characteristic fragments of the rhizome and root powder include crystal druses, cork fragments, pitted xylem vessels and cylindrical sclereids as well as simple starch grains, often arranged in pairs, triples or quadruples. Harvesting and utilizing the rhizomes and roots of *R. japonica* as medicinal raw material would also help to mitigate the negative impact of this species on biodiversity and ecosystems in Serbia.

ACKNOWLEDGMENTS

This research was funded by the Ministry of Science, Technological Development and Innovation, Republic of Serbia through two Grant Agreements with University of Belgrade-Faculty of Pharmacy No 451-03-65/2024-03/200161 and No 451-03-66/2024-03/200161.

CONFLICT OF INTEREST

The authors declare that they have no financial and commercial conflicts of interest.

REFERENCES

Alberternst, B., and Böhmer, H. J. (2011): NOBANIS - invasive alien species fact sheet - fallopia japonica.

Barney, J. N., Tharayil, N., DiTommaso, A., and Bhowmik, P. C. (2006): The Biology of Invasive Alien Plants in Canada. 5. *Polygonum cuspidatum* Sieb. & Zucc. [= *Fallopia japonica* (Houtt.) Ronse Decr.], *Canadian Journal of Plant Science*, **86**(3), 887–906. https://doi.org/10.4141/P05-170

Chen, H., Tuck, T., Ji, X., Zhou, X., Kelly, G., Cuerrier, A., and Zhang, J. (2013): Quality Assessment of Japanese Knotweed (*Fallopia japonica*) Grown on Prince Edward Island as a Source of Resveratrol, *Journal of Agricultural and Food Chemistry*, **61**(26), 6383–6392. https://doi.org/10.1021/jf4019239

Czaja, A. Th. (1978): The structure of starch grains and the classification of vascular plant families, *TAXON*, **27**(5–6), 463–470. https://doi.org/10.2307/1219895

Dong, X., Fu, J., Yin, X., Cao, S., Li, X., Lin, L., Huyiligeqi, and Ni, J. (2016): Emodin: A Review of its Pharmacology, Toxicity and Pharmacokinetics, *Phytotherapy Research*, **30**(8), 1207–1218. https://doi.org/10.1002/ptr.5631

Jovanović, S., Lakušić, D., and Anačkov, G. (2022): *Reynoutria houtt*, *Flora Srbije 3*, 241–249.

Khalil, A. A. K., Akter, K.-M., Kim, H.-J., Park, W. S., Kang, D.-M., Koo, K. A., and Ahn, M.-J. (2020): Comparative Inner Morphological and Chemical Studies on *Reynoutria* Species in Korea, *Plants*, **9**(2), 222. https://doi.org/10.3390/plants9020222

Kovarova, M., Bartunkova, K., Frantik, T., Koblihova, H., Prchalova, K., and Vosatka, M. (2010): Factors influencing the production of stilbenes by the knotweed, *Reynoutria xbohemica*, *BMC Plant Biology*, **10**(1), 19. https://doi.org/10.1186/1471-2229-10-19

Kundaković, T. D., Drobac, M. M., Petrović, S. D., Maksimović, Z. A., and Marčetić, M. D. (2017): *Makroskopska i mikroskopska analiza*

biljnih droga. Priručnik za praktičnu nastavu iz predmeta Farmakognozija, Univerzitet u Beogradu, Farmaceutski fakultet, Beograd.

Liu, S., Zhang, R., Zhang, X., Zhu, S., Liu, S., Yang, J., Li, Z., Gao, T., Liu, F., and Hu, H. (2022): The Invasive Species *Reynoutria japonica* Houtt. as a Promising Natural Agent for Cardiovascular and Digestive System Illness, *Frontiers in Pharmacology*, **13**, 863707. https://doi.org/10.3389/fphar.2022.863707

Lowe, S., Browne, M., Boudjelas, S., and De Poorter, M. (2000): *100 of the world's worst invasive alien species: a selection from the global invasive species database*, Invasive Species Specialist Group (ISSG) a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN), Auckland, 12.

Moon, H. K., Park, J. H., and Park, C. W. (2011): Trichome morphology of *Fallopia* sect. *Reynoutria* (Polygonaceae) in Korea, *Korean Journal of Plant Taxonomy*, **41**(1), 51–57. https://doi.org/10.11110/kjpt.2011.41.1.051

Peng, W., Qin, R., Li, X., and Zhou, H. (2013): Botany, phytochemistry, pharmacology, and potential application of *Polygonum cuspidatum* Sieb.et Zucc.: A review, *Journal of Ethnopharmacology*, **148**(3), 729–745. https://doi.org/10.1016/j.jep.2013.05.007

Schweingruber, F. H., Börner, A., and Schulze, E.-D. (2011): *Atlas of Stem Anatomy in Herbs, Shrubs and Trees*, Springer Berlin Heidelberg, Berlin, Heidelberg, 332–339. https://doi.org/10.1007/978-3-642-11638-4_1

Stojanović, V., Bjedov, I., Jovanović, I., Jelić, I., Nešić, M., Obratov, D., and Nedeljković, D. (2021): Odabrane invazivne strane vrste u flori Srbije, *Zavod Za Zaštitu Prirode Srbije, Beograd*, 151–154.

Vinogradova, Y., Kuklina, A., and Ryabchenko, A. (2021): Taxonomic characteristics of vegetative organs for invasive species of *Reynoutria*Hook, *Agrobiodiversity for Improving Nutrition, Health and Life Quality*, retrieved from internet: https://agrobiodiversity.uniag.sk/scientificpapers/article/view/358, **5**(1).

Antioxidant, antimicrobial and cytotoxic activity of selected macroalgae from the Adriatic Sea (Boka Kotorska Bay, Montenegro)

SANJA ŠOVRAN[1*], ALEKSANDAR KNEŽEVIĆ[1], JASMINA GLAMOČLIJA[2], ANA ĆIRIĆ[2], TATJANA STANOJKOVIĆ[3], VESNA MAČIĆ[4], JELENA KRIZMANIĆ[1] AND DANIJELA VIDAKOVIĆ[5]

[1]*University of Belgrade, Faculty of Biology, Studentski trg 26, 11000 Belgrade, Serbia*
[2]*University of Belgrade, Institute for Biological Research "Siniša Stanković", - National Institute of Republic of Serbia, Bulevar despota Stefana 142, 11000 Belgrade, Serbia*
[3]*Institute for Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Serbia*
[4]*Institute of Marine Biology, put I Bokeljske brigade 68, 85330 Kotor, Montenegro*
[5]*University of Belgrade, Institute of Chemistry, Technology and Metallurgy, - National Institute of Republic of Serbia, Department of Chemistry, Njegoševa 12, 11000 Belgrade, Serbia*
**Corresponding author: sanjaf@bio.bg.ac.rs*

Received: October 28, 2024
Accepted: November 8, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

In this study, the antioxidant, antimicrobial, and cytotoxic activities of four marine macroalgae, *Codium bursa, Codium effusum, Laurencia obtusa* and *Padina pavonica* from the Adriatic Sea were investigated. The antioxidant activities of the extracts were characterized by the DPPH (1,1-diphenyl-2-picrylhydrazyl) method and their total phenolic and flavonoid contents were quantified. The antimicrobial activity of four species against 8 pathogenic bacteria: *Staphylococcus aureus, Bacillus cereus, Micrococcus luteus, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella Typhimurium, Escherichia coli, Enterobacter cloacae* and 8 fungi: *Aspergillus fumigatus, Aspergillus versicolor, Aspergillus ochraceus, Aspergillus niger, Trichoderma viride, Penicillium funiculosum, Penicillium ochrochloron* and *Penicillium verrucosum* var. *cyclopium*) were assessed. The antitumor activities were determined using two different tumor cell lines (HeLa and K562). The results have shown potential antioxidant activity of *Padina pavonica*, antibacterial and antifungal activity of *Codium effusum*, and strong cytotoxic activity of *Laurencia obtusa*. The results of this study show that marine macroalgae *Codium effusum, Padina pavonica* and *Laurencia obtusa* can be a good choice in the search for new compounds with antioxidant, antimicrobial and antitumor effects.

Keywords: macroalgae, extracts, antioxidant activity, antimicrobial activity, cytotoxic activity

https://doi.org/10.61652/leksir2444010S

1. INTRODUCTION

Marine algae, traditionally classified as green algae (Chlorophyta), brown algae (Phaeophyceae), and red algae (Rhodophyta), are rich sources of structurally novel and biologically active metabolites. They are also a potential renewable resource in the marine environment (Barbosa et al., 2014). Research over the past 50 years has led to the isolation of over 15 thousand new compounds. Many of the isolates have been shown to possess biological activity. Some of the bioactive compounds are carotenoids, dietary fiber, proteins, essential fatty acids, vitamins, and minerals (Kelman et al., 2012; Negreanu-Pirjol et al., 2022; Stanojković et al., 2013).

The potential of macroalgae for functional foods or pharmaceutical applications is enormous due to their exceptional richness in bioactive compounds that can exert antioxidant, antimicrobial, anti-inflammatory, and antitumor effects (Negreanu-Pirjol et al., 2022). Reactive oxygen species (ROS) are generated in living organisms during metabolism and cause extensive oxidative damage, which in turn leads to geriatric degenerative conditions, cancer, and a variety of other human diseases (Yangthong et al., 2009). As photosynthetic

organisms, algae are exposed to a combination of light and high oxygen concentration that leads to the formation of free radicals and other oxidative reagents. The finding of no structural damage to algal organs has led the scientific community to believe that their protection from oxidation is due to their natural content of antioxidant substances or their production under stress (Kelman et al., 2012; Negreanu-Pirjol et al., 2022). In recent years, molecules from marine algae have led to promising results in studies of various cancers (Salehi et al., 2019). Numerous macroalgae have shown potent cytotoxic activities, and some authors have suggested consumption of algae as a chemopreventive agent against various cancers (Stanojković et al., 2013).

Bacteria are common pathogens in humans, as demonstrated by the wide clinical use of antibiotics. The use of antibiotics to develop resistance in pathogenic bacteria has increased at an alarming rate. Alternative prevention and treatment methods are needed, and natural sources such as plants and algae are increasingly being used (Rajauria et al., 2013). The antimicrobial activity of algal extracts has been reported by almost all groups and in different geographical areas (Pane et al., 2015). The aim of this work was to investigate the antioxidant, antimicrobial, and cytotoxic effects of methanol extracts from four marine algae: *Codium bursa*, *C. effusum*, *Laurencia obtusa* and *Padina pavonica* collected in the Boka Kotorska Bay the Adriatic Sea, Montenegro.

2. MATERIALS AND METHODS

2.1. Seaweed material

Samples of *Codium bursa, C. effusum, Laurencia obtusa* and *Padina pavonica* were collected in the Adriatic Sea, in the Bay of Kotor (Montenegro). Upon delivery to the laboratory, they were placed on ice. The seaweed samples were thoroughly washed with fresh water to remove salt, sand and epiphytes. Part of the cleaned algae was freeze-dried at -35 °C and then ground into fine powder using a grinder. The other portion of the cleaned algae was air-dried at 50 °C with forced convection for 48 hours and then ground to fine powder with a mill. The samples, both lyophilized (L) and freeze-dried (D), were stored at -20 °C until further use.

2.2. Preparation of seaweed material for *in vitro* antibiological susceptibility testing

Briefly, 30 g of sample powder (lyophilized (L) or freeze-dried (D)) was mixed with
ethanol/methanol in a 10:1 ratio (v/w). The mixture was kept at room temperature in an orbital shaking incubator for 72 hours. Each mixture was clarified by centrifugation at 3000 x for 10 minutes (Knežević et al., 2017). The supernatant was filtered with Whatman No. 4 filter paper and concentrated under reduced pressure in a rotary evaporator (BUCHI R-114, Switzerland). Prior to analyses, extracts were dissolved in appropriate solvent.

2.3. Antioxidative activity (DPPH• radical scavenging assay)

The radical scavenging activity of the extracts was determined spectrophotometrically based on the reduction of a methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) (Blois 1958). 1800.0 µL of a 4% methanol solution of DPPH- and 200.0 µL of an extract of defined concentration (series of double dilutions from 20.0 mg mL^{-1} to 0.312 mg mL^{-1}) were mixed and shaken vigorously. After incubation for 30 min in the dark, the absorbances of the reactive mixtures were measured at 517 nm against methanol as a blank using a spectrophotometer (CECIL CE2501, UK). The negative control contained all reaction reagents except the extract. The scavenging effect was calculated according to the following equation:

$$DPPH^{\bullet} scavenging\ effect\ (\%) = \frac{A_0 - A_{sample}}{A_0} \times 100$$

A_0 - the absorbance of the negative control; A_{sample} - the absorbance of the reaction mixture.

The extract concentration (mg extract mL^{-1}) giving 50% of DPPH reduction (EC 50) was determined by interpolation from linear regression analysis. The commercial antioxidant butylated hydroxyanisole (BHA) at a concentration range of 20.0-0.312 mg/mL was used as a positive control.

2.4. Total phenolic content

Total phenolic compounds in the methanol extracts of the algae were determined with the Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (1965) using gallic acid as a standard. 1000.0 µL of the 10% Folin-Ciocalteu reagent and 200.0 µL of the extract were reacted in the dark for 6 min before 800.0 µL of 7.5% Na_2CO_3 was added. The reaction mixture was shaken vigorously and incubated for 2 h in the dark at room temperature on a rotary shaker (100 rpm). The absorbance was measured spectrophotometrically at 740 nm against the blank (mixture without extract). The total concentration of phenolic compounds in the tested extracts was determined as µg gallic acid equivalents (GAE) per mg dry extract, using an equation obtained from the standard gallic acid diagram as follows:

$$Absorbance = 1.966 \times total\ phenols\ (\mu g\ of\ gallic\ acid) + 5.346\ \ (R^2 = 0.991)$$

2.5. Total flavonoid content

Total flavonoid content was determined according to the method of Park et al. (1997) using quercetin as the standard. 1000.0 µL of the extract was diluted with 4300.0 µL of mixture containing 4100.0 µL of 80% ethanol, 100.0 µL of 10% aluminium nitrate, and 100.0 µL of 1 M aqueous potassium acetate. The reaction mixture was incubated at room temperature for 40 minutes and the absorbance was measured spectrophotometrically at 415 nm. The mixture with the ethanol extract served as a blank. The amount of total flavonoids was expressed as µg of quercetin equivalents (QE) per mg of dry extract, using an equation obtained from the diagram of standard quercetin hydrate as follows:

$$Absorbance = 0.457 \times total\ flavonoid\ (quercetin\ hydrate) - 0.989\ R^2 = (0.957)$$

A total of 16 microbial pathogens (eight bacterial and eight fungal strains), were tested in this study. The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (food isolate), *Micrococcus luteus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella Typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 35210), and *Enterobacter cloacae* (human isolate) were used. The following strains of microfungi were evaluated: *Aspergillus fumigatus* (human isolate), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *A. niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112)

and *P. verrucosum* var. *cyclopium* (food isolate). All the tested microorganisms are deposited in the Mycological Laboratory, Department of Plant Physiology, Institute of Biological Research "Siniša Stanković" - National Institute of the Republic of Serbia, University of Belgrade, Serbia.

2.6. *In vitro* antimicrobial assays

2.6.1. Bacterial and fungal strains tested

A total of 16 microbial pathogens (eight bacterial and eight fungal strains), were tested in this study. The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (food isolate), *Micrococcus luteus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella Typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 35210), and *Enterobacter cloacae* (human isolate) were used. The following strains of microfungi were evaluated: *Aspergillus fumigatus* (human isolate), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *A. niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112) and *P. verrucosum* var. *cyclopium* (food isolate). All the tested microorganisms are deposited in the Mycological Laboratory, Department of Plant Physiology, Institute of Biological Research "Siniša Stanković" - National Institute of the Republic of Serbia, University of Belgrade, Serbia.

2.6.2. Microdilution method

The *in vitro* antimicrobial test was performed using the broth microdilution method (CLSI, 2015) to determine the minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and minimum fungicidal concentrations (MFC) of the tested agents. The tested samples were dissolved in 5% DMSO or physiological solution (antimicrobial drugs). The bacterial suspensions were adjusted to a concentration of 1.0×10^5 CFU/mL using sterile saline. Fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v), and the spore suspension was adjusted to a concentration of 1.0×10^5 with sterile saline. Inocula were prepared daily and stored at +4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. The MIC values obtained in the susceptibility testing of various bacteria to the extracts tested were also determined using a colorimetric assay to determine microbial viability based on the reduction of *p*-iodonitrotetrazolium violet (INT) [2-(4-iodophe nyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; Sigma Aldrich, St. Louis, MO, USA] color and compared with the positive control for the strains of the individual microorganisms. The lowest concentrations without visible growth (under the binocular microscope) were defined as MICs, for testing the various fungi. The minimum bactericidal (MBC) and fungicidal concentrations (MFC) concentrations were determined by serial sub-cultivation of 2 µL into microtiter plates with 100 µL of broth per well and further incubation at 37 °C for 24 hours for bacteria, and at 28 °C for 72 hours for fungal strains. All experiments were performed in duplicate and repeated three times. The final tested concentration of methanol extracts was in a range of 0.0625–16.0 mg/mL for all tested strains. The following synthetic commercial antibiotics, Streptomycin (Sigma- Aldrich S6501) and Ampicillin (Sigma-Aldrich A9393) and the commercial fungicides, Bifonazole (Srbolek, Belgrade, Serbia) and Ketoconazole (Zorkapharma, Šabac, Serbia) were used as positive controls (1 mg/mL in 0.01 M PBS), in final tested concentration ranges of 0.00015–0.03 mg/mL (antibiotics) and 0.1–3.50 mg/mL (antimycotics), respectively. Five percent DMSO and 0.01 M PBS was used as a negative control.

2.7. Cytotoxic activity

2.7.1. Cell lines

The cervical adenocarcinoma cell line (HeLa) and human chronic myeloid leukemia cells (K562) were grown in RPMI-1640 medium (Sigma). The media were supplemented with 10% fetal bovine serum, L-glutamine and penicillin/streptomycin (Sigma).

2.7.2. Treatment of cell lines

Target HeLa cells (2000 cells per well) and K562 (5000 cells per well) were seeded in the wells of a 96-well flat-bottomed microtiter plate. Twenty-four hours later, after attachment of the cells, different concentrations of the extracts studied were added to the wells, except for the control cells, to which only nutrient medium was added. Stock solutions (100 mg/mL) of the extracts prepared in dimethyl sulfoxide (DMSO) were dissolved in the appropriate medium to the required working concentrations. The final concentration range chosen was 1-100 µg/mL (1, 8.25, 16.5, 33 and 100 µg/mL). The final solvent DMSO concentration never exceeded 0.5%, which was not toxic to the cells. Notably, the compounds were applied to the suspension of K562 cells 2 hours after cell seeding. All concentrations were prepared in triplicate. Culture medium containing the appropriate concentrations of the compounds studied, but without cells, was also used in triplicate as a blank. The cultures were incubated for 72 hours.

2.7.3. Treatment of cell lines

The effect of the prepared compounds on cancer cell survival was determined by the microculture tetrazolium test (MTT) of Mosmann (1983) with modification by Ohno and Abe (1991) 72 hours after addition of the compounds as previously described. Briefly, 20 mL of MTT solution (5 mg/mL phosphate-buffered saline) was added to each well. Samples were incubated for an additional 4 hours at 37 °C in a humidified atmosphere of 95% air/5% CO_2 (v/v). Then, 100 mL of 100 g/L sodium dodecyl sulfate was added to extract the insoluble product formazan resulting from the conversion of MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of light absorbance measured in an ELISA plate reader at 570 nm. The absorbance (A) at 570 nm was measured 24 hours later. To determine cell survival (%), the A of a sample containing cells grown in the presence of different concentrations of the compounds studied was divided by the optical density of the control (the A of control cells grown only in culture medium) and multiplied by 100. It was assumed that the A of the blank sample was always subtracted from the A of the corresponding sample with target cells. IC_{50} was defined as the concentration of an agent that inhibited cell survival by 50% compared with a vehicle-treated control. Positive controls were cis-diamine dichloroplatin (cis-DDP). All experiments were performed in triplicate.

2.8. Statistical analysis

For each species, three samples were used and all the assays were carried in triplicate. The results expressed a mean values and standard errors, and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HDS Test with α=0.05. This analysis was carried out using SPSS v. 18.0 program.

3. RESULTS

The extraction yields of the methanolic extracts on a lyophilizates and dry weight basis has shown in Table 1. The extracts

based on freeze-dried weight had higher yields than the extracts based on dry weight for all algal samples. The species with the highest extraction yield was *C. effusum* (lyophilized), 21.07%.

Table 1. Extraction yield of seaweeds methanolic extracts on lyophilized (L) and dry (D) weight basis

Extracts	Yield (%) w/w
Codium bursa (L)	11.83
Codium bursa (D)	10.10
Codium effusum (L)	21.07
Codium effusum (D)	13.83
Laurencia obtusa (L)	14.87
Laurectia obtusa (D)	2.73
Padina pavonica (L)	13.40
Padina pavonica (D)	5.20

3.1. Total phenolic (TP) and flavonoid (TF) content

The results in Table 2 show that flavonoids content was significantly higher in all tested extracts comparing to phenolic content. It has been demonstrated that drying method used during material preparation does not affect the amount of extracted phenolic compounds. In contrast, the amount of extracted flavonoids was significantly higher in extracts obtained from oven-dried material compared to lyophilized samples (Table 2).

Table 2. Total phenolic (TP) and flavonoid (TF) contents of seaweeds lyophilized (L) and dry (D) extracts

Extracts	Total phenols (μg GAE/mg extract)	Total flavonoids (μg QE/mg extract)
Codium bursa (L)	2.89 ± 0.35	10.83 ± 0.10
Codium bursa (D)	3.19 ± 0.14	11.94 ± 0.80
Codium effusum (L)	0.54 ± 0.45	10.08 ± 0.86
Codium effusum (D)	2.81 ± 0.28	22.12 ± 0.74
Laurencia obtusa (L)	ND	7.91 ± 0.21
Laurencia obtusa (D)	0.95 ± 0.49	25.89 ± 3.25
Padina pavonica (L)	10.25 ± 0.94	11.70 ± 1.45
Padina pavonica (D)	10.44 ± 0.94	73.30 ± 2.62

Values are expressed as mean ± standard error
ND, no detected total phenolic and flavonoid contents

3.2. Antioxidative activity

The antioxidant activity of methanolic extracts, lyophilized and dry, determined by DPPH assays, is shown in Table 3. Antioxidative activity expressed in EC_{50} values ranged from 5.88 to 84.05 mg/mL. The extracts *of P. pavonica*, lyophilized, showed the strongest radical scavenging activity (EC50=5.88 mg/mL). The antioxidant activity of the methanolic extract of *C. bursa* (dried) was not detected. The activity of the commercial antioxidant butylated hydroxyanisole (BHA) was 0.06 ± 0.03 mg/mL.

Table 3. Antioxidant activity of seaweeds lyophilized (L) and dry (D) extracts determined by DPPH• assays

Extracts	DPPH• EC_{50} (mg/mL)
Codium bursa (L)	71.41± 4.85
Codium bursa (D)	ND
Codium effusum (L)	63.83 ± 3.22
Codium effusum (D)	36.96 ± 0.40
Laurencia obtusa (L)	60.20 ± 0.97
Laurencia obtusa (D)	32.06 ± 0.30
Padina pavonica (L)	5.88 ± 0.13
Padina pavonica (D)	84.05 ± 10.03

Values are expressed as mean ± standard error
ND, no detected antioxidant activity

3.3. Antibacterial and antifungal activity

The antibacterial activities of all tested extracts of four seaweeds are shown in Table 4. The seaweed extracts showed antibacterial activity against all bacterial species, but at different levels. The minimum inhibitory concentration (MIC) ranged from 0.0625-4.000 mg/mL, while the minimum bactericidal concentration (MBC) was 0.125-8.000 mg/mL. Their antibacterial potential can be presented as follows extracts: *C. effusum* (dried) > *P. pavonica* (dried) > *L. obtusa* (dried) > *P. pavonica* (lyophilized) > *L. obtusa* (lyophilized) > *C. effusum* (lyophilized) > *C. bursa* (dried) > *C. bursa* (lyophilized). The best antibacterial activity was obtained for the dry extract of *C. effusum*, with MIC ranging from 0.0625-2.000 mg/mL and MBC from 0.125-4.000 mg/mL. The lowest antibacterial activity among all extracts tested here was determined for freeze-dried extracts of *C. bursa*. The most sensitive bacterial species was *B. cereus* with an MIC of 0.0625-3.000 mg/mL and an MBC of 0.125-4.000 mg/mL. *L. monocytogenes* was the most resistant species with inhibitory activity between 1.500-4.000 mg/mL and bactericidal activity of 3.000-8.000 mg/mL. Streptomycin possessed inhibitory activity of 0.0003-0.015 mg/mL and bactericidal activity of 0.0006-0.030 mg/mL. The MIC range for ampicillin was 0.00015-0.00045 mg/mL and the MBC was 0.0003-0.0009 mg/mL. Comparing the biological activity of the extracts with the commercially available antibiotics, we found that the tested samples had lower antibacterial potential (Table 4). The results of the antifungal activity of the different types of four algae are shown in Table 5. All the tested extracts showed antifungal activities in the MIC range of 1.000-8.000 mg/mL and in the MFC range of 2.000-18.000 mg/mL. The best antifungal activity was obtained for the extract of *C. effusum* (lyophilized) with inhibitory activity of 1.000-4.000 mg/mL and fungicidal activity of 4.000-8.000 mg/mL. *A. niger*, was the most sensitive fungus tested, while *P. verrucosum* was the most resistant. Bifonazole showed inhibitory activity of 0.1000.200 mg/mL and fungicidal activity of 0.200-0.2500 mg/mL, while ketoconazole showed inhibitory activity of 0.200-2.500 mg/mL and fungicidal activity of 0.500-3.500 mg/mL. The synthetic fungicides showed better antifungal activity than the tested extracts.

The tested methanolic extracts of algae possessed better antibacterial activity than antifungal potential.

3.4. Cytotoxic activity

Screening of methanolic extracts of the studied algae was performed with two human cancer cell lines: the cervical adenocarcinoma cell line (HeLa) and human chronic myeloid leukemia (K562). The cytotoxic effects of the tested extracts estimated by the MTT assay are shown as IC_{50} values in Table 6. First, all tested extracts showed excellent to moderate activity against malignant cells. Very important information that emerged from our results is that all methanolic dry extracts (D) of the algae showed significant or several times better cytotoxic activity compared to the freeze-dried samples (L). Moreover, the HeLa cells were found to be more sensitive than the K562 cell lines. The dry extracts (D) from *L. obtusa* and *P. pavonica* algae showed pronounced cytotoxic activity in both cell lines tested (Table 6). In contrast, the lyophilized (L) extract of *P. pavonica* showed the weakest activity against HeLa cells and moderate activity against K562 when comparing the lyophilized (L) extracts. For all other algal samples, good to moderate cytotoxicity is reported against the malignant cells studied.

Table 4. Minimum inhibitory (MIC) and bacterial (MBC) concentrations of tested methanol extracts of macroalgae (mg/mL)

Extracts	*Codium effusum* L	*Codium effusum* D	*Codium bursa* L	*Codium bursa* D	*Padina pavonica* L	*Padina pavonica* D	*Laurencia obtusa* L	*Laurencia obtusa* D	Streptomycin	Ampicillin
Bacteria	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC
Staphylococcus aureus	1.00±0.07^{a} 2.00±0.07^{d}	0.125±0.30^{b} 0.25±0.01^{ab}	3.00±0.05^{f} 4.00±0.10^{e}	3.00±0.000^{f} 4.00±0.20^{e}	0.50±0.07^{d} 1.00±0.10^{c}	0.25±0.02^{c} 0.35±0.02^{b}	0.50±0.007^{d} 1.00±0.02^{c}	0.50±0.02^{d} 1.00±0.05^{c}	0.0004±0.00005^{a} 0.0008± 0.00007^{a}	0.0002± 0.00007^{a} 0.0004± 0.0001^{a}
Bacillus cereus	1.00±0.10^{d} 2.00±0.000^{f}	0.0625±0.04^{a} 0.125±0.008^{ab}	3.00±0.05^{e} 4.00±0.000^{g}	3.00±0.08^{e} 4.00±0.10^{g}	0.125±0.000^{ab} 0.25±0.02^{b}	1.00±0.000^{d} 1.50±0.10^{e}	0.25±0.02^{b} 0.50±0.08^{c}	0.75±0.08^{c} 1.00±0.07^{d}	0.0003±0.00007^{a} 0.0006±0.00003^{a}	0.00015±0.00002^{a} 0.0003±0.00005^{a}
Micrococcus luteus	2.00±0.000^{d} 4.00±0.20^{d}	1.00±0.80^{b} 2.00±0.10^{c}	4.00±0.10^{f} 6.00±0.20^{e}	3.00±0.10^{e} 4.00±0.000^{d}	2.00±0.000^{d} 4.00±0.07^{d}	0.75±0.08^{b} 1.50±0.10^{b}	3.00±0.10^{e} 4.00±0.10^{d}	1.50±0.07^{c} 2.00±0.10^{c}	0.00035±0.00005^{a} 0.0007±0.0001^{a}	0.0004±0.00007^{a} 0.0008±0.00007^{a}
Listeria monocytogenes	4.00±0.07^{d} 8.00±0.20^{d}	2.00±0.20^{c} 4.00±0.05^{c}	4.00±0.20^{d} 8.00±0.000^{d}	2.00±0.07^{c} 4.00±0.20^{c}	2.00±0.10^{c} 4.00±0.07^{c}	1.50±0.10^{b} 3.00±0.10^{b}	4.00±0.000^{d} 8.00±0.20^{d}	2.00±0.15^{c} 4.00±0.000^{c}	0.015±0.002^{a} 0.030±0.00007^{a}	0.00015±0.000^{d} 0.0003±0.00007^{a}
Pseudomonas aeruginosa	1.00±0.10^{d} 2.00±0.000^{e}	0.25±0.05^{b} 0.50±0.07^{b}	4.00±0.20^{f} 8.00±0.000^{g}	3.00±0.07^{e} 4.00±0.000^{f}	0.50±0.10^{c} 1.00±0.10^{d}	0.35±0.05^{bc} 0.75±0.05^{c}	0.35±0.02^{b} 0.50±0.10^{b}	0.25±0.02^{b} 0.50±0.07^{b}	0.0004±0.000^{a} 0.0008±0.00007^{a}	0.00015±0.00002^{a} 0.0003±0.00007^{a}
Escherichia coli	2.0±0.20^{e} 4.00±0.05^{e}	1.00±0.10^{c} 2.00±0.10^{d}	4.00±0.08^{g} 6.00±0.20^{f}	3.00±0.07^{f} 4.00±0.10^{e}	1.50±0.000^{d} 2.00±0.10^{c}	0.75±0.02^{b} 1.50±0.08^{b}	3.00±0.10^{f} 4.00±0.000^{e}	1.50±0.10^{d} 2.00±0.10^{d}	0.0004±0.0001^{a} 0.0008±0.00007^{a}	0.00015±0.00002^{a} 0.0003±0.000^{a}
Enterobacter cloacae	2.00±0.07^{c} 8.00±0.10^{d}	2.00±0.000^{c} 4.00±0.10^{c}	4.00±0.20^{e} 8.00±0.000^{d}	2.00±0.10^{c} 4.00±0.20^{e}	3.00±0.10^{d} 4.00±0.07^{c}	0.75±0.08^{b} 1.50±0.20^{b}	4.00±0.08^{e} 8.00±0.20^{d}	1.00±0.000^{b} 4.00±0.10^{c}	0.015±0.002^{a} 0.03±0.007^{a}	0.0002±0.0001^{a} 0.0004±0.00007^{a}
Salmonella typhimurium	2.00±0.000^{e} 4.00±0.08^{f}	1.00±0.15^{c} 2.00±0.15^{e}	3.00±0.10^{f} 4.00±0.10^{f}	4.00±0.20^{g} 8.00±0.20^{g}	1.50±0.10^{d} 2.00±0.000^{e}	0.75±0.08^{b} 1.50±0.20^{d}	0.75±0.000^{b} 1.00±0.20^{c}	0.15±0.02^{a} 0.35±0.02^{b}	0.00045±0.00005^{a} 0.0009±0.0001^{a}	0.00045±0.00002^{a} 0.0009±0.0001^{a}

[MIC/MBC (mg/mL); L lyophilized; D dry]

Table 5. Minimum inhibitory (MIC) and fungicidal (MFC) concentrations of tested methanol extracts of macroalgae (mg/mL)

Extracts	*Codium effusum* L	*Codium effusum* D	*Codium bursa* L	*Codium bursa* D	*Padina pavonica* L	*Padina pavonica* D	*Laurencia obtusa* L	*Laurencia obtusa* D	Bifonazole	Ketoconazole
Fungi	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC
Aspergillus fumigatus	4.00±0.000^{cd} 8.00±0.20^{c}	4.00±0.10^{c} 8.00±0.000^{c}	4.00±0.07^{cd} 8.00±0.10^{e}	4.00±0.20^{d} 18.0±1.00^{e}	4.00±0.08^{cd} 8.00±0.10^{c}	4.00±0.000^{cd} 16.0±0.30^{d}	2.00±0.10^{b} 4.00±0.10^{b}	4.00±0.07^{cd} 8.00±0.70^{c}	0.15±0.02^{a} 0.20±0.10^{a}	0.20±0.02^{a} 0.50±0.000^{a}
Aspergillus versicolor	2.00±0.10^{d} 4.00±0.10^{c}	2.00±0.20^{d} 4.00±0.05^{c}	4.00±0.10^{e} 16.0±0.70^{f}	2.00±0.10^{d} 4.00±0.000^{c}	1.00±0.000^{c} 2.00±0.10^{b}	2.00±0.10^{d} 4.00±0.20^{c}	8.00±0.10^{f} 16.0±0.30^{e}	4.00±0.07^{e} 8.00±0.10^{d}	0.10±0.03^{a} 0.20±0.10^{a}	0.20±0.07^{b} 0.50±0.07^{a}
Aspergillus ochraceus	4.00±0.20^{c} 8.00±0.000^{d}	2.00±0.000^{b} 4.00±0.20^{c}	4.00±0.07^{c} 18.0±1.00^{f}	4.00±0.07^{c} 16.0±0.70^{e}	4.00±0.20^{c} 8.00±0.20^{d}	4.00±0.000^{c} 8.00±0.10^{d}	4.00±0.08^{c} 8.00±0.000^{d}	4.00±0.03^{c} 16.0±0.30^{e}	0.15±0.03^{a} 0.20±0.000^{a}	1.50±0.05^{a} 2.00±0.20^{b}
Aspergillus niger	1.00±0.10^{b} 4.00±0.000^{c}	1.00±0.20^{b} 2.00±0.20^{b}	1.00±0.000^{b} 2.00±0.08^{b}	2.00±0.10^{c} 4.00±0.10^{c}	2.00±0.07^{c} 8.00±0.10^{d}	4.00±0.10^{d} 16.0±0.07^{e}	2.00±0.10^{c} 8.00±0.10^{d}	4.00±0.02^{d} 16.0±0.30^{e}	0.15±0.02^{a} 0.20±0.10^{a}	0.20±0.02^{a} 0.50±0.07^{a}
Trichoderma viride	2.00±0.07^{c} 4.00±0.20^{d}	2.00±0.000^{c} 4.00±0.10^{de}	2.00±0.20^{c} 4.00±0.20^{e}	2.00±0.000^{c} 4.00±0.07^{de}	2.00±0.10^{c} 4.00±0.10^{de}	4.00±0.20^{2} 8.00±0.10^{f}	1.00±0.005^{b} 2.00±0.10^{c}	2.00±0.10^{c} 8.00±0.07^{f}	0.15±0.02^{a} 0.20±0.02^{a}	1.0±0.10^{b} 1.0±0.07^{b}
Penicillium funiculosum	1.00±0.20^{b} 4.00±0.08^{b}	2.0±0.07^{c} 4.00±0.000^{b}	2.00±0.20^{c} 4.00±0.07^{b}	2.00±0.000^{c} 4.00±0.10^{b}	4.00±0.08^{d} 16.0±0.30^{d}	2.00±0.08^{c} 4.00±0.10^{b}	2.00±0.10^{c} 4.00±0.10^{c}	4.00±0.03^{d} 8.00±0.20^{c}	0.20±0.10^{a} 0.25±0.02^{a}	0.20±0.07^{a} 0.50±0.000^{a}
Penicillium ochrochloron	4.00±0.07^{c} 8.00±0.10^{c}	4.00±0.10^{c} 8.00±0.10^{c}	4.00±0.20^{c} 16.0±0.000^{de}	4.00±0.05^{c} 16.0±0.70^{d}	4.00±0.000^{c} 16.0±0.70^{e}	2.00±0.20^{b} 4.00±0.20^{b}	2.00±0.10^{b} 4.00±0.08^{b}	4.00±0.03^{c} 16.0±0.70^{e}	0.20±0.000^{a} 0.25±0.02^{a}	2.50±0.20^{b} 3.50±0.20^{b}
Penicillium verrucosum var. cyclopium	4.00±0.08^{d} 8.00±0.000^{c}	4.00±0.07^{d} 16.0±0.70^{d}	4.00±0.20^{d} 16.0±0.20^{de}	8.00±0.08^{e} 16.0±0.30^{e}	8.00±0.000^{e} 16.0±0.70^{d}	2.00±0.10^{c} 4.00±0.20^{b}	1.00±0.10^{b} 8.00±0.10^{c}	4.00±0.07^{d} 16.0±0.000^{de}	0.10±0.007^{a} 0.20±0.03^{a}	0.20±0.03^{a} 0.30±0.02^{a}

[MIC/MFC (mg/mL); L lyophilized; D dry]

Table 6. Cytotoxic activity of seaweeds lyophilized (L) and dry (D) extracts on HeLa and K562 cells.

Extracts	HeLa	K562
	IC50 (μg/mL)	
Padina pavonica (D)	21.27 ± 0.89	15.17 ± 2.34
Padina pavonica (L)	87.27 ± 2.05	68.15 ± 1.02
Codium effusum (D)	37.43 ± 0.87	44.39 ± 0.32
Codium effusum (L)	54.68 ± 2.23	47.91 ± 0.69
Codium bursa (D)	59.03 ± 2.52	77.04 ± 2.65
Codium bursa (L)	67.93 ± 1.35	80.31 ± 1.47
Laurencia obtusa (D)	6.51 ± 0.35	16.95 ± 2.37
Laurencia obtusa (L)	42.07 ± 1.96	20.46 ± 0.75

4. DISCUSION

Previously have been shown that polar solvents are usually the solvent of choice for the extraction of various compounds from macroalgal species. The differences in the yields of the various extracts have been attributed to the polarity of the different compounds present in the plants (Ye et al., 2009). In the present study, methanol was used to extract antioxidant, antimicrobial and antitumor compounds from four species of algae. According to Kelman et al. (2012), methanol was found to have the highest extraction efficiency. The phenolic content of these algae was determined by the Folin-Ciocalteu method. Phenolic compounds have various biological activities, including antioxidant activity. The variation in phenolic content was quite wide, ranging from 0.54 to 10.44 μg GAE/mg of extract (Table 2). The brown algae *P. pavonica* (lyophilized and dried) had significantly higher phenolic content than red algae *L. obtusa* and green algae *C. bursa* and *C. effusum*. It has been reported that brown algae generally contain higher amounts of polyphenols than red and green algae (Heffernan et al., 2015). The range of variation in flavonoid content was also quite wide, ranging from 7.91 to 73.30 μg QE/mg of extract (Table 2). Flavonoids are the largest class of polyphenols and contribute most to the antioxidant capacity of plants. They act by either blocking the formation of hypervalent metal forms, scavenging free radicals, or interrupting chain reactions of lipid peroxidation (Zaragozá et al., 2008). The significantly higher flavonoid content was found in brown algae *P. pavonica* (dried). Zaragoza et al. (2008) and Rajauria et al. (2013) observed flavonoid content in methanolic extracts of brown algae, *Fucus vesiculosus* and *Himanthalia elongate*.

In this study, the antioxidative activities of algal extracts were tested using the DPPH assay. DPPH is a useful reagent to study the radical scavenging activities of compounds (Duan et al., 2006). The antioxidant activity of the methanolic extracts was significantly different among the four tested algae. Among them, *P. pavonica* showed the strongest radical scavenging activity and the highest TP among. Praba and Sumaya (2022) also reported similar results in other species of the genus *Padina* (*P. antillarum*, *P. tetrastomatica*, *P. gymnospora*). *Codium fragile* are known to have relatively low antioxidant activity (KeskiNkaya et al., 2022). We found that *C. bursa* and *C. effusum* have low antioxidant activity. The methanolic extracts of *L. obtusa* showed low antioxidant activity. According to Kelman et al. (2012) and Al-Enazi et al. (2018), *Laurencia obtusa* is a good source of biologically active secondary metabolites but does not exhibit high antioxidant activity.

Results clearly showed that antioxidative activities of *C. effusum* and *L. obtusa* were strongly correlated to flavonoids content. The linear correlation between radical scavenging activity and TP and TF content suggests that algae may also contain other antioxidants such as ascorbic acid, carotenoids, folic acid, and thiamine (Yangthong et al., 2009).

The best antibacterial activity was obtained for the methanolic extract of *C. effusum* (dry) and the lowest antibacterial activity of all extracts tested here was obtained for extracts of *C. bursa* (freeze-dried). According to Albayati et al. (2020) extracts of *C. bursa* showed antimicrobial activity. The tested methanolic extracts of algae possessed better antibacterial activity than antifungal potential. According to Khaled et al. (2012), *P. pavonica* exhibited antifungal activity against four *Candida* species.

Dry extracts (D) from *L.obtusa* and *P. pavonica* algae showed pronounced cytotoxic activity in both cell lines tested (HeLa and K562). Stanojković et al. (2013) reported strong cytotoxic activity of methanolic extract of *P. pavonica* on HeLa cancer cell line. Awad et al. (2008) reported the cytotoxic activity of *P. pavonica* against human lung carcinoma (H460) and liver carcinoma (HepG2) cell lines. According to Ktari and Guyot (1999), dichloromethane extract of *P. pavonica* showed cytotoxic activity against KB cells.

5. CONCLUSION

In the pursuit of new natural compounds with potential antimicrobial and antitumor properties, algae have demonstrated significant promise as a valuable source. This study highlights the potential of algae, particularly *Padina pavonica*, as a rich source of bioactive compounds with antioxidant, antimicrobial, and cytotoxic properties. Methanol was effective in extracting phenolic and flavonoid compounds, with *P. pavonica* exhibiting the highest antioxidant activity among tested species. Antimicrobial effects were more pronounced in antibacterial assays, notably in *C. effusum*, while *L. obtusa* and *P. pavonica* showed strong cytotoxicity against cancer cell lines. These findings suggest that specific algae may serve as promising natural sources for developing therapeutic agents.

ACKNOWLEDGMENTS

This work was financially supported by Ministri of Science, Technological Development and Innovation of the Republic of Serbia (Grants No., 451-03-65/2024-03/200178, 451-03-66/2024-03/200178, 451-03-66/2024-03/ 200007, 451-03-66/2024-03/200026 and 451-03-66/2024-03/200043).

CONFLICT OF INTEREST

The authors declare that they have no financial conflicts of interest.

REFERENCES

Albayati, MAF., Akköz, C., Güneş, E., Keskinkaya, HB., Okudan, EŞ., and Karakurt, S. (2020): Evaluation of antimicrobial activities of *Codium bursa* methanol and chloroform extracts against different test pathogens, 240 *in VI international congres of natural and health sciences, proceedings book*.

Al-Enazi, N. M., Awaad, A. S., Zain, M. E., and Alqasoumi, S. I. (2018): Antimicrobial, antioxidant and anticancer activities of *Laurencia catarinensis* , *Laurencia majuscula* and *Padina pavonica* extracts, *Saudi Pharmaceutical Journal*, **26**(1), 44–52. https://doi.org/10.1016/j.jsps.2017.11.001

Awad, N. E., Selim, M. A., Metawe, H. M., and Matloub, A. A. (2008): Cytotoxic Xenicane Diterpenes from the Brown Alga *Padina pavonia* (L.) Gaill., *Phytotherapy Research*, **22**(12), 1610–1613. https://doi.org/10.1002/ptr.2532

Barbosa, M., Valentão, P., and Andrade, P. (2014): Bioactive Compounds from Macroalgae in the New Millennium: Implications for Neurodegenerative Diseases, *Marine Drugs*, **12**(9), 4934–4972. https://doi.org/10.3390/md12094934

Duan, X.-J., Zhang, W.-W., Li, X.-M., and Wang, B.-G. (2006): Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*, *Food Chemistry*, **95**(1), 37–43. https://doi.org/10.1016/j.foodchem.2004.12.015

Heffernan, N., Smyth, T. J., Soler-Villa, A., Fitzgerald, R. J., and Brunton, N. P. (2015): Phenolic content and antioxidant activity of fractions obtained from selected Irish macroalgae species (*Laminaria digitata*, *Fucus serratus*, *Gracilaria gracilis* and *Codium fragile*), *Journal of Applied Phycology*, **27**(1), 519–530. https://doi.org/10.1007/s10811-014-0291-9

Kelman, D., Posner, E. K., McDermid, K. J., Tabandera, N. K., Wright, P. R., and Wright, A. D. (2012): Antioxidant Activity of Hawaiian Marine Algae, *Marine Drugs*, **10**(2), 403–416. https://doi.org/10.3390/md10020403

KeskïNkaya, H. B., Devecï, E., Güneş, E., Okudan, E. Ş., Akköz, C., Gümüş, N. E., and Karakurt, S. (2022): Deniz Makroalgi *Codium fragile* (Suringar) Hariot 'in Kimyasal Bileşimi, *In-Vitro* Antimikrobiyal ve Antioksidan Aktivitelerinin Analizi, *Commagene Journal of Biology*, 94–104. https://doi.org/10.31594/commagene.1084336

Khaled, N., Hiba, M. R., and Asma, C. (2012): Antioxidant and antifungal activities of *Padina pavonica* and s*Argassum vulgare* from the lebanese mediterranean coast, *Advances in Environmental Biology*, **6**(1), 42–48.

Knežević, A., Stajić, M., Živković, L., Milovanović, I., Spremo-Potparević, B., and Vukojević, J. (2017): Antifungal, Antioxidative, and Genoprotective Properties of Extracts from the Blushing Bracket Mushroom, *Daedaleopsis confragosa* (Agaricomycetes), *International Journal of Medicinal Mushrooms*, **19**(6), 509–520. https://doi.org/10.1615/IntJMedMushrooms.v19.i6.30

Ktari, L., and Guyot, M. (1999): A cytotoxic oxysterol from the marine alga *Padina pavonica* (L.) Thivy, *Journal of Applied Phycology*, **11**(6), 511–513. https://doi.org/10.1023/A:1008162624027

Mosmann, T. (1983): Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *Journal of Immunological Methods*, **65**(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Negreanu-Pirjol, B.-S., Negreanu-Pirjol, T., Popoviciu, D. R., Anton, R.-E., and Prelipcean, A.-M. (2022): Marine Bioactive Compounds Derived from Macroalgae as New Potential Players in Drug Delivery Systems: A Review, *Pharmaceutics*, **14**(9), 1781. https://doi.org/10.3390/pharmaceutics14091781

Ohno, M., and Abe, T. (1991): Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), *Journal of Immunological Methods*, **145**(1–2), 199–203. https://doi.org/10.1016/0022-1759(91)90327-C

Pane, G., Cacciola, G., Giacco, E., Mariottini, G., and Coppo, E. (2015): Assessment of the Antimicrobial Activity of Algae Extracts on Bacteria Responsible of External Otitis, *Marine Drugs*, **13**(10), 6440–6452. https://doi.org/10.3390/md13106440

Park, Y. K., Koo, M. H., Ikegaki, M., and Contado, J. L. (1997): Comparison of the flavonoid aglycone contents of *Apis mellifera* propolis from various regions of Brazil, *Brazilian Archives of Biology and Technology*, **40**, 97–106.

Praba, N., and Sumaya, Dr. S. (2022): Study On Phytochemical and Antioxidant Properties of *Padina gymnospora* and *Ulva lactuca*: Life Sciences-Marine Biology, *International Journal of Life Science and Pharma Research*. https://doi.org/10.22376/ijpbs/lpr.2022.12.6.L155-160

Rajauria, G., Jaiswal, A. K., Abu-Gannam, N., and Gupta, S. (2013): The antimicrobial, antioxidant and free radical-scavenging capacity of brown seaweed *Himanthalia elongata* from western coast of Ireland: antimicrobial and antioxidant properties of Irish seaweed, *Journal of Food Biochemistry*, **37**(3), 322–335. https://doi.org/10.1111/j.1745-4514.2012.00663.x

Salehi, B., Sharifi-Rad, J., Seca, A. M. L., Pinto, D. C. G. A., Michalak, I., Trincone, A., Mishra, A. P., Nigam, M., Zam, W., and Martins, N. (2019): Current Trends on Seaweeds: Looking at Chemical Composition, Phytopharmacology, and Cosmetic Applications, *Molecules*, **24**(22), 4182. https://doi.org/10.3390/molecules24224182

Singleton, V. L., and Rossi, J. A. (1965): Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *American Journal of Enology and Viticulture*, **16**(3), 144–158.

Stanojković, P. T., Šavikin, K., Zdunić, G., Kljajić, Z., Grozdanić, N., and Antić, J. (2013): *In vitro* antitumoral activities of *Padina pavonia* on human cervix and breast cancer cell lines, *Journal of Medicinal Plants Research*, **7**(8), 419–424.

Yangthong, M., Hutadilok-Towatana, N., and Phromkunthong, W. (2009): Antioxidant Activities of Four Edible Seaweeds from the Southern Coast of Thailand, *Plant Foods for Human Nutrition*, **64**(3), 218–223. https://doi.org/10.1007/s11130-009-0127-y

Ye, H., Zhou, C., Sun, Y., Zhang, X., Liu, J., Hu, Q., and Zeng, X. (2009): Antioxidant activities *in vitro* of ethanol extract from brown seaweed *Sargassum pallidum*, *European Food Research and Technology*, **230**(1), 101–109. https://doi.org/10.1007/s00217-009-1147-4

Zaragozá, M. C., López, D., P. Sáiz, M., Poquet, M., Pérez, J., Puig-Parellada, P., Màrmol, F., Simonetti, P., Gardana, C., Lerat, Y., Burtin, P., Inisan, C., Rousseau, I., Besnard, M., and Mitjavila, M. T. (2008): Toxicity and Antioxidant Activity i*n Vitro* and *in Vivo* of Two *Fucus vesiculosus* Extracts, *Journal of Agricultural and Food Chemistry*, **56**(17), 7773–7780. https://doi.org/10.1021/jf8007053

Turning Agricultural Waste into a Powerful Solution: Enhanced Lead Removal via Chemically Modified Oat Straw

JELENA DIMITRIJEVIĆ[1*], SANJA JEVTIĆ[2], MARIJA SIMIĆ[1], MARIJA KOPRIVICA[1], ALEKSANDAR JOVANOVIĆ[1], MILICA MIŠIĆ[1] AND JELENA PETROVIĆ[1]

[1]*Institute for Technology of Nuclear and Other Mineral Raw Materials, 86 Franchet d'Esperey St., 11000 Belgrade, Serbia;*
[2]*Faculty of Technology and Metallurgy, University of Belgrade, 4 Carnegie, 11000 Belgrade, Serbia*
**Corresponding author: j.dimitrijevic@itnms.ac.rs*

Received: October 29, 2024
Accepted: November 12, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

The release of industrial effluents and agricultural runoff containing heavy metals, like lead (Pb), poses serious environmental risks and significant impact on human health. This study explores the adsorption capacity of potassium hydroxide (KOH)-modified oat straw (KOS) as an effective, low-cost biosorbent for lead removal from contaminated water. The modification process increased the surface area and improved the availability of functional groups, enhancing adsorption performance compared to unmodified oat straw. Structural analysis was conducted using Scanning Electron Microscopy (SEM) and Fourier Transform Infrared spectroscopy (FTIR). Batch experiments evaluated the effects of contact time, initial Pb^{2+} concentration, and pH on lead removal efficiency. The adsorption process followed pseudo-second-order kinetics, with chemisorption as the main mechanism. Isotherm studies indicated that the Langmuir and Redlich-Peterson models offered the best fit, showing a maximum adsorption capacity of 191.41 mg/g. These findings highlight the potential of KOH-modified oat straw as a sustainable solution for heavy metal removal from wastewater, effectively valorizing agricultural waste biomass.

Keywords: *Avena sativa* L; biomass; heavy metals; wastewater treatment; adsorption, ecofriendly process

https://doi.org/10.61652/leksir2444011D

1. INTRODUCTION

The release of industrial effluents and agricultural run-off into water bodies has resulted in significant global concerns regarding water pollution. Pollutants like heavy metals, dyes, pesticides, and pharmaceuticals have a profound impact on the environment, causing risks to human health, animals, and ecosystems (Dimitrijević et al., 2023; Jovanović et al., 2023; Simić et al., 2022). Due to their persistence, non-degradability, and high toxicity even at low concentrations, heavy metals in particular, such as Pb, cadmium (Cd), and mercury (Hg), represent a critical threat (Zhang et al., 2022). These metals accumulate in living organisms, leading to serious health risks. For this reason, finding effective methods to treat wastewater before its discharge into the environment has become an urgent priority.

Various conventional methods, including coagulation, flocculation, membrane filtration, and chemical precipitation, have been employed to treat wastewater. However, these methods often face limitations such as high operational costs, complexity, and inefficiency in removing pollutants at low concentrations (Dimitrijević et al., 2023; Simić et al., 2022; Zhang et al., 2022). As a result, there is a growing interest in more sustainable and efficient solutions, particularly in the form of biosorbents.

Adsorption has emerged as a highly selective, efficient, and cost-effective technique for removing pollutants from water (Gollakota et al., 2022). Over the past few decades, the application of natural materials, particularly agricultural waste, has gained significant attention due to its sustainability, availability, and low cost (Borrega et al., 2022). Agricultural residues like oat straw, waste coffee pulp, corn cob, and banana pseudo-stems have been investigated for their potential use as biosorbents due to their abundance and renewable nature (Dimitrijević et al., 2023; Fan et al., 2022). Biomass materials are particularly attractive because they contain functional groups, such as carboxyl, phenolic, and amine groups, which interact with pollutants through mechanisms such as hydrogen bonding and electrostatic interactions (Fan et al., 2022). To improve the adsorption capacity of biosorbents, chemical modifications are often per-

formed. Alkali treatments, with KOH are commonly used to enhance the surface area and introduce additional binding sites. For example, Simić et al. (2022) demonstrated that KOH modification of corn silk increased its capacity to remove cadmium Cd (II) ions by more than twofold.

In this context, we explore oat straw, a low-cost byproduct left after oat harvesting, which is available in large quantities. Oat (*Avena sativa* L.) is well known as a quality source of antioxidants such as E-vitamers (α and β tocopherol, α and β tocotrienol), free or esterified phenolic acids (e.g. *p*-coumaric, caffeic and ferulic acids) and avenanthramides (avenanthramides 1, 3, and 4) (Bryngelsson et al., 2002; Ren et al., 2011). These phenols exhibit antioxidant, anti-inflammatory, and antiproliferative activity, which distinguishes this plant as very useful (Kim et al., 2021). Additionally, oat compounds have shown benefits in reducing hyperglycemia, hyperinsulinemia, hypercholesterolemia, and hypertension (Dong et al., 2011). Despite its potential, oat straw remains largely underutilized, making it an ideal candidate for valorization. The lignocellulosic composition of oat straw includes cellulose, hemicellulose, and lignin, providing multiple active sites for adsorption (Gómez Aguilar et al., 2020). While previous studies have investigated oat husks, there is a gap in the research regarding the potential of oat straw itself as a biosorbent. Its composition also includes minerals like magnesium, calcium, and sodium, which enhance its ion-exchange capacity, making it particularly effective for heavy metals like Pb (Hatiya et al., 2022).

In this study, we have focused on modifying oat straw using potassium hydroxide (KOH), a practical and cost-effective treatment. Our primary objective is to evaluate the efficacy of KOH-modified oat straw in adsorbing Pb from contaminated water, given the frequent presence of lead in industrial effluents and its lethal effects on human health. The second objective lies in the potential to turn a widely available agricultural byproduct into an efficient biosorbent for environmental remediation (Kosiorek and Wyszkowski, 2019). This approach offers a scalable, environmentally friendly option for treating contaminated water while simultaneously addressing pollution and waste management challenges.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals and reagents used in this study were of analytical purity. Lead nitrate ($Pb(NO_3)_2$) was used to prepare stock solutions (1000 mg/L) for the adsorption tests. The stock solutions were diluted with ultra-distilled water to achieve desired working concentrations. KOH was utilized for the chemical modification of the biosorbent.

2.2. Biomass Preparation

Oat straw waste was used as the base biomass material, collected from a local field in Banat, Serbia, after the 2021 harvest. The biomass was thoroughly washed to remove any remaining soil and surface impurities, air-dried at room temperature, and then ground and sieved to obtain a particle size fraction of 63-125 μm. The material was subsequently oven-dried at 105 °C to a constant weight. For the chemical modification process, the prepared OS was treated with KOH to improve its adsorptive properties by modifying its surface structure, specifically targeting hemicellulose breakdown. The modified biomass (KOS) was stored in sealed containers for further use in the adsorption experiments.

2.3. Biomass Modification

The modification of the OS biosorbent was carried out by treating 2 g of the raw material with 50 mL of 0.1 mM potassium hydroxide (KOH) solution(Petrović et al., 2016). The mixture was stirred on a magnetic stirrer for 4 hours to ensure thorough interaction between the KOH and the biomass. After the reaction, the modified biomass was filtered through filter paper, and the residue was rinsed with distilled water until the filtrate reached a neutral pH of 7, effectively removing any excess KOH remaining on the material surface. The neutralized biomass was then dried and stored for further adsorption experiments and characterization.

2.4. Biomass Characterization

To evaluate the structural changes resulting from the modification process, a detailed characterization of both OS and KOS samples was conducted. Surface analysis was performed using Fourier Transform Infrared (FTIR) spectroscopy on a Thermo Scientific Nicolet iS50 FT-IR spectrometer. The samples were prepared by mixing 0.8 mg of either OS or KOS with 80 mg of KBr, and the spectra were recorded within the range of 4000 to 400 cm^{-1}.

The structural properties of the biosorbents were examined using Scanning Electron Microscopy (SEM) on a MIRA TESCAN microscope, operating at 20 keV. Before imaging, all samples were coated with a thin layer of gold, and mounted on adhesive carbon discs to ensure conductivity during analysis.

2.5. Adsorption Studies

To understand the impact of the modification process on the efficiency of the adsorbent, a preliminary adsorption test was conducted using both materials. For the test, 0.1 g/L of each biosorbent was added to a 1 mM Pb solution. The contact time was 24 hours, after which the concentration of remaining lead ions in the filtrates was measured by Atomic Absorption Spectrophotometry (AAS) at Perkin Elmer 900T.

Further adsorption studies were conducted using KOS as the adsorbent to optimize adsorption conditions and investigate the Pb^{2+} removal mechanism. Several key operational parameters were varied, including the initial pH of the solution (2-5), contact time (15-1440 min), and initial concentration of lead ions (40-400 mg/L). Adsorption experiments were carried out using a sorbent dose of 1 g/L and a lead solution at pH 5.

The batch adsorption experiments were performed in 100 mL Erlenmeyer flasks and placed on an orbital shaker at 220 rpm and room temperature. After the specified contact time, the remaining concentration of lead ions in the solution was measured using AAS.

The amount of lead adsorbed onto the biosorbent was calculated using the following equation:

$$q_e = \frac{(Ci - Ce)\cdot V}{m} \quad (1)$$

where: q_e is the amount of lead adsorbed (mg/g), Ci and Ce are the initial and equilibrium concentrations of lead (mg/L), V is the volume of the solution (L), m is the mass of the adsorbent (g).

Adsorption kinetics and isotherm models were evaluated using both linear and non-linear fitting methods with Origin software. Additionally, to investigate the potential ion-exchange mechanism during lead adsorption, the concentrations of released cations (such as Ca^{2+}, Mg^{2+}, K^+, Na^+) were monitored after adsorption by AAS.

3. RESULTS

3.1. Material Characterization

3.1.1. SEM

SEM was employed to closely examine the morphological

changes in OS and KOS. The SEM micrograph of native OS (Figure 1a) reveals a smooth, continuous surface characteristic of untreated lignocellulosic biomass (Dimitrijević et al., 2023). Notably, well-defined channels and occasional cracks are observed across the surface, which is typical for plant-based structures (Simić et al., 2022). These features suggest that the native OS has limited surface area and adsorption capacity due to its relatively unaltered and compact structure. Following modification with KOH, significant morphological transformations are evident, as shown in the SEM image of KOS (Figure 1b).

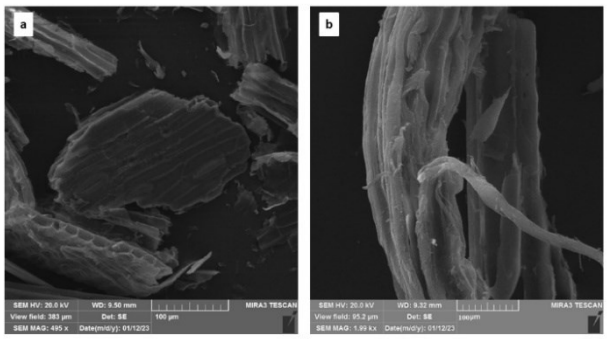

Fig. 1: SEM micrographs of OS (a) and KOS (b)

The previously smooth surface becomes highly disordered, with numerous cracks, channels, and rough textures (Lai et al., 2023). This pronounced surface disruption is indicative of the chemical action of KOH, which likely leads to partial degradation of cellulose and hemicellulose components within the lignocellulosic matrix. Such degradation results in the formation of new surface pores and an overall increase in surface roughness, thereby creating a more porous structure (Gollakota et al., 2022).

The increase in surface heterogeneity and the formation of additional microchannels in KOS are crucial for enhancing the adsorption properties of the material. The new cracks and pores provide improved pathways for ion diffusion, facilitating greater interaction between the material's active sites and metal ions in solution. This observation aligns with previous research, which has demonstrated that chemical modifications, such as alkaline treatments, significantly improve the adsorption performance of biomass by increasing the number of accessible binding sites (Dimitrijević et al., 2023; Gollakota et al., 2022).

3.1.1. FTIR Analysis

To investigate the chemical structure changes in native OS caused by KOH modification, FTIR analysis was conducted, and the spectra before and after modification are shown in Figure 2. The FTIR spectrum of OS exhibits characteristic peaks for lignocellulosic biomass. The broad peak around 3300 cm^{-1} is attributed to O-H stretching vibrations from hydroxyl groups as expected for cellulose and hemicellulose (Babicka et al., 2022; Lawal et al., 2017). After modification with KOH, this peak diminishes, indicating a reduction in free hydroxyl groups due to their participation in chemical reactions.

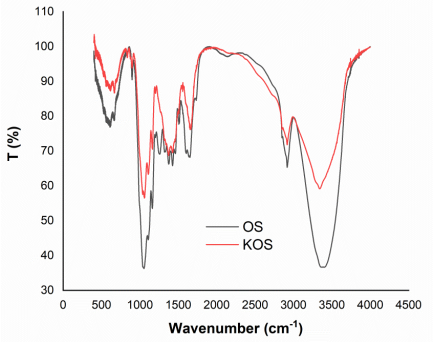

Fig. 2: FTIR spectra of OS and KOS

The peak around 2918 cm^{-1}, associated with C-H stretching vibrations from cellulose and hemicellulose, also decrease after modification, suggesting partial degradation of hemicellulose. This is further supported by changes in the region between 1000 and 1100 cm^{-1}, where C-O-C stretching vibrations occur (Dehkhoda et al., 2022; Kubovský et al., 2020). The shift and weakening of these peaks in the KOS spectrum confirm structuralalterationsin the ether bonds of cellulose and hemicellulose, likely enhancing the material's adsorption capacity.

Additionally, the peaks around 1600–1650 cm^{-1}, corresponding to C=O stretching in lignin and cellulose, are less pronounced in the KOS spectrum, suggesting modifications in the carbonyl groups. In the region between 500 and 800 cm^{-1}, the increase in intensity points to the possible incorporation of new functional groups or minerals after KOH treatment, which could play a role in enhancing ion exchange properties (Lawal et al., 2017; Ying et al., 2017).

In summary, the FTIR analysis confirms significant structural changes in OS after modification with KOH. These changes, including the reduction of hydroxyl groups and degradation of hemicellulose, contribute to the improved adsorptive properties of KOS (Simić et al., 2022).

In addition, FTIR analysis was performed to identify the structural changes in KOS before and after Pb adsorption. The broad peak around 3300 cm^{-1}, corresponding to O-H stretching, shows a decrease in intensity after Pb^{2+} adsorption (KOS-Pb), indicating the interaction between lead ions and hydroxyl groups on the biosorbent surface. The C-H stretching vibrations at 2918 cm^{-1}, associated with cellulose and hemicellulose, also decrease in intensity, suggesting that these components may be affected by lead binding. The C=O stretching vibrations around 1600–1650 cm^{-1}, which represent carbonyl groups of lignin and cellulose, show reduced intensity after adsorption, implying their involvement in the adsorption process (Lawal et al., 2017; Ying et al., 2017). In the C-O- C stretching region (1000–1100 cm^{-1}), slight shifts and a reduction in peak intensity suggest structural changes in the cellulose and hemicellulose ether linkages. Furthermore, the increased intensity in the 500–800 cm^{-1} region after Pb^{2+} adsorption points to the possible formation of new bonds or interactions with lead ions (Petrović et al., 2023).

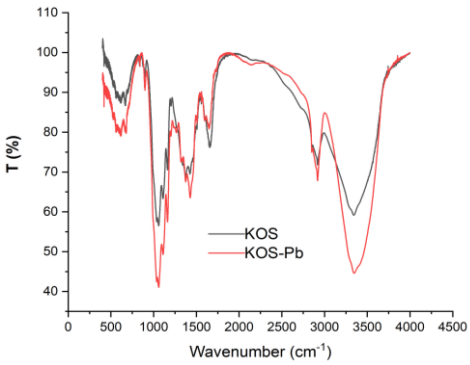

Fig. 3: FTIR spectra of KOS before and after Pb^{2+} adsorption

Overall, the FTIR spectra reveal that lead adsorption on KOS involves interactions with hydroxyl, carbonyl, and ether groups, confirming the role of both chemisorption and ion exchange in the adsorption mechanism (Petrović et al., 2016; Simić et al., 2022).

3.2. Batch adsorption tests

3.2.1. Preliminary Adsorption Test

The preliminary adsorption test was performed to evaluate the effectiveness of OS and KOS in removing Pb from aqueous

solutions. The biosorbent OS, showed an adsorption capacity of 38 mg/g, while the KOS, showed a significantly higher capacity of 89 mg/g. This clear increase in performance highlights the effectiveness of the KOH modification, which enhances the biosorbent's surface properties and provides more active sites for lead binding. Due to this substantial improvement, all subsequent adsorption studies were carried out using the modified KOS biosorbent to ensure optimal results.

3.2.2. *Influence of Contact Time and Kinetic Studies*

The impact of contact time on the adsorption of Pb ions by the KOS was investigated to establish the equilibrium time for sorption. The decrease in Pb ion concentration was observed over a time period ranging from 15 to 1440 minutes. As shown in Figure 4, the adsorption process began rapidly, with approximately 50% of lead ions being removed within the first 60 minutes. This fast initial uptake can be attributed to the abundance of accessible active sites on the KOS surface, where lead ions quickly interacted with these sites (Dimitrijević et al., 2023; Petrović et al., 2016).

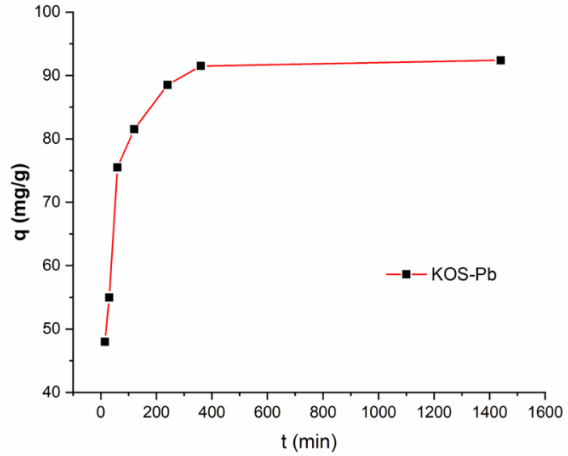

Fig. 4: Effect of contact time on Pb adsorption on KOS

After this initial period, the rate of adsorption slowed down, and the adsorption capacity gradually increased until equilibrium was reached. The equilibrium capacity for Pb^{2+} adsorption was found to be 93.45 mg/g, with equilibrium being achieved after approximately 240 minutes. This behavior suggests that after the rapid adsorption phase, the remaining unoccupied sites required more time for diffusion and interaction with the lead ions, likely due to intraparticle diffusion limitations (Petrović et al., 2016).

3.2.3. *Adsorption Kinetic Study*

Kinetic studies provide valuable information on the adsorption mechanisms, the rate of sorption, and potential rate-limiting steps. To interpret the kinetics of Pb^{2+} adsorption on KOS, three models were applied: the pseudo-first-order model (Lagergren, 1898), the pseudo-second-order model (Ho and McKay, 1999), and the Weber-Morris intraparticle diffusion model (Weber and Morris, 1963). These models help elucidate whether the process is controlled by physical adsorption, chemical adsorption (chemisorption), or diffusion mechanisms.
The pseudo-first-order model, based on the assumption that the rate of adsorption is proportional to the number of available adsorption sites, is described by the equation:

$$\frac{1}{q_t} = \left(\frac{k_1}{q_{eq}}\right)\left(\frac{1}{t}\right) + \left(\frac{1}{q_{eq}}\right) \quad (2)$$

where: q_{eq} is the equilibrium adsorption capacity (mg/g), q_t is the amount of adsorbate adsorbed at time t (mg/g), and k_1 is the pseudo-first-order rate constant (1/min). In this study, the pseudo-first-order model provided a poor fit to the experimental data, with a low correlation coefficient, indicating that the adsorption of lead onto the biosorbent is not primarily controlled by physical adsorption (Table 1). This suggests that the interaction between the lead ions and the biosorbent surface involves more complex mechanisms, such as chemical bonding, which is better captured by other models.
The pseudo-second-order kinetic model assumes that chemisorption is the rate-limiting step in the adsorption process. This model is represented by the equation:

$$\frac{t}{q_t} = \left(\frac{1}{k_2 q_{eq}^2}\right) + \left(\frac{1}{q_{eq}}\right) t \quad (3)$$

where: q_{eq} is the amount of lead adsorbed at equilibrium (mg/g), q_t is the amount of lead adsorbed at time t (mg/g), and k_2 is the pseudo-second-order rate constant (g/mg min). In this case, the pseudo-second-order model provided an excellent fit to the experimental data, with a correlation coefficient $R^2 = 0.9999$ (Table 1). The calculated equilibrium adsorption capacity $q_{eq} = 93.45$ mg/g closely matched the experimental value, confirming the validity of the model. The rate constant $k_2 = 0.0071$ g/mg min indicates that chemisorption is the primary controlling step, involving the ion exchange or sharing of electrons between Pb ions and the active functional groups on the KOS surface, which is the dominant mechanism.
The Weber-Morris intraparticle diffusion model was applied to evaluate whether diffusion into the pores of the biosorbent particles was a limiting factor in the adsorption process. The Weber-Morris model is represented by the equation:

$$q_t = K_{id} t^{0.5} + C \quad (4)$$

where: K_{id} is the intraparticle diffusion rate constant mg/g·min$^{1/2}$, and C is the intercept, which indicates the boundary layer thickness.

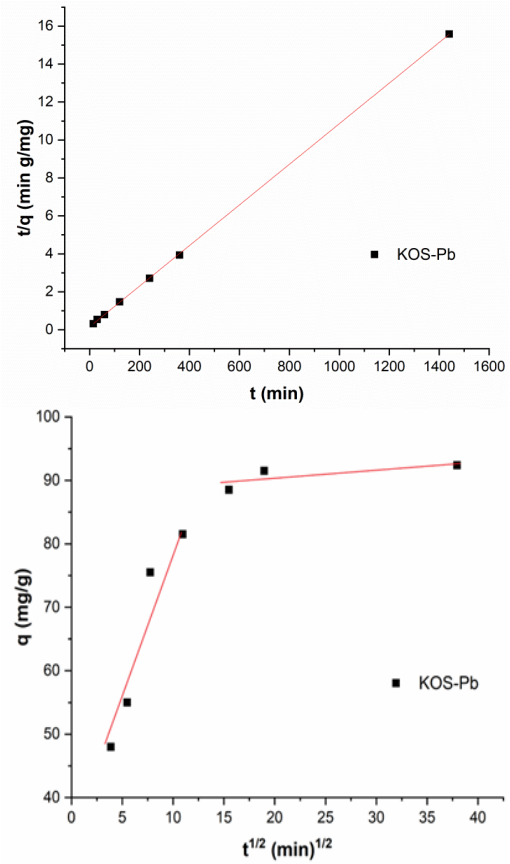

Fig.5: Kinetic models of Pb adsorption on KOS

The plot of q_t versus $t^{1/2}$ (Figure 5) revealed two distinct linear regions, indicating a multi-stage adsorption process. During the initial phase, the adsorption was dominated by surface interactions, as indicated by the first linear segment with a k_{id} value of 3.5512 mg/g·min$^{1/2}$ and an intercept C_1 = 27.9739 mg/g. In the later stages, the adsorption slowed down, with a lower k_{id2} = 0.14901 mg/g·min$^{1/2}$ suggesting that intraparticle diffusion became a more significant factor.

The results from the Weber-Morris model suggest that while external surface adsorption controls the initial rate of lead removal, intraparticle diffusion plays a role in the later stages of the process (Simić et al., 2022). However, the non-zero intercepts indicate that intraparticle diffusion is not the sole rate-limiting step. This finding supports the conclusion that the adsorption process is primarily controlled by chemisorption, with diffusion processes contributing to the overall mechanism (Dimitrijević et al., 2023).

Table 1: Kinetic parameters of Pb adsorption on KOS

Adsorbent KOS	KOS-Pb
$q_{eq,\,exp}$ [mg/g]	92.45±0.71
Pseudo-First-Order Model	
$q_{eq,cal}$ [mg/g]	87.49±0.84
k_1 [1/min]	8.95±0.25
R^2	0.9503 ±0.086
Pseudo-Second-Order Model	
$q_{eq,cal}$ [mg/g]	93.45±0.38
k_2 [g/mg min^{-1}]	0.0071±0.0017
R^2	0.9999±0.0001
Weber-Morris diffusion Model	
K_{id1} [mg/g min$^{-1/2}$]	3.5512 ±0.032
C_1 [mg/g]	27.9739 ±0.014
R^2	0.9173 ±0.022
K_{id2} [mg/g min$^{-1/2}$]	0.14901 ±0.0124
C_2 [mg/g]	86.8698 ±1.251
R^2	0.8527 ±0.0671

3.3. Adsorption of Lead on a Modified Biosorbent: Isotherm

The adsorption behavior of Pb onto a KOS was analyzed using multiple isotherm models to elucidate the underlying adsorption mechanisms. These models, including Langmuir (Langmuir, 1918), Freundlich (Freundlich, 1907), Sips (Sips, 1948), and Redlich-Peterson (Peterson and Redlich, 1962), each offered insights into different aspects of the adsorption. They provided Figure 6. perspectives on whether adsorption followed a monolayer or multilayer formation, the surface homogeneity of the biosorbent, and the possible coexistence of physical and chemical adsorption.

Langmuir isotherm is based on the assumption that adsorption occurs uniformly on a monolayer across a homogeneous surface, where all active sites have the same energy and each site can adsorb only one ion. This isotherm model is expressed as follows:

$$q_e = q_{max} K_L C_e / (1 + K_L C_e) \quad (5)$$

Where: q_e is the equilibrium adsorption capacity (mg/g), q_{max} is the maximum adsorption capacity (mg/g), K_L is the Langmuir constant (L/mg), and C_e is the equilibrium concentration of lead ions (mg/L).

In this study, the maximum adsorption capacity q_{max} was determined to be 191.41 mg/g, with a Langmuir constant K_L = 0.0072 L/mg (Table 2). This relatively high q_{max} value indicates that the KOS has a substantial capacity to remove Pb, reflecting a favorable interaction between Pb ions and the surface functional groups. Additionally, the high R^2 value of 0.9647 suggests that the Langmuir model fits the experimental data well, supporting the idea of monolayer adsorption (Table 2) which is characteristic of chemisorption. The favorability of this adsorption process is further confirmed by the dimensionless separation factor R_L = 0.970 (Table 2), indicating that adsorption is most favorable at lower Pb concentrations.

Table 2: Isotherm parameters for Pb^{2+} adsorption on KOS

Models	Parameters	KOS-Pb
Langmuir	q_m (mg/g)	191.41±2.15
	K_L (L/mg)	0.0072±0.0016
	R^2	0.9647±0.0021
	R_L	0.970±0.014
	χ^2	44.34 ±0.08
Freundlich	K_F (mg/g)(L/mg)$^{1/n}$	5.63±1.18
	1/n	1.78±0.24
	R^2	0.9442±0.0127
	χ^2	96.76±1.59
Sips	q_m (mg/g)	178.21±1.98
	K_S (L/mg)	0.0644±0.0125
	n_s	0.99±0.14
	R^2	0.9016±0.14
	χ^2	227.46±0.23
Redlich-Peterson	K_{RP} (L/g)	1.158±0.305
	a_{RP} (L/mg)	8.53±0.21
	β	1.33±0.67
	R^2	0.9777±0.0313
	χ^2	51.82±0.45

$$R_L = \frac{1}{1+K_L C_0} \quad (6)$$

Freundlich isotherm describes adsorption on a heterogeneous surface, where different sites exhibit varying affinities for the adsorbate. It is represented by the following equation:

$$q_e = K_F C_e^{1/n} \quad (7)$$

Where: K_F is the Freundlich constant, 1/n indicates the intensity of adsorption, with 1/n < 1 suggesting favorable adsorption.

In this study, the Freundlich constant K_F was 5.63, and the parameter 1/n = 1.78, indicating that the adsorption process was moderately favorable and occurred on a heterogeneous surface (Table 2). The R^2 = 0.9442 value was slightly lower than for the Langmuir model, suggesting that although the surface is somewhat heterogeneous, the assumption of monolayer adsorption remains more appropriate.

The Sips isotherm is a hybrid model that combines features of both the Langmuir and Freundlich isotherms. It predicts Freundlich behavior at lower concentrations and Langmuir behavior at higher concentrations. The Sips equation is:

$$q_e = q_{max}(K_S C_e)^{1/ns} / (1+(K_S C_e)^{1/nS}) \quad (8)$$

where: q_{max} is the maximum adsorption capacity, K_S is the Sips constant, and nS is the heterogeneity factor.

The Redlich-Peterson isotherm is an empirical model that combines elements of both the Langmuir and Freundlich models, making it suitable for systems involving both monolayer and multilayer adsorption. The equation is:

$$q_e = K_{RP} C_e / (1+a_{RP} C_e^g) \quad (9)$$

where: K_{RD} and a_{RP} represent Redlich–Peterson constants (L/g), and ((mg/L)$^{-g}$), respectively, and g is an empirical Redlich–Peterson parameter (g ≤ 1)

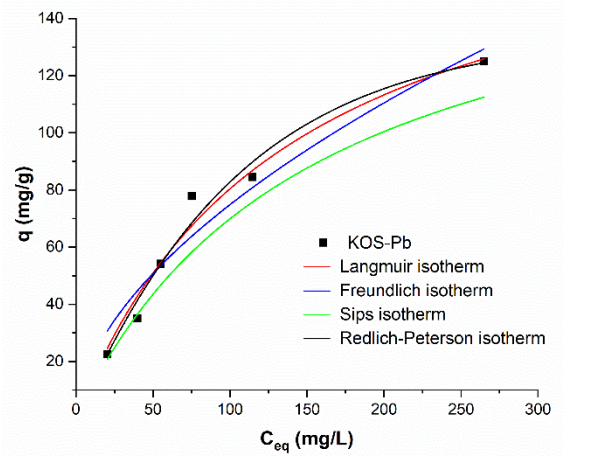

Fig.6: Adsorption isotherms of Pb removal on KOS

The Redlich-Peterson model provided the best fit to the experimental data, with an R^2 = 0.9777 (Table 2). The parameters were K_{RP}= 1.158 L/g, a_{RP} = 8.53 L/mg, and g = 1.33, suggesting that chemisorption plays a significant role in the adsorption process, though physical adsorption may also contribute at higher concentrations. This is in accordance with the Kinetic study.

The strong performance of the Redlich-Peterson model, as indicated by its high R^2 value, suggests that this model effectively captures the complexity of the adsorption process. The g parameter points to a combination of chemisorption and physical adsorption, with chemisorption being the dominant mechanism.

As can be concluded from results, the KOS demonstrates better adsorption properties for Pb removal compared to similar materials available in literature (Table 3).

Table 3. Adsorption capacity of different sorbents towards Pb(II) ions

Adsorbent	q_m (mg/g)	Reference
	Pb(II)	
Phoenix dactylifera	5.15	(Yazid and Maachi, 2008)
Black cumin seeds biochar	17.70	(Birgili et al., 2024)
Carbons derived of chestnut kernel	27.53	(Momčilović, 2012)
Carbon derived of Black pine cones	25.51	(Momčilović, 2012)
Simplicillium chinense QD10	57.80	(Torres, 2020)
Pseudomonas sp.	60.00	(Kaleem et al., 2023)
Rice husk biochar	72.00	(Birgili et al., 2024)
Nostoc sp. MK-11	83.96	(Kaleem et al., 2023)
Olive cake biochar	102.00	(Birgili et al., 2024)
Sargassum sp.	112.00	(Putri et al., 2021)
Lemna minor	142.86	(Kaya et al., 2024)
Allium scorodoprasum L.	190.41	(Şenol and Arslanoğlu, 2024)
KOS	191.41	This study
OS	38.00	This study

3.4. Ion-exchange mechanism

The ion exchange mechanism plays a crucial role in the adsorption of heavy metal ions from aqueous solutions. In this study, the removal of Pb ions was investigated in relation to the release of cations (Ca^{2+}, Na^+, K^+, and Mg^{2+}) from the biosorbent surface during the adsorption process. The results, indicate that with increasing Pb concentration, there is a corresponding release of Ca^{2+}, Na^+, K^+, and Mg^{2+} ions from the biosorbent, confirming that ion exchange is one of the mechanisms involved in the adsorption process (Figure 7). The higher release of Ca^{2+}, followed by K^+ and Mg^{2+}, indicates that calcium and potassium play a more significant role in the ion-exchange process, while sodium shows relatively minor participation. The exchange of these cations for Pb ions supports the formation of ionic bonds between the biosorbent surface and the lead ions (Petrović et al., 2016; Simić et al., 2022). The results also suggest that the quantity of released cations is lower than the total amount of adsorbed Pb^{2+} ions, indicating that additionalmechanisms, suchas surfacecomplexation or chemisorption, may also contribute to the overall removal process.

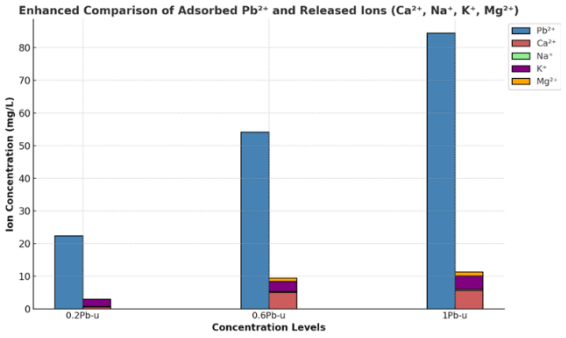

Fig.7: Ion exchange of $M^{+/2+}$ during the Pb adsorption on KOS

4. CONCLUSION

This study successfully demonstrated that the modification of OS with KOH significantly enhances its adsorption capacity for Pb^{2+} from contaminated water. The chemical modification increased the surface area and exposed functional groups that improved the interaction with pollutants, leading to a substantial increase in adsorption efficiency. The pseudo-second-order kinetic model confirmed that chemisorption was the primary mechanism involved. Langmuir isotherm model revealed a maximum adsorption capacity of 191.41 mg/g.

The results indicate that KOS is a highly effective biosorbent for Pb removal, offering a solution to both water pollution and agricultural waste management. Its application can be further expanded to other environmental contaminants, providing a scalable, eco-friendly option for wastewater treatment.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract No. 451-03-66/2024-03/200023 and 451-03-65/2024-03/200135).

CONFLICT OF INTEREST

The authors declare that they have no financial or commercial conflict of interest.

REFERENCES

Babicka, M., Woźniak, M., Bartkowiak, M., Peplińska, B., Waliszewska, H., Zborowska, M., Borysiak, S., and Ratajczak, I. (2022): Miscanthus and Sorghum as sustainable biomass sources for nanocellulose production, *Industrial Crops and Products*, **186**, 115177. https://doi.org/10.1016/j.indcrop.2022.115177

Birgili, B., Haykiri-Acma, H., and Yaman, S. (2024): Biosorption of lead, hexavalent chrome and cadmium from aqueous solution by torrefied biomass, *International Journal of Environmental Science and Technology*, **21**(12), 8049–8062. https://doi.org/10.1007/s13762-024-05507-w

Borrega, M., Hinkka, V., Hörhammer, H., Kataja, K., Kenttä, E., Ketoja, J. A., Palmgren, R., Salo, M., Sundqvist-Andberg, H., and Tanaka, A. (2022): Utilizing and Valorizing Oat and Barley Straw as an Alternative Source of Lignocellulosic Fibers, *Materials*, **15**(21), 7826. https://doi.org/10.3390/ma15217826

Bryngelsson, S., Dimberg, L. H., and Kamal-Eldin, A. (2002): Effects of Commercial Processing on Levels of Antioxidants in Oats (*Avena sativa* L.), *Journal of Agricultural and Food Chemistry*, **50**(7), 1890–1896. https://doi.org/10.1021/jf011222z

Dehkhoda, S., Bagheri, M., Heydari, M., and Rabieh, S. (2022): Extraction of carboxylated nanocellulose from oat husk: Characterization, surface modification and in vitro evaluation of indomethacin drug release, *International Journal of Biological Macromolecules*, **212**, 165–171. https://doi.org/10.1016/j.ijbiomac.2022.05.125

Dimitrijević, J., Jevtić, S., Marinković, A., Simić, M., Koprivica, M., and Petrović, J. (2023): Ability of Deep Eutectic Solvent Modified Oat Straw for Cu(II), Zn(II), and Se(IV) Ions Removal, *Processes*, **11**(5), 1308. https://doi.org/10.3390/pr11051308

Dong, J., Cai, F., Shen, R., and Liu, Y. (2011): Hypoglycaemic effects and inhibitory effect on intestinal disaccharidases of oat beta-glucan in streptozotocin-induced diabetic mice, *Food Chemistry*, **129**(3), 1066–1071. https://doi.org/10.1016/j.foodchem.2011.05.076

Fan, L., Miao, J., Yang, J., Zhao, X., Shi, W., Xie, M., Wang, X., Chen, W., An, X., Luo, H., Ma, D., and Cheng, L. (2022): Invasive plant-crofton weed as adsorbent for effective removal of copper from aqueous solution, *Environmental Technology & Innovation*, **26**, 102280. https://doi.org/10.1016/j.eti.2022.102280

Freundlich, H. (1907): Über die Adsorption in Lösungen, *Zeitschrift Für Physikalische Chemie*, **57U**(1), 385–470. https://doi.org/10.1515/zpch-1907-5723

Gollakota, A. R. K., Subbaiah Munagapati, V., Shu, C.-M., and Wen, J.-C. (2022): Adsorption of Cr (VI), and Pb (II) from aqueous solution by 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide functionalized biomass Hazel Sterculia (*Sterculia Foetida* L.), *Journal of Molecular Liquids*, **350**, 118534. https://doi.org/10.1016/j.molliq.2022.118534

Gómez Aguilar, D. L., Rodríguez Miranda, J. P., Astudillo Miller, M. X., Maldonado Astudillo, R. I., and Esteban Muñoz, J. A. (2020): Removal of Zn(II) in Synthetic Wastewater Using Agricultural Wastes, *Metals*, **10**(11), 1465. https://doi.org/10.3390/met10111465

Hatiya, N. A., Reshad, A. S., and Negie, Z. W. (2022): Chemical Modification of Neem (*Azadirachta indica*) Biomass as Bioadsorbent for Removal of Pb^{2+} Ion from Aqueous Waste Water, *Adsorption Science & Technology*, **2022**, 7813513. https://doi.org/10.1155/2022/7813513

Ho, Y. S., and McKay, G. (1999): Pseudo-second order model for sorption processes, *Process Biochemistry*, **34**(5), 451–465. https://doi.org/10.1016/S0032-9592(98)00112-5

Jovanović, A., Stevanović, M., Barudžija, T., Cvijetić, I., Lazarević, S., Tomašević, A., and Marinković, A. (2023): Advanced technology for photocatalytic degradation of thiophanate-methyl: Degradation pathways, DFT calculations and embryotoxic potential, *Process Safety and Environmental Protection*, **178**, 423–443. https://doi.org/10.1016/j.psep.2023.08.054

Kaleem, M., Minhas, L. A., Hashmi, M. Z., Ali, M. A., Mahmoud, R. M., Saqib, S., Nazish, M., Zaman, W., and Samad Mumtaz, A. (2023): Biosorption of Cadmium and Lead by Dry Biomass of Nostoc sp. MK-11: Kinetic and Isotherm Study, *Molecules*, **28**(5), 2292. https://doi.org/10.3390/molecules28052292

Kaya, S., Çetinkaya, S., Jalbani, N. S., Yenidünya, A. F., Kütük, N., Kasaka, E., and Maslov, M. M. (2024): Biosorption of lead ions (Pb^{2+}) from water samples using dried *Lemna minor* biomass: experimental and density functional theory studies, *Biomass Conversion and Biorefinery*, **14**(15), 17603–17617. https://doi.org/10.1007/s13399-023-03914-6

Kim, I.-S., Hwang, C.-W., Yang, W.-S., and Kim, C.-H. (2021): Multiple Antioxidative and Bioactive Molecules of Oats (*Avena sativa* L.) in Human Health, *Antioxidants*, **10**(9), 1454. https://doi.org/10.3390/antiox10091454

Kosiorek, M., and Wyszkowski, M. (2019): Content of macronutrients in oat (*Avena sativa* L.) after remediation of soil polluted with cobalt, *Environmental Monitoring and Assessment*, **191**(6), 389. https://doi.org/10.1007/s10661-019-7529-6

Kubovský, I., Kačíková, D., and Kačík, F. (2020): Structural Changes of Oak Wood Main Components Caused by Thermal Modification, *Polymers*, **12**(2), 485. https://doi.org/10.3390/polym12020485

Lagergren, S. (1898): Zur theorie der sogenannten adsorption gelöster stoffe, **24**, 1–39. https://doi.org/10.1007/BF01501332

Lai, P., Zhou, H., Niu, Z., Li, L., Zhu, W., and Dai, L. (2023): Deep eutectic solvent-mediated preparation of solvothermal carbon with rich carboxyl and phenol groups from crop straw for high-efficient uranium adsorption, *Chemical Engineering Journal*, **457**, 141255. https://doi.org/10.1016/j.cej.2022.141255

Langmuir, I. (1918): The adsorption of gases on plane surfaces of glass, mica and platinum, *Journal of the American Chemical Society*, **40**(9), 1361–1403. https://doi.org/10.1021/ja02242a004

Lawal, I. A., Chetty, D., Akpotu, S. O., and Moodley, B. (2017): Sorption of Congo red and reactive blue on biomass and activated carbon derived from biomass modified by ionic liquid, *Environmental Nanotechnology, Monitoring & Management*, **8**, 83–91. https://doi.org/10.1016/j.enmm.2017.05.003

Momčilović, M. Z. (2012): *Kinetic and equilibrium parameters of adsorption processes under removal of certain harmful cationic ingrediants from aqueous solutions using activated carbons derived by thermochemical treatment of chestnut kernel and Black pine cones*, PhD thesis, Prirodno-Matematički Nis.

Peterson, D. L., and Redlich, O. (1962): Sorption of Normal Paraffins by Molecular Sieves Type 5A., *Journal of Chemical & Engineering Data*, **7**(4), 570–574. https://doi.org/10.1021/je60015a043

Petrović, J., Ercegović, M., Simić, M., Kalderis, D., Koprivica, M., Milojković, J., and Radulović, D. (2023): Novel Mg-doped pyrohydrochars as methylene blue adsorbents: Adsorption behavior and mechanism, *Journal of Molecular Liquids*, **376**, 121424. https://doi.org/10.1016/j.molliq.2023.121424

Petrović, J., Stojanović, M., Milojković, J., Petrović, M., Šoštarić, T., Laušević, M., and Mihajlović, M. (2016): Alkali modified hydrochar of grape pomace as a perspective adsorbent of Pb^{2+} from aqueous solution, *Journal of Environmental Management*, **182**, 292–300. https://doi.org/10.1016/j.jenvman.2016.07.081

Putri, L. S. E., Syafiqa, E., and Apriliyani, E. (2021): Biosorption of lead by biomass of dried sargassum, *IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)*, **15**(4), 58–63.

Ren, Y., Yang, X., Niu, X., Liu, S., and Ren, G. (2011): Chemical Characterization of the Avenanthramide-Rich Extract from Oat and Its Effect on D -Galactose-Induced Oxidative Stress in Mice, *Journal of Agricultural and Food Chemistry*, **59**(1), 206–211. https://doi.org/10.1021/jf103938e

Şenol, Z. M., and Arslanoğlu, H. (2024): Influential biosorption of lead ions from aqueous solution using sand leek (*Allium scorodoprasum* L.) biomass: kinetic and isotherm study, *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-024-05539-9

Simić, M., Petrović, J., Šoštarić, T., Ercegović, M., Milojković, J., Lopičić, Z., and Kojić, M. (2022): A Mechanism Assessment and Differences of Cadmium Adsorption on Raw and Alkali-Modified Agricultural Waste, *Processes*, **10**(10), 1957. https://doi.org/10.3390/pr10101957

Sips, R. (1948): On the Structure of a Catalyst Surface, *The Journal of Chemical Physics*, **16**(5), 490–495. https://doi.org/10.1063/1.1746922

Torres, E. (2020): Biosorption: A Review of the Latest Advances, *Processes*, **8**(12), 1584. https://doi.org/10.3390/pr8121584

Weber, W. J., and Morris, J. C. (1963): Kinetics of Adsorption on Carbon from Solution, *Journal of the Sanitary Engineering Division*, **89**(2), 31–59. https://doi.org/10.1061/JSEDAI.0000430

Yazid, H., and Maachi, R. (2008): Biosorption of Lead (II) Ions From Aqueous Solutions by Biological Activated Dates Stems, *Journal of Environmental Science and Technology*, **1**(4), 201–213. https://doi.org/10.3923/jest.2008.201.213

Ying, D., Hlaing, M. M., Lerisson, J., Pitts, K., Cheng, L., Sanguansri, L., and Augustin, M. A. (2017): Physical properties and FTIR analysis of rice-oat flour and maize-oat flour based extruded food products containing olive pomace, *Food Research International*, **100**, 665–673. https://doi.org/10.1016/j.foodres.2017.07.062

Zhang, T., Jiang, W., Cao, Y., Zhu, C., Toukouki, S., and Yao, S. (2022): A facile one-pot synthesis of ionic liquid@porous organic frameworks for rapid high-capacity removal of heavy metal ions, pesticides and aflatoxin from two non-food bioactive products, *Industrial Crops and Products*, **181**, 114859. https://doi.org/10.1016/j.indcrop.2022.114859

Cyclodextrin-aided green extraction from *Fagopyrum esculentum* Moench – an effective strategy for the sustainable recovery of polyphenols

SNEŽANA KUZMANOVIĆ NEDELJKOVIĆ[1*], MILICA RADAN[1], DUBRAVKA BIGOVIĆ[1] AND KATARINA ŠAVIKIN[1]

[1]*Institute for Medicinal Plants Research „Dr. Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia*
**Corresponding author: skuzmanovic@mocbilja.rs*

Received: November 13, 2024
Accepted: December 4, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

As a rich source of polyphenolic compounds, *Fagopyrum esculentum* Moench herb, is a valuable subject for a numerous industries, such as pharmaceutical, chemical, food, or cosmetic. Given the biological potential that is closely related to the polyphenolic content, this study aimed to develop ecologically suitable cyclodextrin-aided extraction from the aerial parts of *F. esculentum*. To maximize the polyphenolic content, highly-reliable mathematical and statistical method – response surface methodology (RSM) was used for the evaluation of crucial extraction parameters: temperature, extraction time, concentrations of ethanol and two types of cyclodextrins (*β*-cyclodextrin (*β*-CD), and (2-hydroxypropyl)-*β*-cyclodextrin (HP-*β*-CD)). While temperature and ethanol concentration significantly influenced the total phenolic content (TPC) in both models, the impact of cyclodextrin concentration was observed when HP-*β*-CD was used. According to the RSM analysis, optimal conditions for the maximization of TPC were noted for the extraction at 80 °C for 40 min, using 50% ethanol and 1.50% HP-*β*-CD. Thus, under optimized extraction conditions, the measured TPC was 42.16 ± 1.01 mg GAE/g DW, among rutin was the most abundant with content of 29.55 mg/g DW, followed by the quercetin and quercitrin in slightly scarcer content. This study confirmed that cyclodextrin-aided extraction is an ecologically friendly, effective method for the recovery of *F. esculentum* polyphenolics.

Keywords: common buckwheat; ultrasound-assisted extraction; cyclodextrin; extraction optimization; response surface methodology; rutin

https://doi.org/10.61652/leksir2444012K

1. INTRODUCTION

For centuries, medicinal plants and their products have built the foundation for the treatment of various diseases. Considering that nearly 80% of the population is still dependent on herbal medicine, the demand for medicinal herbs, including minor grain crops and their products, is currently increasing on the global level (Purkait et al., 2023). Among them, buckwheat, as a member of the genus *Fagopyrum*, family Polygonaceae, has been widely grown mainly in the cold regions, with China and Russia being the main producers (Li, 2019). The most frequently used, common buckwheat (*Fagopyrum esculentum* Moench), has been recognized for significant health benefits, including its potential for the prevention of chronic diseases (Radan et al., 2023). For example, the consumption of this herb or its products has been shown to express anti-inflammatory, hypoglycemic, hypocholesterolemic, and anticancer properties (Giménez-Bastida and Zieliński, 2015). According to the randomized, double-blind, single-center, placebo-controlled clinical trial carried out by (Ihme et al., 1996), the buckwheat herb tea proved its safety and favorable effects against further edema development in chronic venous insufficiency as well. Polyphenolic components have been described as the carriers of the buckwheat's bioactive potential, where rutin, quercetin, hyperin, chlorogenic acid, and catechins, have been commonly identified among various extracts (Hinneburg and Neubert, 2005; Inglett et al., 2010; Radan et al., 2023). Numerous biological effects have been described for these polyphenolics, such as antimicrobial, anti-hypertensive, antioxidant, and anti-inflammatory potential (Ahmed et al., 2014; Noreen et al., 2021).

Not only that, rutin has benefits in the treatment and prevention of cardiovascular diseases, with its potential to lower plasma cholesterol and decrease the fragility of capillaries (Salvamani et al., 2014). Besides the potential pharmaceutical use, it has been shown that it could be also used as a natural pigment, food preservative, and stabilizer, making it an interesting compound in the food and chemical industries (Kim et al., 2005). Furthermore, in the cosmetic industry rutin showed promising effects as a component of suncare products as well, due to its potential to scavenge free radicals and absorb UV radiation (Hinneburg and Neubert, 2005). On the other hand, quercetin was reported to evince neuroprotective, antineoplastic, anti-inflammatory, anti-allergic, and antihistamine effects (Rakha et al., 2022; Zalpoor et al., 2022).

Embracing the great potential of buckwheat's bioactive compounds in various industries, optimal extraction techniques must be employed, covering not only the highest yield and quality of the final product but also choosing the sustainable methods following green chemistry principles (Radan et al., 2024). Proper extraction method is of the key importance for both qualitative and quantitative composition of bioactive compounds (Azmir et al., 2013). While being usually used on the small research and manufacturing level, traditional methods such as Soxhlet extraction and maceration have certain disadvantages, related to low reproducibility, low production efficiency, and a demand for a significant amount of time (Pilkington et al., 2014). Thus, ultrasound-assisted extraction (UAE) stands out as a proper, sustainable, and more feasible method, capable of providing high quality products, with a significant reduction in solvent and time consumption (Pandey et al., 2018; Pilkington et al., 2014). This particular method potentiates the solvent's mass transport into the treated plant material and simultaneously facilitates the recovery of bioactive compounds by applying the acoustic cavitation effect that increases the contact surface between plant cells and solvent (Azwanida, 2015). Additionally to selecting the optimal extraction method, the green chemistry principles embolden the use of eco-friendly, safe, and non-toxic solvents as well (Alibante et al., 2021). Polyphenolic compounds are usually extracted using various organic solvents, which raise some toxicological and environmental concerns (Cai et al., 2018). However, despite being the most green solvent of all, pure water proved to be significantly less potent in extracting bioactives from buckwheat, mainly due to the poor solubility of main polyphenolics – quercetin and rutin (Çelik et al., 2015; Chemat et al., 2019; Hinneburg and Neubert, 2005; Radan et al., 2023). For example, (Zieliński and Kozłowska, 2000) revealed that 80% methanol as an extraction solvent provided a 64 times greater yield of polyphenolics from buckwheat and four times more powerful antioxidant potential than pure water. Thus, the mixture of water with diverse portions of ethanol accouter to provide multiple benefits – adequate environmental status, non-toxic behavior, and satisfactory potential for the extraction of quercetin glycosides (Hinneburg and Neubert, 2005).

For the enhancement of bioactives' solubility and bioavailability, a novel, cutting-edge trend of cyclodextrin-based extraction proved to be the green and safe alternative to the overuse of organic, toxic solvents (Bozinou et al., 2021). Some research revealed the beneficial use of cyclodextrins (CDs) for the solubility improvement of rutin and quercetin as well (Carlotti et al., 2011; Nguyen et al., 2013; Paczkowska et al., 2015; Savic et al., 2016). The specific cone structure of these molecules, with primary and secondary hydroxyl functional groups facing outwards, provides improved solubility in polar solvents, embracing hydrophobic molecules encapsulated into the cone cavity at the same time (Alibante et al., 2021). The β-cyclodextrin (β-CD), which has the approved GRAS (generally recognized as safe) status, is the most commonly used natural CD due to its low price and accessibility, while its modified molecule – (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) has been described as the more water-soluble and safer than its precursor (Cai et al., 2018). This straightforward approach proved to be simple and easily implemented at both laboratory and industrial scales (Bozinou et al., 2021).

However, as the final yield of bioactive compounds is closely dependent on several factors, such as extraction temperature, time of the extraction process, and the concentration of the used solvent, their optimization is the essential step (Cai et al., 2018; Radan et al., 2024). While being time-consuming and incompetent to evaluate the interactions between the observed factors, the conventional "one-factor-at-a-time" optimization approach has been widely replaced with more sophisticated and accurate mathematical and statistical techniques such as response surface methodology (RSM) (Radan et al., 2023). The RSM has been applied to a great extent for the optimization of bioactives' extraction (Mudrić et al., 2020; Setyani et al., 2023; Weremfo, Abassah-Oppong, et al., 2023). This particular method investigates the relative importance of each varied factor on the dependent variables (monitored responses) and provides information on the most optimal conditions for the maximized desired outcomes (Yu et al., 2019).

Therefore, hypothesizing that CDs may improve the recovery of poor-soluble valuable bioactive compounds from the *F. esculentum* Moench aerial parts, and reduce the ethanol content and duration of the extraction process, this study aimed to reveal the novel, environmentally friendly, and cost-effective extraction method for the maximum recovery of buckwheat's polyphenolics. In that manner, the extraction temperature, extraction time, ethanol concentration, simultaneously with the concentration of CDs – β-CD and HP-β-CD, were varied, and their individual and interactive influence on the polyphenols content have been analyzed by the RSM approach. This is the novel method for the extraction of *F. esculentum* polyphenols, using a green extraction technique and potent and safe complexation agents. To the best of the authors' knowledge, the optimization of the environmentally-friendly UAE method of bioactive compounds from common buckwheat, by using the RSM modeling approach evaluating several crucial factors on the final yield of polyphenols, including the influence of two CDs, has not been investigated so far.

2. MATERIALS AND METHODS

2.1. Plant material

The dried aerial parts in the flowering stage of *F. esculentum* Moench (control number: 01540120), were provided from the Production sector of the Institute for Medicinal Plants Research "Dr. Josif Pančić", Belgrade, Serbia. The plant material was grinded using the industrial mill and then sieved through a 5-sieve set of standardized pore size according to the Yugoslavian Pharmacopoeia, 2000 (*V. Yugoslavian Pharmacopoeia*, 2000) Yug. V). The fraction of 0.75–2 mm particle size was selected for further experiments.

2.2. Chemicals

Both CDs – β-CD and HP-β-CD, were provided by Acros Organics (Geel, Belgium). Ortho-phosphoric acid and acetonitrile were of analytical grade, and purchased from Sigma Aldrich (St Louis, MO, USA), while the following standards: rutin, quercetin, and quercitrin, were provided from Extrasynthese (Genay, France). The Milli-Q water purification system (Millipore, Molsheim, France) was used to provide the ultrapure distilled water used for the preparation of the mobile phase.

2.3. Ultrasound-assisted extraction of bioactive compounds

Recovery of polyphenols from *F. esculentum* dried aerial parts was obtained by the UAE method in the ultrasonic water bath (Bandelin Sonorex, Berlin, Germany). The 1.0 g of sieved plant material was mixed with 20 mL of CD aqueous ethanolic solution in a 50 mL glass flask (1:20 w/v), and exposed to the sonification under constant power and frequency (320 W and 35 kHz, respectively). To reveal the optimal extraction conditions for the maximized polyphenolic content, four crucial parameters according to the literature (Cai et al., 2018; Mudrić et al., 2020) were varied, including extraction time (10–70 min), temperature (20–80 °C), ethanol concentration (0–80%), and the concentration of used CD (0–1.6%). Looking into perspective that this study aimed to maximize total phenolic content (TPC) under the most possible "green" extraction conditions, including the reduction of ethanol concentration and duration of extraction process, and taking into account that cyclodextrins have been attributed with significant potential to enhance the solubility of poorly dissolved bioactives (Pinho et al., 2014), the influence of CD was evaluated simultaneously with time, temperature, and solvent concentration. As the difference between the solubility of *β*-CD and HP-*β*-CD can be expected (Cai et al., 2018), two separate experimental protocols were applied for each CD. Obtained mixtures were filtered through filter paper, and properly stored at 4 °C until further experiments.

2.4. Chemical characterization of *F. esculentum* extracts

2.4.1. Total phenolic content

The TPC in the obtained *F. esculentum* extracts was measured spectrophotometrically by the Folin-Ciocalteu (FC) method (Waterman and Mole, 1994) with modifications related to the volume of diluted extract, sodium carbonate solution, and FC reagent described in the study of (Radan et al., 2024). After 2 h of incubation at room temperature and hidden from light, the absorbance was measured at 765 nm. All experiments were performed in three repetitions, and the results were expressed as a mean value of milligrams of gallic acid equivalent (GAE) per gram of dry weight (DW) of *F. esculentum* herb (mg GAE/g DW).

2.4.2. HPLC analysis

Quantification of the main polyphenols from the optimal *F. esculentum* extract (extract with the highest TPC) was performed by HPLC analysis on Agilent Technologies 1260 Series with the Lichrospher RP-18 (250 × 4.0 mm) analytical column, with particle size 5 μm, and DAD detector, following the method described in the study of (Radan et al., 2023). After the UV detection at 270 nm and 340 nm, the identification of the most abundant polyphenols – rutin, quercetin, and quercitrin, was carried out by comparison of obtained UV spectra and retention time, with the same parameters corresponding to the reference standards. The final content of polyphenolics, presented as milligrams per gram of dry weight of *F. esculentum* herb (mg/g DW), was calculated using the generated calibration curves for each compound ($R^2 > 0.99$).

2.5. Experimental design using Response surface methodology (RSM)

The RSM was employed for the optimization of the UAE recovery of polyphenols from *F. esculentum* herb, with applied central-composite design for the evaluation of the impact of

Table 1. Central-composite design of four independent variables at five levels, using *β*-CD as a complexation agent, and experimentally observed values of TPC as an investigated response.

	Independent variables				*Dependent variable*
Run	X_1: Temperature (°C)	X_2: Time (min)	X_3: EtOH concentration (%)	X_4: *β*-CD concentration (%)	TPC (mg GAE/g DW)
1	35	55	20	0.4	15.11
2	65	25	60	0.4	35.83
3	50	40	40	1.6	28.81
4	50	40	40	0.8	28.83
5	50	40	40	0.8	33.57
6	35	55	20	1.2	17.56
7	50	40	40	0	31.82
8	35	55	60	0.4	30.35
9	35	55	60	1.2	30.56
10	50	40	40	0.8	32.43
11	65	55	60	0.4	35.09
12	35	25	20	0.4	15.41
13	20	40	40	0.8	21.74
14	65	55	20	1.2	25.03
15	50	10	40	0.8	31.39
16	50	70	40	0.8	29.83
17	80	40	40	0.8	36.51
18	65	25	20	0.4	24.75
19	35	25	60	1.2	29.62
20	50	40	0	0.8	12.23
21	50	40	40	0.8	30.69
22	65	55	20	0.4	28.44
23	50	40	40	0.8	30.60
24	50	40	80	0.8	29.93
25	65	25	60	1.2	34.32
26	35	25	60	0.4	30.99
27	35	25	20	1.2	19.73
28	65	25	20	1.2	27.10
29	50	40	40	0.8	30.89
30	65	55	60	1.2	34.24

β-CD – *β*-cyclodextrin; TPC – Total phenolic content.

different extraction conditions. Four process variables, including extraction time, temperature, ethanol concentration, and the concentration of CD were varied at five levels, resulting in 30 runs with 5 central points, per each used CD (β-CD or HP-β-CD) (Tables 1 and 2). The relationship between the varied factors (independent variables) and the TPC as the tracked response is explained with Equation (1):

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad (1)$$

where Xi and Xj represent the independent variables, Y the dependent variable, while β_0, β_i, β_{ii}, and β_{ij}, stand for the intercept, linear, quadratic, and interactive regression coefficients, respectively. Design-Expert 13 program (Stat-Ease, Minneapolis, Minnesota, USA) was used for the central-composite experimental design and statistical analysis, and analysis of variance (ANOVA) was used to evaluate the models' significance. The adequacy of the model was observed by the evaluation of coefficient of determination (R^2), root-mean-square error (RMSE), lowest mean absolute deviation (MAD), models' p values, and finally, the lack-of-fit testing. In order to verify the capability of the model's prediction, the validation analysis was performed in triplicates, under optimized conditions.

3. RESULTS AND DISCUSSION

Exploring the opportunities of the *F. esculentum* herb's polyphenolics in various industries, such as pharmaceutical, food, chemical, and cosmetic, this study investigated the optimal extraction method for the maximized recovery of bioactive compounds, respecting the green chemistry principles. Being the main carriers of the common buckwheat's bioactive potential, the recovery of polyphenols was potentiated through extraction optimization using a highly competent mathematical and statistical method – RSM.

3.1. Total phenolic content in the obtained extracts

Following the matrix provided by the RSM, thirty separate extractions for each model were performed, varying crucial factors that may affect the yield of total phenolics. As presented in Tables 1 and 2, the obtained results revealed that the TPC ranged from 12.23 to 36.51 mg GAE/g DW when β-CD was used, while the HP-β-CD provided the content of 13.35–39.67 mg GAE/g DW. These results are slightly higher than the obtained TPC range of 9.87–25.87 mg GAE/g DW in our previous study, when the conventional method for extraction (maceration), was used for the recovery of bioactives from the same plant material (Radan et al., 2023). Given that the mass transport of the solvent into the treated plant material is potentiated by the generation of ultrasound in the UAE (Azwanida, 2015), the superior yield obtained in this study is not surprising. The highest yield (39.6730 mg GAE/g DW) was accomplished by the extraction at 80 °C for 40 min, using 40% ethanol solution with the addition of 0.8% HP-β-CD, while lower TPC was obtained with β-CD under the same conditions, which could be related to a greater solubility of hydroxypropyl derivate (Cai et al., 2018). A similar observation was reported by (Diamanti et al., 2017) when TPC

Table 2. Central-composite design of four independent variables at five levels, using HP-β-CD as a complexation agent, and experimentally observed values of TPC as an investigated response.

	Independent variables				*Dependent variable*
Run	**X_1: Temperature (°C)**	**X_2: Time (min)**	**X_3: EtOH concentration (%)**	**X_4: HP-β-CD concentration (%)**	**TPC (mg GAE/g DW)**
1	35	55	20	0.4	16.34
2	65	25	60	0.4	35.99
3	50	40	40	1.6	33.61
4	50	40	40	0.8	29.14
5	50	40	40	0.8	31.56
6	35	55	20	1.2	17.11
7	50	40	40	0	29.48
8	35	55	60	0.4	30.13
9	35	55	60	1.2	30.92
10	50	40	40	0.8	33.10
11	65	55	60	0.4	34.05
12	35	25	20	0.4	17.54
13	20	40	40	0.8	22.57
14	65	55	20	1.2	27.91
15	50	10	40	0.8	30.35
16	50	70	40	0.8	33.45
17	80	40	40	0.8	39.67
18	65	25	20	0.4	24.83
19	35	25	60	1.2	28.74
20	50	40	0	0.8	13.35
21	50	40	40	0.8	32.55
22	65	55	20	0.4	27.29
23	50	40	40	0.8	32.12
24	50	40	80	0.8	31.07
25	65	25	60	1.2	37.55
26	35	25	60	0.4	28.76
27	35	25	20	1.2	20.04
28	65	25	20	1.2	26.83
29	50	40	40	0.8	32.21
30	65	55	60	1.2	37.40

HP-β-CD – HP-β-cyclodextrin; TPC – Total phenolic content.

obtained after polyphenolic extraction from pomegranate fruit with HP-β-CD (71.70 mg GAE/ g DW) was significantly higher than by using the β-CD-aided extraction (58.70 mg GAE/g DW). On the other hand, the poorest TPC of 12.23 mg GAE/g DW was obtained with 0.8% β-CD, using pure water (0% ethanol) for 40 min at 50 °C. Slightly higher TPC was noted under the same conditions using HP-β-CD (13.35 mg GAE/g DW).

3.2. RSM analysis

3.2.1. Model fitting

As polyphenols represent the carriers of common buckwheat's bioactivity, the optimization of UAE was focused on the maximization of TPC in both models. The individual influence of each varied independent variable, as well as the influence of their interactions were evaluated by RSM technique based on the obtained results on the TPC, and the summary of statistically significant factors for each model is presented in Table 3.

Table 3. RSM model fitting of β-CD- and HP-β-CD-aided extractions.

β-CD-aided extraction		**HP-β-CD-aided extraction**	
TPC		TPC	
	p-value		*p*-value
Model	< 0.0001	**Model**	< 0.0001
X_1	< 0.0001	X_1	< 0.0001
X_3	< 0.0001	X_3	< 0.0001
X_1X_3	0.0042	X_4	0.0174
$(X_1)^2$	0.0715	$(X_3)^2$	< 0.0001
$(X_3)^2$	< 0.0001		
Lack-of-fit - not significant		**Lack-of-fit - not significant**	
R^2 = 0.9522		**R^2 = 0.9501**	
R^2 adj = 0.9422		**R^2 adj = 0.9421**	
R^2 pred = 0.9145		**R^2 pred = 0.9119**	

TPC – total phenolic content; X_1 – Extraction temperature (°C); X_3 – Ethanol concentration (%); X_4 – CD concentration (%); $p > 0.10$ – not significant; $0.05 < p \leq 0.10$ – moderately significant; $0.01 < p \leq 0.05$ – significant; $p < 0.01$ – highly significant; β-CD – β-cyclodextrin; HP-β-CD – hydroxypropyl-β-cyclodextrin; R^2 – coefficient of determination; R^2 adj – adjusted coefficient of determination; R^2 pred – predicted coefficient of determination.

Based on the obtained results, the total polyphenols recovered by both β-CD and HP-β-CD-aided extraction from aerial parts of *F. esculentum* were appropriately described with highly significant quadratic regression models ($p < 0.0001$). Additionally, a statistically non-significant parameter of lack-of-fit confirmed the adequacy of the models. Strong predictive capability and robustness were confirmed by high values of coefficients of determination (0.9522 and 0.9501 for the β-CD and HP-β-CD-aided extraction models, respectively), indicating a good fitting between the experimentally obtained and predicted values, which is graphically shown in Figure 1.

3.2.2. Effect of UAE extraction parameters on the TPC

As noticeably presented in Table 3, the recovery of polyphenols from *F. esculentum* herb by the UAE method in both models was highly influenced by the linear terms of the temperature and ethanol concentration with a positive effect, as well as the quadratic term of the solvent concentration ($p < 0.0001$). Also, the β-CD-aided extraction model revealed the moderately significant negative influence of the quadratic effect of temperature ($p < 0.1$), as well as the highly significant interaction term between ethanol concentration and temperature ($p < 0.01$). Under studied experimental conditions, the influence of CD concentration was statistically significant ($p < 0.05$) only when HP-β-CD was used, with the positive influence in the linear term. In addition, variations in extraction time did not show a significant impact on polyphenol extraction in either model. Predicted regression models for TPC are presented by the following equations based on the statistically significant independent variables, for β-CD- (Eq. (2)) and HP-β-CD-aided extraction (Eq. (3)):

$$\mathrm{TPC}_{\beta\text{-CD}}\ (\mathrm{mg\ GAE/g\ DW}) = 30.5996 + 3.5419X_1 + 5.1366X_3 - 1.2224X_1X_3 - 0.5471(X_1)^2 - 2.5587(X_3)^2 \quad (2)$$

$$\mathrm{TPC}_{\text{HP-}\beta\text{-CD}}\ (\mathrm{mg\ GAE/g\ DW}) = 30.8955 + 4.0194X_1 + 5.0454X_3 + 0.8270X_4 - 2.5500(X_3)^2 \quad (3)$$

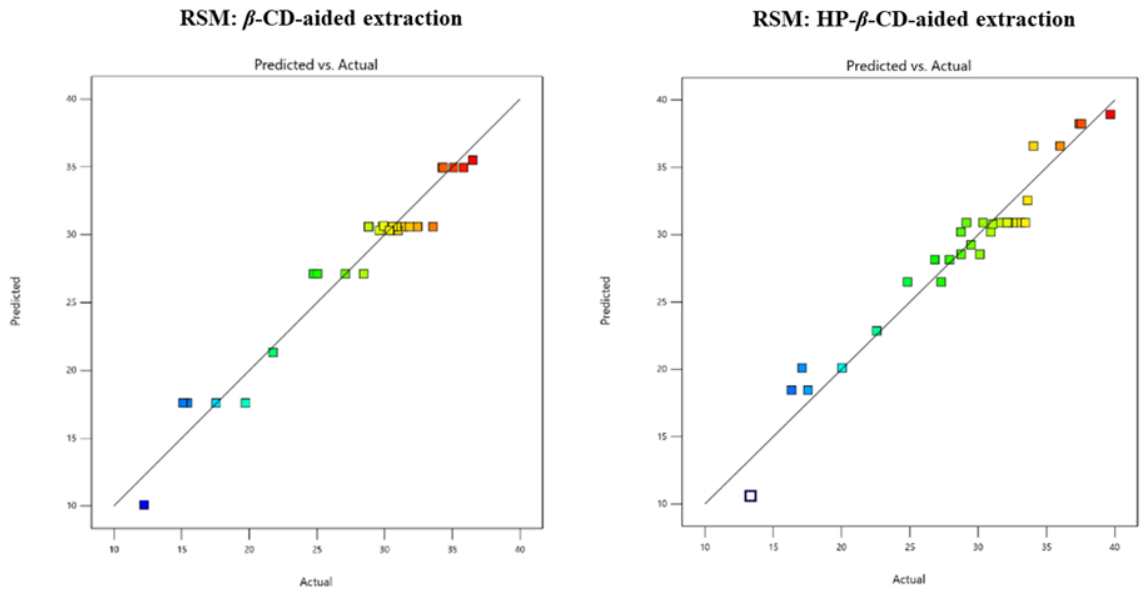

Fig. 1. Plots of experimental versus predicted values for each response in the created RSM models.

Based on the obtained results, it can be observed that the temperature highly influenced the yield of total polyphenols. In both models, the increase of the extraction temperature resulted in the following augmentation of TPC (Figures 2A$_1$ and 2A$_2$), which can be explained by the improvement of phenolic solubility, extraction, and diffusion rate, as well as the reduction of surface tension under higher extraction temperatures (Prasad et al., 2011). Moreover, the UAE method provides additional temperature-augmented compound solubility, as the heating evokes an increase in the amount of cavitation bubbles favoring the diffusivity of the solvent (Chemat et al., 2017). However, under relatively high-temperature conditions, polyphenols could be degraded due to their insufficient stability, resulting in a decrease in the total yield (Durling et al., 2007). This particular outcome was revealed only for a model with β-CD that indicated negative quadratic influence ((X_1)^2) of extraction temperature on the polyphenols recovery. Notably, while the formation of inclusion complexes with CDs and polyphenols may be improved by the increasing temperature, at 50–60 °C the decomposition of the formed inclusion complexes may happen (Cai et al., 2018). Thus, as the positive effect of CDs may be weakened with the temperature rise (Del Valle, 2004), these results may indicate the supremacy of HP-β-CD-formed inclusion complexes on the stability endurance compared with β-CD.

Ethanol concentration also revealed the significant positive linear (X_3) and negative quadratic (X_3)^2 influence in both CD models (Figures 2B$_1$ and 2B$_2$), highlighting the importance of identifying the optimal solvent's concentration to achieve the best TPC. While the increasing ethanol content to certain values leads to the reduction of the dielectric constant of the solvent, and subsequently to the better attraction of the dissolved fraction, i.e. improved solubility (Pompeu et al., 2009), higher concentrations may lead to dehydration of the plant material and cause the lower solubility and decreased recovery of polyphenolic compounds (Kumar et al., 2021).

While the linear influence of temperature and ethanol content revealed a positive impact, their interaction (X_1X_3) showed a negative influence on the polyphenols recovery in the β-CD-aided extraction model, which is presented in Figure 2C. These results implied that the extraction temperature exerted a positive effect more evident at lower concentrations of ethanol, and vice versa – the ethanol content presented a significantly greater impact at low extraction temperatures. Other studies confirmed the negative influence of this particular interaction on the extraction of polyphenols from different plant materials as well (Ilaiyaraja et al., 2015; Mašković et al., 2024; Radan et al., 2024; Živković et al., 2019).

Lastly, as CDs are described as powerful agents for the improvement of poorly soluble bioactive compounds, the positive linear influence of CD concentration (X_4) was observed in the HP-β-CD model, which is presented in Figure 2D. Therefore, this model predicted that recovery of polyphenols may be enhanced by the increasing HP-β-CD content. This can be explained by the fact that with the increase of CD molecules' amount, the probability of successful interactions between poorly-soluble bioactive compounds and the cavity of CDs is improved as well (Radan et al., 2023).

3.3. Model validation

As the RSM approach was used for the optimization of the UAE extraction of polyphenolic compounds from *F. esculentum* herb, the obtained analysis pointed out that the TPC can be maximized under the following conditions: extraction temperature (X_1) of 80 °C, extraction time (X_2) of 30 min, ethanol concentration (X_3) of 50%, and β-CD concentration (X_4) of 0.80% (Table 4). Slightly different optimal conditions were provided for the HP-β-CD-aided extraction, emphasizing the influence of CD used in this model: extraction temperature (X_1) of 80 °C, extraction time (X_2) of 40 min, ethanol concentration (X_3) of 50%, and HP-β-CD concentration (X_4) of 1.50%. The validation step is crucial for strategic decision-making and possible industrial application, as it confirms the process's reliability (Mayer and Butler, 1993a). Thus, to validate the accuracy of both models, a new set of experiments was conducted under the predicted optimal conditions, in three repetitions.

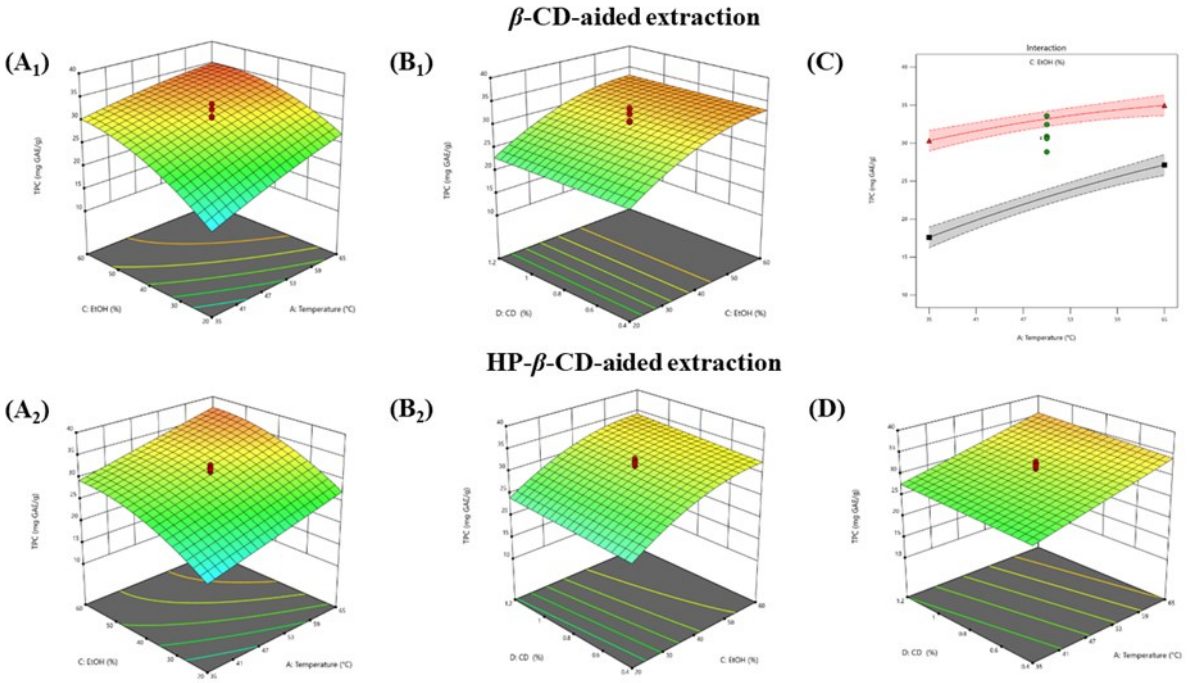

Fig. 2. Response surface plots of β-CD- (the upper half) and HP-β-CD-assisted extractions (the lower half) showing the impact of independent variables on the extraction efficiency of total polyphenols. Independent variables with statistically significant influence: temperature (A$_1$ and A$_2$), ethanol concentration (B$_1$ and B$_2$), interaction between temperature and ethanol concentration (C), and HP-β-CD concentration (D).

Table 4. The optimized conditions for β-CD- and HP-β-CD-aided extractions.

***B*-CD-aided extraction**		**HP-*β*-CD-aided extraction**	
Optimal conditions		**Optimal conditions**	
X_1	80 °C	X_1	80 °C
X_2	30 min	X_2	40 min
X_3	50%	X_3	50%
X_4	0.80%	X_4	1.50%
Predicted TPC = 36.20 mg GAE/g DW		**Predicted TPC** = 42.26 mg GAE/g DW	
Validated TPC = 36.19 ± 0.99 mg GAE/g DW		**Validated TPC** = 42.16 ± 1.01 mg GAE/g DW	

β-CD – β-cyclodextrin; HP-β-CD – hydroxypropyl-β-cyclodextrin; X_1 – Extraction temperature; X_2 – Extraction time; X_3 – Ethanol concentration; X_4 – CD concentration; TPC – total phenolic content.

As the validated responses (36.19 ± 0.99 and 42.16 ± 1.01 mg GAE/g DW for β-CD- and HP-β-CD-aided extractions, respectively) highly corresponded to the predicted values, it could be concluded that the created models were reliable and accurate.

3.4. HPLC analysis of the optimal *F. esculentum* extract with maximal TPC

Numerous health benefits have been reported for the use of common buckwheat and its products, such as hypoglycemic, hypocholesterolemic, anti-inflammatory, and anticancer effects (Giménez-Bastida and Zieliński, 2015), which prompted the interest for the formulation of novel dietary products and functional food. Regarding this medicinal plant's pharmaceutical and nutraceutical potential which is derives from polyphenolic compounds, this study aimed to optimize the TPC using CD-aided extraction. As the optimal extract obtained by the HP-β-CD-aided extraction model resulted in a higher content of polyphenolic compounds, compared to β-CD, it was selected for the final chemical characterization.

The HPLC method revealed that the most abundant compound in the obtained extract was rutin with 29.56 mg/g DW, followed by quercetin and quercitrin, which were detected in smaller quantities (Table 5). Other studies also reported rutin as a main component in the *F. esculentum* extracts (Hinneburg and Neubert, 2005; Radan et al., 2023; Tian et al., 2002; Vollmannová et al., 2021). In fact, obtained rutin yield was similar to the study of (Vollmannová et al., 2021), where *F. esculentum* leaves were treated with 80% methanol for 8 h, resulting in the rutin yield range of 17.742–31.069 mg/g DM. Given the high concentration of methanol, a toxic organic solvent, and the prolonged extraction time used in the study by (Vollmannová et al., 2021), the HP-β-CD-aided extraction protocol featuring a significantly shorter extraction time of 40 min and a 50% ethanolic solution opens up new avenue for the application of sustainable practices in the recovery of potent phenolic compounds.

The significance of the evaluated chemical profile lies in the abundance of rutin, a bioactive compound with valuable bioactive potential lying in its' anticancer, anti-inflammation, hepatoprotective, and antioxidant activity (Sofi et al., 2023). Moreover, given the rising rate of non-communicable diseases worldwide, especially hypertension and diabetes, its' biological potential could be the key to the prevention and/or treatment. The mass expansion of these diseases threatens to affect a significant share of the population. Namely, by the year 2025, even 1.56 billion people will be affected only by hypertension, while approximately 642 million people could get diabetes type 2 (Oguntibeju, 2019; Sofi et al., 2023). Hence, the inhibitory potential of rutin towards the diabetes-related enzymes - glucosidase and amylase, as well as the antihypertension-associated effects, such as the improvement of blood circulation, prevention of blood vessels hardening, elimination of toxins, and microcirculation improvement, could be beneficial in the prevention on these threatening diseases (Hou et al., 2017; Jadhav and Puchchakayala, 2012; Sofi et al., 2023). Moreover, the second-abundant polyphenol in the optimal extract – quercetin, has been reported to have positive effects on the regulation of vasoconstriction and heart diastole, resulting in blood pressure lowering (Giménez-Bastida and Zieliński, 2015).

Additionally, the cosmetic, food, and chemical industry potential of rutin should not be unplaced as well, considering the reported benefits (Hinneburg and Neubert, 2005; Kim et al., 2005).

Table 5. The content of individual polyphenolics of *F. esculentum* herb optimal extract.

Rutin (mg/g DW)	Quercitrin (mg/g DW)	Quercetin (mg/g DW)
29.56	1.42	0.35

4. CONCLUSION

Currently, with the growing demand for medicinal plants and their products in the numerous fields of industry, the development of green and sustainable extraction methods occupies the focus of innovative research. Thus, aiming to contribute to that common goal, this study investigated the optimal extraction conditions for the maximized recovery of *F. esculentum* herb's polyphenols, given their significant bioactive potential. In that manner, the green, feasible, and effective UAE, aided with cutting-edge complexation agents – cyclodextrins, were used for the recovery of bioactive compounds.

Accountable method for the extraction optimization – RSM, which is often used to reduce the time and solvent over excessive consumption, was successfully applied, revealing statistically highly significant models for each used CD ($p < 0.0001$), describing the relationship between the varying factors and the TPC as the dependent variable. Obtained lack-of-fit and coefficients of determination confirmed the models' adequacy, robustness, and strong predictive capability as well. Based on the obtained results of the TPC in *F. esculentum* extracts, and the statistical analysis by the RSM approach, it was concluded that extraction temperature and the content of ethanol had a highly significant impact. Their interaction resulted in a moderately significant negative influence when β-CD was used, while the CD content significantly impacted the TPC only when HP-β-CD was used, pointing out the differences between used CDs on their potential to preserve the stability of the formed inclusion complexes. Optimal conditions for the total polyphenols maximal recovery in the extracts with β-CD

were as follows: 80 °C temperature of the extraction performed for 30 min, using 50% ethanol and 0.80% β-CD, while the HP-β-CD-aided extraction suggested the slightly different optimal conditions: extraction temperature of 80 °C, extraction time of 40 min, ethanol concentration of 50%, and β-CD concentration of 1.50%. Under optimized conditions, the HP-β-CD-aided extract had a superior TPC of 42.16 ± 1.01 mg GAE/g DW, composed of a significant content of rutin, quercitrin, and quercetin.

Overall, the CD-aided extraction revealed a promising approach for the recovery of *F. esculentum* polyphenolic compounds, following the principles of green chemistry and circular economy. In light of the rising industrial demand, this polyphenolic-rich extract, with significant rutin content, could be valuable for the formulation of dietary supplements, functional food, or cosmetic products that could be used in prevention or treatment of various diseases, as well as for the upkeep of the general wellbeing of the modern society.

ACKNOWLEDGMENTS

This research was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, grant number 451-03-66/2024-03/200003.

CONFLICT OF INTEREST

The authors declare that they have no financial and commercial conflicts of interest.

REFERENCES

Ahmed, A., Khalid, N., Ahmad, A., Abbasi, N. A., Latif, M. S. Z., and Randhawa, M. A. (2014): Phytochemicals and biofunctional properties of buckwheat: a review, *The Journal of Agricultural Science*, **152**(3), 349–369. https://doi.org/10.1017/S0021859613000166

Alibante, A., Lakka, A., Bozinou, E., Chatzilazarou, A., Lalas, S., and Makris, D. P. (2021): Integrated green process for the extraction of red grape pomace antioxidant polyphenols using ultrasound-assisted pretreatment and β-cyclodextrin, *Beverages*, **7**(3). https://doi.org/10.3390/beverages7030059

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N., and Omar, A. K. M. (2013): Techniques for extraction of bioactive compounds from plant materials: A review, *Journal of Food Engineering*, **117**(4), 426–436. https://doi.org/10.1016/j.jfoodeng.2013.01.014

Azwanida, N. (2015): A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation, *Medicinal & Aromatic Plants*, **04**(03), 3–8. https://doi.org/10.4172/2167-0412.1000196

Bozinou, E., Lakka, A., Poulianiti, K., Lalas, S., and Makris, D. P. (2021): Cyclodextrins as high-performance green co-solvents in the aqueous extraction of polyphenols and anthocyanin pigments from solid onion waste, *European Food Research and Technology*, **247**(11), 2831–2845. https://doi.org/10.1007/s00217-021-03839-2

Cai, R., Yuan, Y., Cui, L., Wang, Z., and Yue, T. (2018): Cyclodextrin-assisted extraction of phenolic compounds: Current research and future prospects, *Trends in Food Science and Technology*, **79**, 19–27. https://doi.org/10.1016/j.tifs.2018.06.015

Carlotti, M. E., Sapino, S., Ugazio, E., and Caron, G. (2011): On the complexation of quercetin with methyl-β-cyclodextrin: Photostability and antioxidant studies, *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, **70**(1–2), 81–90. https://doi.org/10.1007/s10847-010-9864-7

Çelik, S. E., Özyürek, M., Güçlü, K., and Apak, R. (2015): Antioxidant capacity of quercetin and its glycosides in the presence of β-cyclodextrins: Influence of glycosylation on inclusion complexation, *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, **83**(3–4), 309–319. https://doi.org/10.1007/s10847-015-0566-z

Chemat, Abert Vian, Ravi, Khadhraoui, Hilali, Perino, and Tixier (2019): Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects, *Molecules*, **24**(16), 3007. https://doi.org/10.3390/molecules24163007

Chemat, F., Rombaut, N., Sicaire, A. G., Meullemiestre, A., Fabiano-Tixier, A. S., and Abert-Vian, M. (2017): Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review, *Ultrasonics Sonochemistry*, **34**, 540–560. https://doi.org/10.1016/j.ultsonch.2016.06.035

Del Valle, E. M. M. (2004): Cyclodextrins and their uses: A review, *Process Biochemistry*, **39**(9), 1033–1046. https://doi.org/10.1016/S0032-9592(03)00258-9

Diamanti, A. C., Igoumenidis, P. E., Mourtzinos, I., Yannakopoulou, K., and Karathanos, V. T. (2017): Green extraction of polyphenols from whole pomegranate fruit using cyclodextrins, *Food Chemistry*, **214**, 61–66. https://doi.org/10.1016/j.foodchem.2016.07.072

Durling, N., Catchpole, O., Grey, J., Webby, R., Mitchell, K., Foo, L., and Perry, N. (2007): Extraction of phenolics and essential oil from dried sage (*Salvia officinalis*) using ethanol–water mixtures, *Food Chemistry*, **101**(4), 1417–1424. https://doi.org/10.1016/j.foodchem.2006.03.050

Giménez-Bastida, J. A., and Zieliński, H. (2015): Buckwheat as a Functional Food and Its Effects on Health, *Journal of Agricultural and Food Chemistry*, **63**(36), 7896–7913. https://doi.org/10.1021/acs.jafc.5b02498

Hinneburg, I., and Neubert, R. H. H. (2005): Influence of Extraction Parameters on the Phytochemical Characteristics of Extracts from Buckwheat (*Fagopyrum esculentum*) Herb, *Journal of Agricultural and Food Chemistry*, **53**(1), 3–7. https://doi.org/10.1021/jf049118f

Hou, Z., Hu, Y., Yang, X., and Chen, W. (2017): Antihypertensive effects of Tartary buckwheat flavonoids by improvement of vascular insulin sensitivity in spontaneously hypertensive rats, *Food & Function*, **8**(11), 4217–4228. https://doi.org/10.1039/C7FO00975E

Ihme, N., Kiesewetter, H., Jung, F., Hoffmann, K. H., Birk, A., Müller, A., and Grützner, K. I. (1996): Leg oedema protection from a buckwheat herb tea in patients with chronic venous insufficiency: a single-centre, randomised, double-blind, placebo-controlled clinical trial, *European Journal of Clinical Pharmacology*, **50**(6), 443–447. https://doi.org/10.1007/s002280050138

Ilaiyaraja, N., Likhith, K. R., Sharath Babu, G. R., and Khanum, F. (2015): Optimisation of extraction of bioactive compounds from *Feronia limonia* (wood apple) fruit using response surface methodology (RSM), *Food Chemistry*, **173**, 348–354. https://doi.org/10.1016/j.foodchem.2014.10.035

Inglett, G. E., Rose, D. J., Chen, D., Stevenson, D. G., and Biswas, A. (2010): Phenolic content and antioxidant activity of extracts from whole buckwheat (*Fagopyrum esculentum* Möench) with or without microwave irradiation, *Food Chemistry*, **119**(3), 1216–1219. https://doi.org/10.1016/j.foodchem.2009.07.041

Jadhav, R., and Puchchakayala, G. (2012): Hypoglycemic and antidiabetic activity of flavonoids: Boswellic acid, Ellagic acid, Quercetin, Rutin on streptozotocin-nicotinamide induced type 2 diabetic rats, *International Journal of Pharmacy and Pharmaceutical Sciences*, **4**(2), 251–256.

Kim, K. H., Lee, K. W., Kim, D. Y., Park, H. H., Kwon, I. B., and Lee, H. J. (2005): Optimal recovery of high-purity rutin crystals from the whole plant of *Fagopyrum esculentum* Moench (buckwheat) by extraction, fractionation, and recrystallization, *Bioresource Technology*, **96**(15), 1709–1712. https://doi.org/10.1016/j.biortech.2004.12.025

Kumar, K., Srivastav, S., and Sharanagat, V. S. (2021): Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review, *Ultrasonics Sonochemistry*, **70**, 105325. https://doi.org/10.1016/j.ultsonch.2020.105325

Li, H. (2019): Buckwheat, 137–149 *in Bioactive Factors and Processing Technology for Cereal Foods*, Springer Singapore, Singapore. https://doi.org/10.1007/978-981-13-6167-8_8

Mašković, J. M., Jakovljević, V., Živković, V., Mitić, M., Kurćubić, L. V., Mitić, J., and Mašković, P. Z. (2024): Optimization of Ultrasound-Assisted Extraction of Phenolics from *Satureja hortensis* L. and Antioxidant Activity: Response Surface Methodology Approach, *Processes*, **12**(9), 2042. https://doi.org/10.3390/pr12092042

Mayer, D. G., and Butler, D. G. (1993): Statistical validation, *Ecological Modelling*, **68**(1–2), 21–32. https://doi.org/10.1016/0304-3800(93)90105-2

Mudrić, J., Janković, T., Šavikin, K., Bigović, D., Đukić-Ćosić, D., Ibrić, S., and Đuriš, J. (2020): Optimization and modelling of gentiopicro-

side, isogentisin and total phenolics extraction from *Gentiana lutea* L. roots, *Industrial Crops and Products*, **155**, 112767. https://doi.org/10.1016/j.indcrop.2020.112767

Nguyen, T. A., Liu, B., Zhao, J., Thomas, D. S., and Hook, J. M. (2013): An investigation into the supramolecular structure, solubility, stability and antioxidant activity of rutin/cyclodextrin inclusion complex, *Food Chemistry*, **136**(1), 186–192. https://doi.org/10.1016/j.foodchem.2012.07.104

Noreen, S., Rizwan, B., Khan, M., and Farooq, S. (2021): Health Benefits of Buckwheat (Fagopyrum Esculentum), Potential Remedy for Diseases, Rare to Cancer: A Mini Review, *Infectious Disorders - Drug Targets*, **21**(6). https://doi.org/10.2174/1871526520999201224122605

Oguntibeju, O. O. (2019): Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links., *International Journal of Physiology, Pathophysiology and Pharmacology*, **11**(3), 45–63.

Paczkowska, M., Mizera, M., Piotrowska, H., Szymanowska-Powałowska, D., Lewandowska, K., Goscianska, J., Pietrzak, R., Bednarski, W., Majka, Z., and Cielecka-Piontek, J. (2015): Complex of rutin with β-cyclodextrin as potential delivery system, *PLoS ONE*, **10**(3), 1–16. https://doi.org/10.1371/journal.pone.0120858

Pandey, A., Belwal, T., Sekar, K. C., Bhatt, I. D., and Rawal, R. S. (2018): Optimization of ultrasonic-assisted extraction (UAE) of phenolics and antioxidant compounds from rhizomes of *Rheum moorcroftianum* using response surface methodology (RSM), *Industrial Crops and Products*, **119**(April), 218–225. https://doi.org/10.1016/j.indcrop.2018.04.019

Pilkington, J. L., Preston, C., and Gomes, R. L. (2014): Comparison of response surface methodology (RSM) and artificial neural networks (ANN) towards efficient extraction of artemisinin from *Artemisia annua*, *Industrial Crops and Products*, **58**, 15–24. https://doi.org/10.1016/j.indcrop.2014.03.016

Pinho, E., Grootveld, M., Soares, G., and Henriques, M. (2014): Cyclodextrins as encapsulation agents for plant bioactive compounds, *Carbohydrate Polymers*, **101**(1), 121–135. https://doi.org/10.1016/j.carbpol.2013.08.078

Pompeu, D. R., Silva, E. M., and Rogez, H. (2009): Optimisation of the solvent extraction of phenolic antioxidants from fruits of *Euterpe oleracea* using Response Surface Methodology., *Bioresource Technology*, **100**(23), 6076–82. https://doi.org/10.1016/j.biortech.2009.03.083

Prasad, K. N., Hassan, F. A., Yang, B., Kong, K. W., Ramanan, R. N., Azlan, A., and Ismail, A. (2011): Response surface optimisation for the extraction of phenolic compounds and antioxidant capacities of underutilised *Mangifera pajang* Kosterm. peels, *Food Chemistry*, **128**(4), 1121–1127. https://doi.org/10.1016/j.foodchem.2011.03.105

Purkait, M. K., Haldar, D., and Duarah, P. (2023): Pharmacologic and therapeutic aspects of various medicinal plants, 197–217 *in Advances in Extraction and Applications of Bioactive Phytochemicals*, Elsevier. https://doi.org/10.1016/B978-0-443-18535-9.00002-8

Radan, M., Jovanović, M., Ćujić Nikolić, N., Mudrić, J., Janković, T., Bigović, D., and Šavikin, K. (2024): Cyclodextrin-assisted extraction as a green alternative for the recovery of phenolic compounds from *Helichrysum plicatum* DC. flowers, *Sustainable Chemistry and Pharmacy*, **39**, 101547. https://doi.org/10.1016/j.scp.2024.101547

Radan, M., Živković, J., Nedeljković, S. K., Janković, T., Lazarević, Z., Bigović, D., and Šavikin, K. (2023): Influence of hydroxypropyl-β-cyclodextrin complexation on the extraction efficiency of rutin, quercetin and total polyphenols from *Fagopyrum esculentum* Moench, *Sustainable Chemistry and Pharmacy*, **35**, 101220. https://doi.org/10.1016/j.scp.2023.101220

Rakha, A., Umar, N., Rabail, R., Butt, M. S., Kieliszek, M., Hassoun, A., and Aadil, R. M. (2022): Anti-inflammatory and anti-allergic potential of dietary flavonoids: A review, *Biomedicine & Pharmacotherapy*, **156**, 113945. https://doi.org/10.1016/j.biopha.2022.113945

Salvamani, S., Gunasekaran, B., Shaharuddin, N. A., Ahmad, S. A., and Shukor, M. Y. (2014): Antiartherosclerotic Effects of Plant Flavonoids, *BioMed Research International*, **2014**, 1–11. https://doi.org/10.1155/2014/480258

Savic, I. M., Savic-Gajic, I. M., Nikolic, V. D., Nikolic, L. B., Radovanovic, B. C., and Milenkovic-Andjelkovic, A. (2016): Enhencemnet of solubility and photostability of rutin by complexation with β-cyclodextrin and (2-hydroxypropyl)-β-cyclodextrin, *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, **86**(1–2), 33–43. https://doi.org/10.1007/s10847-016-0638-8

Setyani, W., Murwanti, R., Sulaiman, T. N. S., and Hertiani, T. (2023): Application of Response Surface Methodology (RSM) for the Optimization of Ultrasound-Assisted Extraction (UAE) of *Moringa oleifera*: Extraction Yield, Content of Bioactive Compounds, and Biological Effects In Vitro, *Plants*, **12**(13), 2455. https://doi.org/10.3390/plants12132455

Sofi, S. A., Ahmed, N., Farooq, A., Rafiq, S., Zargar, S. M., Kamran, F., Dar, T. A., Mir, S. A., Dar, B. N., and Mousavi Khaneghah, A. (2023): Nutritional and bioactive characteristics of buckwheat, and its potential for developing gluten-free products: An updated overview, *Food Science & Nutrition*, **11**(5), 2256–2276. https://doi.org/10.1002/fsn3.3166

Tian, Q., Li, D., and Patil, B. S. (2002): Identification and determination of flavonoids in buckwheat (*Fagopyrum esculentum* Moench, Polygonaceae) by high-performance liquid chromatography with electrospray ionisation mass spectrometry and photodiode array ultraviolet detection, *Phytochemical Analysis*, **13**(5), 251–256. https://doi.org/10.1002/pca.649

V. Yugoslavian Pharmacopoeia (2000): , National Institute for Health Protection, Belgrade, Serbia.

Vollmannová, A., Musilová, J., Lidiková, J., Árvay, J., Šnirc, M., Tóth, T., Bojňanská, T., Čičová, I., Kreft, I., and Germ, M. (2021): Concentrations of phenolic acids are differently genetically determined in leaves, flowers, and grain of common buckwheat (*Fagopyrum esculentum* Moench), *Plants*, **10**(6). https://doi.org/10.3390/plants10061142

Waterman, P. G., and Mole, S. (1994): *Analysis of phenolic plant metabolites* (viii ed.), Blackwell Scientific, Oxford; Boston, 238.

Weremfo, A., Abassah-Oppong, S., Adulley, F., Dabie, K., and Seidu-Larry, S. (2023): Response surface methodology as a tool to optimize the extraction of bioactive compounds from plant sources, *Journal of the Science of Food and Agriculture*, **103**(1), 26–36. https://doi.org/10.1002/jsfa.12121

Yu, H. C., Huang, S. M., Lin, W. M., Kuo, C. H., and Shieh, C. J. (2019): Comparison of artificial neural networks and response surface methodology towards an efficient ultrasound-assisted extraction of chlorogenic acid from *Lonicera japonica*, *Molecules*, **24**(12), 1–15. https://doi.org/10.3390/molecules24122304

Zalpoor, H., Nabi-Afjadi, M., Forghaniesfidvajani, R., Tavakol, C., Farahighasreaboonasr, F., Pakizeh, F., Dana, V. G., and Seif, F. (2022): Quercetin as a JAK–STAT inhibitor: a potential role in solid tumors and neurodegenerative diseases, *Cellular & Molecular Biology Letters*, **27**(1), 60. https://doi.org/10.1186/s11658-022-00355-3

Zieliński, H., and Kozłowska, H. (2000): Antioxidant Activity and Total Phenolics in Selected Cereal Grains and Their Different Morphological Fractions, *Journal of Agricultural and Food Chemistry*, **48**(6), 2008–2016. https://doi.org/10.1021/jf990619o

Živković, J., Janković, T., Menković, N., and Šavikin, K. (2019): Optimization of ultrasound-assisted extraction of isogentisin, gentiopicroside and total polyphenols from gentian root using response-surface methodology, *Industrial Crops and Products*, **139**, 111567. https://doi.org/10.1016/j.indcrop.2019.111567

Vitamin C content and antioxidant activity of red currant (*Ribes rubrum* L.) juices

BOJANA MILADINOVIĆ[1*], MILICA RANDJELOVIĆ[1], SLAVOLJUB ŽIVANOVIĆ[2], MILICA MILUTINOVIĆ[1], MILOŠ JOVANOVIĆ[1], NEMANJA KITIĆ[3], SUZANA BRANKOVIĆ[3] AND DUŠANKA KITIĆ[1]

[1]*Department of Pharmacy, Faculty of Medicine, University of Niš, Dr. Zoran Djindjić Ave 81, 18000 Niš, Serbia*
[2]*Biomedical Research Centre, Faculty of Medicine, University of Niš, Dr. Zoran Djindjić Ave 81, 18000 Niš, Serbia*
[3]*Department of Physiology, Faculty of Medicine, University of Niš, Dr. Zoran Djindjić Ave 81, 18000 Niš, Serbia*
**Corresponding author: ajovanovic@inep.co.rs*

Received: November 19, 2024
Accepted: December 12, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

Red currants (*Ribes rubrum* L.) belong to Grossulariaceae family and the *Ribes* genus. They are sweet and sour, nutrient rich berries that have shown antioxidant, antibacterial, antiseptic, cardioprotective, and anti-inflammatory effects. The aim of this study was to determine the vitamin C content and antioxidant potential of fruit juices from six varieties of red currants - Redpoll, Makosta, Stanza, Jonkheer van Tets, Rolan, and Rondom. Fresh, undamaged fruits were pressed into juice and stored at -18 °C. Ascorbic and dehydroascorbic acid were determined by high-pressure liquid chromatography. Antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) system as well as β-carotene bleaching assay. The results showed that the juice of the Redpoll variety had the highest vitamin C content (66.52±2.9 mg/100 g juice), while the juice of the Stanza variety had the lowest content (6.23±0.28 mg/100 g). The Redpoll variety juice also showed the strongest antioxidant activity (IC_{50} = 1.76±0.25 mg/mL), while the juice of the Rolan variety showed the weakest antioxidant activity (IC_{50} = 6.65±0.84 mg/mL). Thanks to its favorable cultivation properties and numerous potential and proven positive health effects, red currant is becoming the subject of increasing research. The results of this study can serve as an incentive for the use of red currants and their products in nutrition, as well as for the selection of nutritionally high-quality varieties.

Keywords: red currants; vitamin C; antioxidative activity; berries; juice

https://doi.org/10.61652/leksir2444013M

1. INTRODUCTION

Currants (*Ribes* sp.) are berries popular for their sour-sweet taste and high nutrient content. Currants belong to the family Grossulariaceae, genus *Ribes*, which counts around 150 species. They are originally from the northern hemisphere and are grown worldwide as fruit and for their decorative features. The most famous types of currants are white (*Ribes album* L.), red (*Ribes rubrum* L.) and black (*Ribes nigrum* L.).
Red currants are a rich source of vitamins, minerals, sugar, as well as flavonoids and other polyphenolic compounds (USDA, 2024; Zdunić et al., 2016). Thanks to the presence of polyphenolic compounds and partly vitamin C, red currants show antioxidant activity (Benvenuti et al., 2004). In addition, antibacterial, antiseptic, cardioprotective and anti-inflammatory activity of red currant fruit has been established (Berk and Tuna, 2017; Cvetković et al., 2023). One study proved the antioxidant, anti-inflammatory and antiplatelet activity of *R. rubrum* fruit extract in mice with diabetes (Gülmez et al., 2022). The results of this study indicate that red currant extract may be useful in preventing diabetes complications.
Red currant is widely used in the food industry to obtain various products such as juices, jams, dessert toppings, ice cream, and liqueurs. Also, it is increasingly used in the cosmetic industry to obtain care products and perfumes (Milivojevic et al., 2012). Due to the high content of bioactive compounds, there is a possibility of its use in medicine and pharmacy.
In addition to fresh fruits, red currant fruit juices are a significant source of bioactive compounds. Consequently, there is an increasing need to examine the chemical composition and biological effects of these products. Red currants represent a valuable source of primary metabolites, sugars and organic acids, which play a major role in defining the distinctive flavor of the fruit. Additionally, they are rich in secondary metabo-

lites, particularly polyphenols, where three primary classes can be identified: flavonoids, tannins, and phenolic acids. Among the flavonoids, anthocyanins—specifically cyanidin- and delphinidin-3-O-glucoside—stand out as dominant compounds, along with key flavonols like quercetin and kaempferol, and notable flavanols such as catechin and epicatechin (Benvenuti et al., 2004; Djordjević et al., 2010; Milivojevic et al., 2012; Podskalská et al., 2024). The most abundant vitamin in red currant is vitamin C, the amount of which in fresh fruits ranges from 45.80 mg/100 g to 67.50 mg/100 g (Djordjević et al., 2010). Environmental conditions, cultivation systems, and genotypes have the greatest influence on the content of vitamin C in red currant fruits. The amount of vitamin C in fruits can also change depending on the ripening stage. The intensity and quantity of sunlight during the vegetative period can also affect the amount of vitamin C in the fruits because vitamin C is synthesized from sugars that are produced in the process of photosynthesis. Fruits exposed to sunlight contain higher amounts of vitamin C than those in the shade. Vitamin C primarily exists in its reduced form, known as ascorbic acid (ASC), with a smaller amount (about 5-10%) present in its oxidized form - dehydroascorbic acid (DASC). The oxidation of vitamin C occurs readily in aqueous solutions, leading to an increase in the proportion of dehydroascorbic acid. Factors such as the presence of oxygen, enzymes, heavy metal ions (particularly Cu^{2+}, Ag^{+}, and Fe^{3+}), along with high temperatures and alkaline pH, can promote this oxidation. The oxidized form of vitamin C can be easily converted back to ASC both in the human body and in foods through the action of various reducing agents such as 1,4-dithiothreitol (DTT). However, DASC is utterly unstable and it can also undergo irreversible degradation, resulting in 2,3-diketogulonic acid, which does not possess vitamin activity (Russell, 2004).

This work aims to determine the content of vitamin C, before and after reduction, as well as the antioxidant potential of the juices of six varieties of red currant - Redpoll, Makosta, Stanza, Jonkheer van Tets, Rolan and Rondom. The results will offer valuable insight into which variety(s) has the highest vitamin C content and antioxidant activity providing possible guidance for future selection practice.

2. MATERIALS AND METHODS

2.1. Preparation of materials

Six red currant varieties juices were analyzed in this work. Currants were grown with minimal use of chemicals (integrative protection system) in Radmilovac, Serbia and harvested in the period of full maturity. Juices were made from fresh, undamaged fruits by manual squeezing, and in this way, the membranes and seeds were separated from the pulp. Juices were stored in sealed vials at -18 °C for further testing.

2.2. Reagents and standards

The ascorbic acid standard and butylated hydroxytoluene (BHT) was purchased from Supelco, Buchs, Switzerland. α-linoleic acid, 1,1 diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), and 1,4-dithiothreitol (DTT) were obtained from Sigma Chemicals Co (St Louis, Mo., U.S.A.); (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2 carboxylic acid (Trolox) and β-carotene from Fluka, Buchs, Switzerland; chloroform from Carlo Erba Reagents; meta-phosphoric acid p.a from Honeywell Reidel-de Haën. Acetonitrile (HPLC grade) used for analysis was J.T.Baker. The water used was purified with TKA Smart 2 pure deionization system (Thermoscience, Niederelbert, Germany).

2.3. Determination of vitamin C content in red currant juices

Ascorbic acid was quantified by a high-pressure liquid chromatography (HPLC) (Brubacher et al., 1985; Ohta and Harada, 1996). The amount of 2.5 g of juice was dissolved in 25 mL of 4.5% metaphosphoric acid. The sample was additionally dissolved in an ultrasonic bath for 10 minutes and centrifuged for 15 minutes at 10,000 rpm. The supernatant obtained in this way was used to determine ASC. The amount of DASC was determined when 1 mL of a reducing agent (50 μM 1,4 dithiothreitol - DTT) was added to 1 mL of the previously prepared sample. After a 10-minute reaction at room temperature, this solution was filtered through a membrane filter (0.45 μm) and 20 μL was injected into the HPLC. The amount of ascorbic acid quantified after reduction represents the total amount of ascorbic acid (TASC) in the sample. Dehydroascorbic acid was calculated as the difference between content of total ascorbic acid and ascorbic acid (DASC=TASC-ASC). All the results are expressed in mg of ascorbic acid per 100 g of juice.

Analysis of vitamin C content in juice was performed on an Agilent 1100 (Agilent Technologies, Palo Alto, Calif, U.S.A.) equipped with a diode array (DAD) detector, automatic sampler and control system. The separation of the components was performed on a Merck Purospher STAR RP-18e analytical column (150×4.6 mm i.d., 5 μm particle size). Phosphate buffer (40 mM) and methanol in a ratio of 92:8 under isocratic conditions were used as the mobile phase. The flow rate was 0.8 mL/min at room temperature and values were detected at an absorbance of 244 nm. Standard solutions of ascorbic acid (1, 10, 50, 100, 150 and 200 μg/mL) were injected in triplicate for each concentration to obtain a calibration curve.

2.4. Determination of antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl system

The method involves the reduction of DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals in the presence of the examined antioxidant, whereby a non-radical compound is formed and the absorbance decreases due to the discoloration of the radicals. Determining the antioxidant activity of red currant juice in the DPPH system involves testing the ability of the juice to "catch" free radicals, and is performed according to the method described by Cuendet et al (1997). One mL of the DPPH ethanolic solution (0.05 mM) was added to the juices of the tested varieties of red currant (4 mL) with vigorous shaking. The resulting solutions stand for 30 minutes in a dark place at room temperature. The absorbance of the samples was measured on a UV-VIS spectrophotometer (Evolution 60 Thermo Scientific, Madison, USA) at 550 nm, as well as the control consisting of a mixture of solvent and DPPH solution, using the solvent as a blank. Inhibition of free radicals was calculated according to the formula:

$$\% \text{ inhibition of DPPH} = (A_C - A_S/A_C) \times 100$$

In the equation, A_C represents the absorbance of the control reagent, and A_S represents the absorbance of the sample. Lines were constructed showing the dependence of increasing juice concentrations and their inhibition percentages. Using the resulting equations, the results are expressed as IC_{50} (sample concentration that neutralizes 50% of free radicals). The antioxidant activity was compared with the well-known antioxidant ascorbic acid.

2.5. β-carotene bleaching assay

This method determines the antioxidant capacity of the sample by measuring their ability to prevent oxidative loss of β-carotene in the β-carotene/linoleic acid emulsion. The method was developed based on the spectrophotometric measurements of Koleva et al (2002). The emulsion was prepared in

the following way: 2 mg of crystalline β-carotene was dissolved in 10 mL chloroform. Linoleic acid (25 μL) and 180 mg of Tween® 20 are added to one mL of this solution. After complete evaporation of chloroform with a vacuum evaporator at 40 °C, 50 mL of oxygenated water was added and the emulsion thus obtained is shaken until it becomes clear. Aliquots of the emulsion (0.16 mL) were pipetted into the wells of the microtiter plates in which previously added dilution series of juices (0.04 mL). The microtiter plates were then stirred on a mixer for microtiter plates. After mixing, the initial absorbance (A0) was read on an ELISA reader (Multiskan Ascent Thermo Labsystems Elisa No 354, Thermo Fischer scientific) at a wavelength of 450 nm, the plates were incubated for 2 hours at 55 °C, after which absorbance (A_2h) was read again. The antioxidative activity was calculated according to the following formula:

$$\% \text{ inhibition} = (A_2h/A0) \times 100$$

BHA, BHT, Trolox, and ascorbic acid were used as positive controls. The results were expressed as the concentration of the sample that inhibits the loss of 50% of β-carotene (IC_{50}) and it was calculated from the concentration/% inhibition curve.

2.6. Statistical analysis

All results of antioxidant activity and vitamin C content of red currant juices were presented as three individual measurements ± standard deviation. The data were analyzed with the ANOVA method and the Tukey post-hoc test ($p<0.05$) to show statistically significant difference between the samples. Statistical data processing was performed using the SPSS20.0 program.

3. RESULTS

3.1. Vitamin C content in red currant juices

The vitamin C content of the tested juices is shown in Table 1. Based on the obtained results, the juice of the Redpoll variety had the highest amount of vitamin C before and after reduction (33.14 ± 2.21 and 66.52 ± 2.90 mg/100 g of juice, respectively). The chromatograms of vitamin C content in the Redpoll juices were presented in Figure 1a and Figure 1b, respectively.

Table 1. Vitamin C content (mg/100 g) in red currant juices before and after reduction (TASC) and calculated quantity of dehydroascorbic acid (DASC)

Red currant juice samples	Before reduction	After reduction (TASC)	DASC (DASC=TASC-ASC)
Redpoll	33.14±2.20	66.52±2.90a	33.38
Rolan	5.41±0.13	10.96±0.92b	5.55
Jonkheer van Tets	11.62±0.66	23.52±1.45c	11.90
Stanza	3.21±0.10	6.23±0.28b	3.02
Rondom	15.68±1.09	31.68±1.56c	16.00
Makosta	17.46±0.86	35.46±1.44c	18.00

[a,b,c]Different letters in columns indicate a statistically significant difference between samples (Tukey test, $p<0.05$). Results are presented as the mean value of three individual measurements ± standard deviation (except DASC).

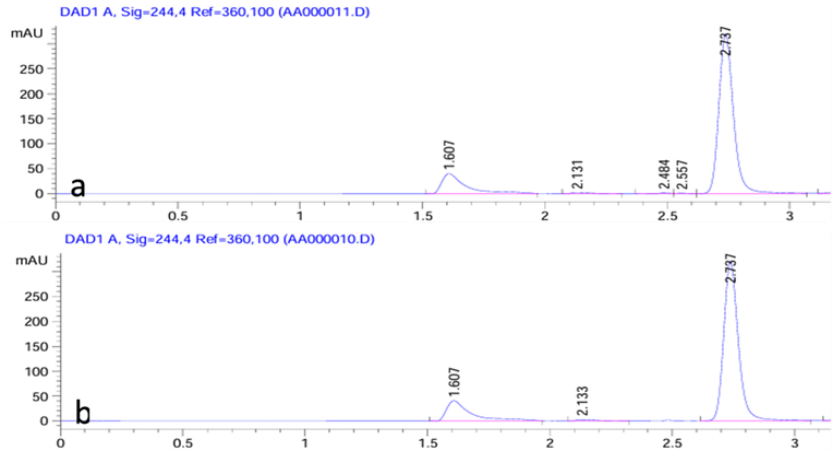

Fig. 1. HPLC chromatogram of vitamin C before (a) and after reduction (b) in Redpoll juice

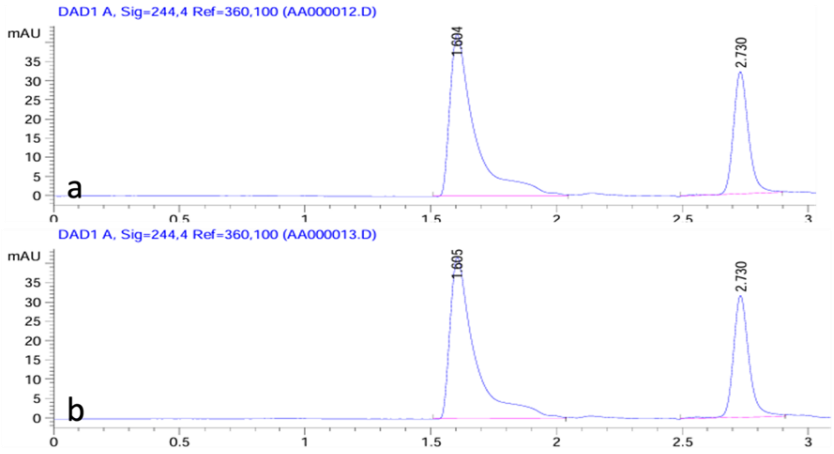

Fig 2. HPLC chromatogram of vitamin C before (a) and after reduction (b) in Stanza juice

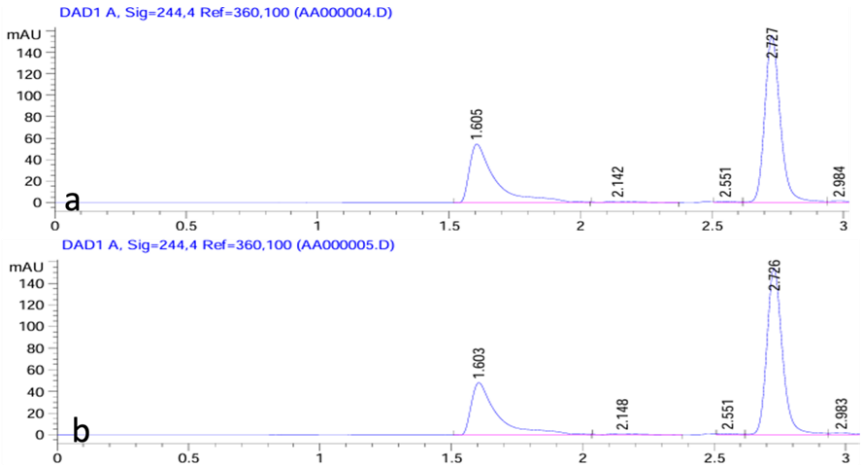

Fig3. HPLC chromatogram of vitamin C before (a) and after reduction (b) in Rondom juice

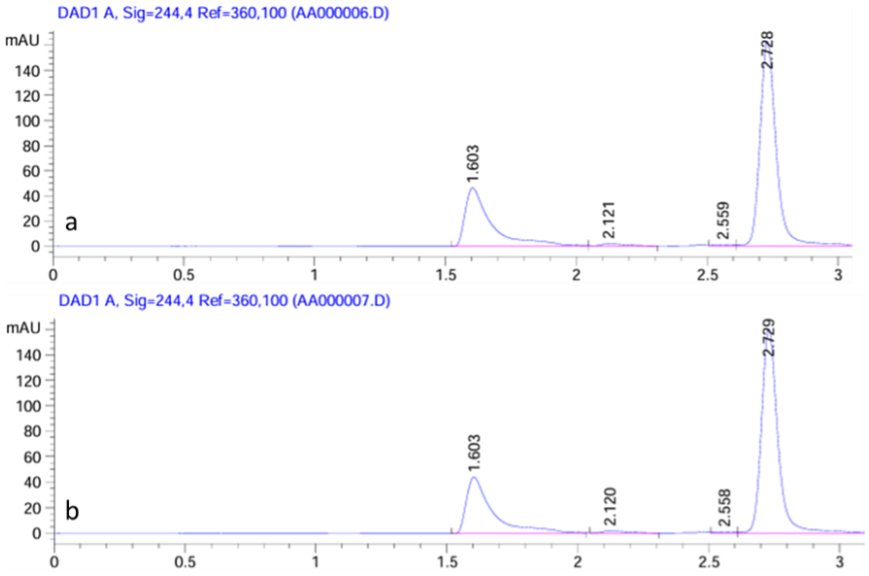

Fig 4. HPLC chromatogram of vitamin C before (a) and after reduction (b) in Makosta juice

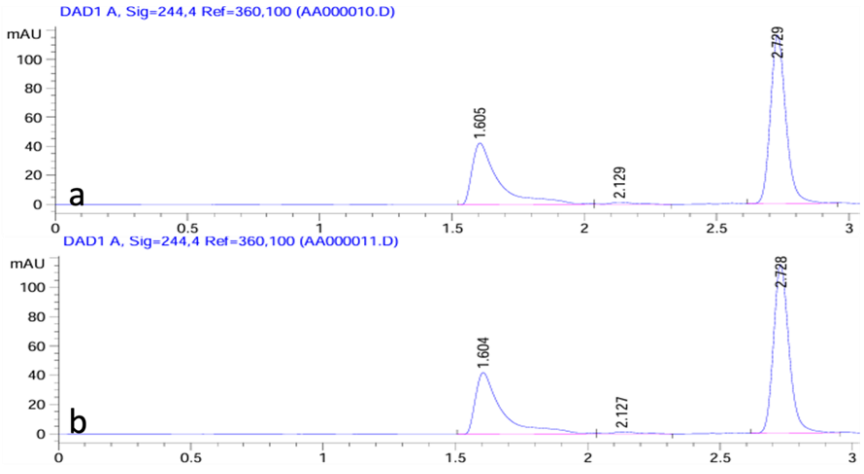

Fig 5. HPLC chromatogram of vitamin C before (a) and after reduction (b) in Jonkheer van Tets juice

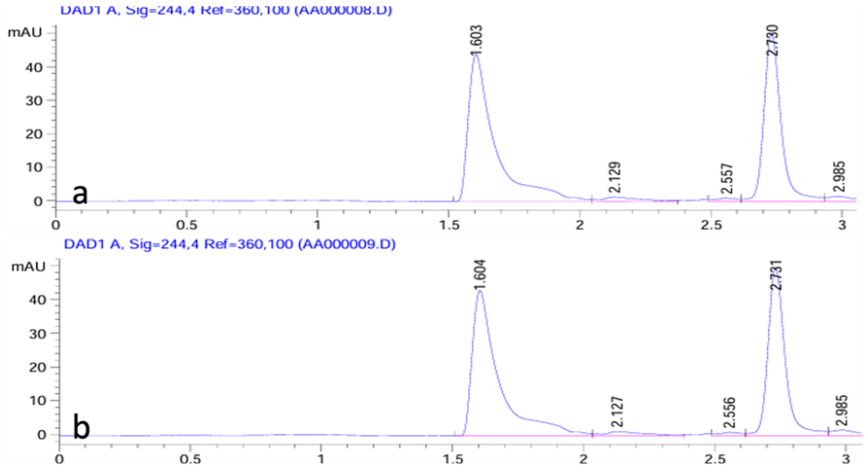

Fig 6. HPLC chromatogram of vitamin C before (a) and after reduction (b) in Rolan juice

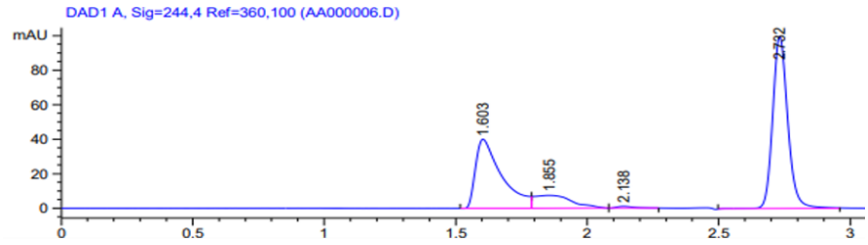

Fig 7. HPLC chromatogram of ascorbic acid standard (10 ppm)

The lowest vitamin C content had the juice of the Stanza variety. It was 3.21 ± 0.1 mg/100 g of juice before reduction (Figure 2a) and 6.23 ± 0.28 mg/100 g of juice after reduction (Figure 2b). HPLC chromatograms of all tested varieties before and after reduction are shown in Figures 1-6. Figure 7 shows the HPLC chromatogram of the ascorbic acid standard.

3.2. Antioxidant activity of red currant juices

The results of the antioxidant activity of the juices are shown in Table 2. The juice of the Redpoll variety showed the strongest free radical scavenging activity (IC_{50} = 1.76 ± 0.25 mg/mL), and the weakest expressed the juice of the Rolan variety (IC_{50} = 6.65 ± 0.84 mg/mL).

The best ability to inhibit lipid peroxidation also expressed Redpoll variety (IC_{50} = 2.52 ± 0.90 mg/mL), and the weakest expressed the juice of Stanza variety (IC_{50} = 6.23 ± 1.28 mg/mL).

Statistical analysis revealed that the antioxidant activity in the DPPH system differs between the juices of certain varieties of red currant and that the varieties Redpoll and Makosta are significantly different from each other, but also the other four tested varieties with a statistical significance of 95%.

4. DISCUSSION

This research shows that different varieties of red currants have different vitamin C content as well as distinct antioxidant activity. The amount of ascorbic acid in juices of 6 varieties of red currant ranged from 3.21 to 33.14 mg/100 g of juice, and from 6.23 to 66.52 mg/100 g of juice after the reduction of the samples. The juice of the Redpoll variety had the highest amount of ascorbic acid, and the juice of the Stanza variety had the lowest amount.

Examining the chemical composition of fresh fruits of 11 varieties of red currant, Djordjević et al. (2010), showed that the fruit of the Jonkheer variety had the lowest content of vitamin C (52.8 mg/100 g), and the fruit of the Rondom variety had the highest content (66.5 mg/100 g). The results of vitamin C content of fresh fruits of Rolan, Stanza, Redpoll and Makosta varieties were also in that range. The amount of vitamin C was lower in juices analyzed in this study compared to fresh fruits in Djordjević et al. (2010). A possible reason might be processing fruits into juices, during which the content of vitamin C decreases, due to the oxidation of ascorbic acid into dehy-

Table 2. Antioxidant activity of red currant juices (IC_{50} mg/mL)

Red currant juice sample	DPPH	β-carotene
Redpoll	1.76±0.25a	2.52±0.90a
Rolan	6.65±0.84b	3.96±0.92b
Jonkheer van Tets	6.44±0.90b	4.52±1.45c
Stanza	5.30±0.34b	6.23±1.28d
Rondom	5.10±0.43b	4.68±1.56c
Makosta	3.31±0.26c	4.46±1.44c

[a,b,c,d]Different letters in columns indicate a statistically significant difference between samples (Tukey test, $p<0.05$).

Results are presented as the mean value of three individual measurements ± standard deviation.

droascorbic acid (Kampuse et al., 2002). The oxidized form of vitamin C can be easily reduced to ascorbic acid under the influence of reducing agents, such as DTT. The results showed that the content of ascorbic acid was significantly higher after the reduction, and that during the processing of fruits into juices, a significant amount of vitamin C was oxidized. The highest increase in vitamin C content occurred in the juice of the Makosta variety, by 103.1%, and the least in the juice of the Stanza variety, by 94%, which shows that during the processing of fruits into juices, there was a partial loss of ascorbic acid content.

Podskalská et al. (2024) evaluated the vitamin C content in some red and black currants juices, and among them was Jonkheer. Although results were expressed as the mean value of several samples of red currants, ascorbic acid content was 24.1 mg/100 g. As the authors highlighted, these differences arise from climatic conditions, higher maturity at harvest, as well as the samples themselves.

Kidoń and Narasimhan (2022) analyzed the ascorbic acid content of Jonkheer van Tets juice obtained with different lengths of ultrasound treatments. The ascorbic acid content was 13.2 to 16.5 mg per 100 mL depending the time used for the ultrasound treatment, and the authors used DTT to measure complete vitamin C. Nevertheless, the vitamin C content was lower compared to our Jonkheer sample after reduction (23.52 mg/100 g) but higher than before the reduction (11.62 mg/100 g). Since the preparation and determination are almost the same, we can conclude that geographic position and climate might be essential parts for the ascorbic acid production in red currants.

Red currant shows antioxidant activity and has the potential to be a good source of antioxidants. The antioxidant activity of red currant is determined by phenolic compounds, mostly anthocyanins, and partly by ascorbic acid (Benvenuti et al., 2004). The antioxidant activity in this work was carried out by two methods. The DPPH method measures the ability of the sample to capture free radicals. The other one is the β-carotene bleaching assay that evaluates the capability of the sample to prevent loss of β-carotene in the β-carotene/linoleic acid model. The results showed that the DPPH antioxidant activity was in the range of 1.76 mg/mL (Redpoll variety) to 6.65 mg/mL (Roland variety). Djordjević et al. (2010) also determined the antioxidant activity of juices of 11 varieties of red currant using this method and it was in the range of 1.9 mg/mL (Redpoll variety) to 8.4 mg/mL (Jonkheer variety). The IC_{50} values obtained by DPPH method in our research were lower for the varieties Redpoll, Jonkheer, Stanza, Rondom and Makosta, which means they showed stronger antioxidant activity.

The differences in the antioxidant value between our research and the work of Djordjević et al. can also be explained by the different preparations of the samples. These samples were previously diluted with water, centrifuged and the supernatant was used for further analysis. Also, in this research, the samples were stored at a lower temperature.

The variety Rolan, which in our research showed the weakest antioxidant activity (6.65 mg/mL), in the work of Djordjević et al. (2010) showed better antioxidant activity (5.5 mg/mL) and it is ahead of the varieties Jonkheer, Stanza and Makosta.

Konić-Ristić et al. (2011) determined the antioxidant activity of the juices of 5 types of berries and for the red currant juice, they obtained the IC_{50} value = 3.14 mg/mL, although the variety used was not specified.

The antioxidant activity which measures the prevention of the β-carotene loss of these red currant juices, has not been determined before.

5. CONCLUSION

Red currants (*Ribes rubrum* L.) are widely grown and used in European countries and beyond, both fresh and processed. Although they are widely used, there is significantly less data on them compared to black currants. Thanks to favorable properties for cultivation and numerous potential and proven positive effects on health, red currant is becoming the subject of an increasing number of studies.

Examining the vitamin C content and antioxidant activity of the juices showed that the best results were obtained from the Redpoll variety juice. The data obtained in this work were in accordance with the already available results on the antioxidant activity of red currant juice but also indicate that the antioxidant potential varies to the variety of red currant. It was also established that processing into juices leads to a significant loss of vitamin C due to its oxidation. The results of this work can serve as an incentive for the use of red currant and its products in the diet, as well as for the selection of varieties with favorable nutritional characteristics.

FUNDING

This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant no. 451-03-66/2024-03/200003 and 451-03-65/2024-03/200113). The authors are also grateful for the support of the Internal project of the Faculty of Medicine, University of Nis, No. 68.

CONFLICT OF INTEREST

The authors declare that they have no financial and commercial conflicts of interest.

REFERENCES

Benvenuti, S., Pellati, F., Melegari, M., and Bertelli, D. (2004): Polyphenols, Anthocyanins, Ascorbic Acid, and Radical Scavenging Activity of Rubus, Ribes, and Aronia, *Journal of Food Science*, **69**(3). https://doi.org/10.1111/j.1365-2621.2004.tb13352.x

Berk, S., and Tuna, S. (2017): Biological activities of currant (*Ribes* sp.) and its effects on human health, *Bahçe*, **46**(1), 113–118.

Brubacher, G., Müller-Mulot, W., and Southgate, D. A. T. (Eds.) (1985): *Methods for the Determination of Vitamins in Food*, Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-009-4944-7

Cuendet, M., Hostettmann, K., Potterat, O., and Dyatmiko, W. (1997): Iridoid Glucosides with Free Radical Scavenging Properties from *Fagraea blumei*, *Helvetica Chimica Acta*, **80**(4), 1144–1152. https://doi.org/10.1002/hlca.19970800411

Cvetković, M., Mihajilov Krstev, T., Šavikin, K., Zdunić, G., Kitić, D., Stojanović, D., Ranđelović, M., Milutinović, M., Ilić, D., and Miladinović, B. (2023): Antimicrobial activity evaluation of lyophilized juice and waste extract of red currant variety Redpoll, *Acta Medica Medianae*, **62**(02), 38–44. https://doi.org/10.5633/amm.2023.0205

Djordjević, B., Šavikin, K., Zdunić, G., Janković, T., Vulić, T., Oparnica, Č., and Radivojević, D. (2010): Biochemical Properties of Red Currant Varieties in Relation to Storage, *Plant Foods for Human Nutrition*, **65**(4), 326–332. https://doi.org/10.1007/s11130-010-0195-z

Gülmez, G., Şen, A., Şekerler, T., Algül, F. K., Çilingir-Kaya, Ö. T., and Şener, A. (2022): The antioxidant, anti-inflammatory, and antiplatelet effects of *Ribes rubrum* L. fruit extract in the diabetic rats, *Journal of Food Biochemistry*, **46**(7). https://doi.org/10.1111/jfbc.14124

Kampuse, S., Kampuss, K., and Pizika, L. (2002): Stability of anthocyanins and ascorbic acid in raspberry and blackcurrant cultivars during frozen storage, *Acta Horticulturae*, (585), 507–510. https://doi.org/10.17660/ActaHortic.2002.585.81

Kidoń, M., and Narasimhan, G. (2022): Effect of Ultrasound and Enzymatic Mash Treatment on Bioactive Compounds and Antioxidant Capacity of Black, Red and White Currant Juices, *Molecules*, **27**(1), 318. https://doi.org/10.3390/molecules27010318

Koleva, I. I., Van Beek, T. A., Linssen, J. P. H., Groot, A. D., and Evstatieva, L. N. (2002): Screening of Plant Extracts for Antioxidant Activity: a Comparative Study on Three Testing Methods, *Phytochemical Analysis*, **13**(1), 8–17. https://doi.org/10.1002/pca.611

Konić-Ristić, A., Šavikin, K., Zdunić, G., Janković, T., Juranic, Z., Menković, N., and Stanković, I. (2011): Biological activity and chemical composition of different berry juices, *Food Chemistry*, **125**(4), 1412–1417. https://doi.org/10.1016/j.foodchem.2010.10.018

Milivojevic, J., Slatnar, A., Mikulic-Petkovsek, M., Stampar, F., Nikolic, M., and Veberic, R. (2012): The Influence of Early Yield on the Accumulation of Major Taste and Health-Related Compounds in Black and Red Currant Cultivars (*Ribes* spp.), *Journal of Agricultural and Food Chemistry*, **60**(10), 2682–2691. https://doi.org/10.1021/jf204627m

Ohta, K., and Harada, K. (1996): Studies on the Measurement of Ascorbic Acid and Dehydroascorbic Acid in Tea Plants, *Nippon Nōgeikagaku Kaishi*, **70**(8), 873–882. https://doi.org/10.1271/nogeikagaku1924.70.873

Podskalská, T., Bhujel, N. K., Hraničková, M., Beňo, F., Tobolka, A., and Čížková, H. (2024): Compositional Characteristics of Currant Juices Prepared by Different Processes and Other Selected Currant Products, *Applied Sciences*, **14**(14), 6029. https://doi.org/10.3390/app14146029

Russell, L. (2004): Water-Soluble Vitamins (Food Science and Technology), 487–571 *in* L. Nollet, ed., *Handbook of Food Analysis, Second Edition -3 Volume Set*, CRC Press. https://doi.org/10.1201/b11081-17

USDA (2024): National nutrient database for standard reference release (2024).

Zdunić, G., Šavikin, K., Pljevljakušić, D., and Djordjević, B. (2016): Black (Ribes nigrum L.) and Red Currant (Ribes rubrum L.) Cultivars, 101–126 *in Nutritional Composition of Fruit Cultivars*, Elsevier. https://doi.org/10.1016/B978-0-12-408117-8.00005-2

Traditional use of pot marigold (*Calendula officinalis* L.) in the Balkan Peninsula

MARIJA S. MARKOVIĆ[1*], DEJAN S. PLJEVLJAKUŠIĆ[2] AND ŁUKASZ J. ŁUCZAJ[3]

[1]*Institute of Forestry, Belgrade, Kneza Višeslava 3, 11030 Belgrade, Serbia*
[2]*Institute for Medicinal Plants Research "Dr. Josif Pančić", Tadeuša Koščuška 1, 11000 Belgrade, Serbia*
[3]*Institute of Biology, University of Rzeszów, Zelwerowicza 4, 35-601 Rzeszów, Poland*
**Corresponding author: markovicsmarija9@gmail.com*

Received: October 23, 2024
Accepted: November 25, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

The objective of this study was to compile and systematize traditional knowledge regarding the medicinal uses of pot marigold (*Calendula officinalis* L.) among the Balkan population, based on existing literature. The ethnobotanical studies reviewed encompass various regions of the Balkan Peninsula, including Bosnia and Herzegovina, Bulgaria, Croatia, and Serbia. These studies identified several categories of disorders commonly treated with pot marigold flowers in Balkan folk medicine, such as autoimmune disorders, cardiovascular conditions, dermatological issues, digestive ailments, neurological complaints, reproductive system disorders, urinary conditions, and other health issues. Additionally, fresh pot marigold leaves are traditionally used as a nutritional supplement. This synthesis of traditional knowledge on the medicinal uses of pot marigold flowers may serve as a valuable foundation for future studies, potentially leading to the development of new medicinal applications.

Keywords: ethnobotany, pot marigold, Balkans, phytotherapy

https://doi.org/10.61652/leksir2444007M

1. INTRODUCTION

The traditional use of medicinal plants has been practiced worldwide, particularly in developing countries, and is increasingly gaining popularity in developed nations (Džamić and Matejić, 2017; Marković et al., 2022). Conventional medicine does not fully address a variety of human health disorders, which has led to a rising global interest in phytotherapy (Marković et al., 2022). According to these authors, the long history of herbal drug use has demonstrated its relative safety and efficacy. Medicinal herbs are often free from hazardous side effects (Schultz et al., 2001). However, Džamić and Matejić (2017) warn that while pharmacologically active compounds in plants benefit health, they may also pose risks due to potential toxic components. Thus, further scientific research is needed to confirm the safety of plants intended for medicinal use.

In the Balkan Peninsula, the population traditionally relies on medicinal plants as common alternatives or complements to conventional treatments (Džamić and Matejić, 2017). The simultaneous use of medicinal herbs alongside official drug therapies is also prevalent in this region (Živković et al., 2020). Traditional knowledge of natural medicinal plant products has been documented extensively through ethnobotanical studies, especially over the past two decades. This review aims to highlight the significance of the traditional use of *Calendula officinalis* L. within the Balkan region.

Calendula officinalis L. (pot marigold) is an annual plant species belonging to the Asteraceae family (Figure 1), native to the Mediterranean region (Ramos et al., 1998). As a wild species, it grows in warm Mediterranean habitats and is cultivated in other parts of the Balkan Peninsula (Sarić, 1989). Initially cultivated as an ornamental plant, it has also been grown extensively in Europe for its medicinal properties and long-standing use in traditional medicine. According to Ramos et al. (1998) and Ashwlayan et al., (2018), pot marigold flowers have commonly been used externally to treat wounds, ulcers, herpes, scars, skin injuries, burns, frostbite, and skin eruptions. Internally, they serve as bactericidal, diuretic, tonic, analgesic, antidiabetic, antitumor, anti-ulcer, and anti-inflammatory agents, and are also used for gastrointestinal disorders, gynecological issues, and eye diseases. Additionally, pot marigold extract has shown immunostimulant properties, as well as antifungal and antiviral activity, including effectiveness against HIV (Patil et al., 2022).

Fig. 1. Pot marigold (*Calendula officinalis* L.)

In Serbia, pot marigold flower oil macerate is commonly applied topically to treat skin disorders, as noted by Gostuški (1973), Sarić (1989), and Tucakov (1990). Additionally, an infusion of the flowers can be used to soothe bee stings (Tucakov, 1990). Pot marigold flower tea is known to induce perspiration (Gostuški, 1973; Sarić, 1989; Tucakov, 1990). It can also be used for kidney ailments, abdominal organ disorders, spleen swelling, and liver diseases (Gostuški, 1973), as well as for stomach and bile issues (Tucakov, 1990). It is reportedly used to ease menstrual pain, provides a calming effect for anemic women (Gostuški, 1973; Sarić, 1989) and can be applied externally as a vaginal wash (Tucakov, 1990). Tucakov (1990) have highlighted the antiseptic properties of *Calendula officinalis* flowers, while Sarić (1989) has also mentioned its use in treating hysteria, chronic illnesses, and even malignant tumors. Dried pot marigold flowers are the component of different tea products, packed as tea mixtures for the treatment of kidney inflammation, constipation, and candidiasis symptoms by the Institute for Medicinal Plant Research "Dr Josif Pančić" in Belgrade (Filipović and Ugrenović, 2015). Preparations from pot marigold flowers are frequently used topically in moisturizing creams or tinctures to help stimulate blood circulation and hydrate the skin (Pavlović and Marković, 2024).

2. ETHNOBOTANICAL STUDIES IN THE BALKAN PENINSULA INCLUDED IN THIS REVIEW

The rural areas of the Balkan Peninsula are notable for ethnobotanical studies due to their unique mountain ranges, rich biodiversity, and cultural diversity. This part of Europe reflects a history of diverse cultural influences (Živković et al., 2020), and extensive ethnobotanical research has been conducted here over the past twenty years. Each studied region features populations of various ethnic backgrounds who have traditionally utilized local plants for medicinal purposes.

This review includes ethnobotanical studies documenting the traditional uses of pot marigold (*Calendula officinalis* L.) across various Balkan regions: Kopaonik Mt (Jarić et al., 2007), central, southern, and western Bosnia and Herzegovina (Šarić-Kundalić et al., 2010), Pešter Plateau in Sandžak (Pieroni et al., 2011), Deliblato Sands (Popović et al., 2012), Zlatibor District (Šavikin et al., 2013), the Suva Planina Mt (Jarić et al., 2015), Bulgaria (Koleva et al., 2015), southern Kosovo and Metohija (Mustafa et al., 2015), northeastern Bosnia and Herzegovina (Saric-Kundalic et al., 2016), Svrljig and Timok (Matejić et al., 2020), Štrpce area in southern Kosovo and Metohija (Mustafa et al., 2020), Pčinja District (Živković et al., 2020), Kuršumlija (Đelić et al., 2021), the Adriatic Islands in Croatia (Łuczaj et al., 2021), the Stara Planina Mt (Jarić et al., 2024), Pirot District (Marković et al., 2024), and Rujan Mt (Simić et al., 2024) (Table 1).

3. THE USE OF POT MARIGOLD IN ETHNOBOTANICAL STUDIES ON THE BALKAN PENINSULA

In the Kopaonik Mt of Serbia, pot marigold has traditionally been used topically in the form of an oil-based cream (ointment) to treat fungal foot infections, wounds, burns, frostbite, leg swelling, and painful veins (Jarić et al., 2007). It is also taken internally as a vermifuge in tea form. In Bosnia and Herzegovina, pot marigold is used in ointments for skin injuries, burns, varicose veins, and leg pain, while the infusion is taken for increased vaginal secretion (Šarić-Kundalić et al., 2010). On the Sandžak region's Pešter Plateau, pot marigold is traditionally used to treat hepatitis (Pieroni et al., 2011), and in Deliblato Sands, it has been noted as an emmenagogue, mild purgative, and diuretic (Popović et al., 2012).

In Serbia's Zlatibor District, pot marigold is applied externally for skin issues, burns, wounds, hemorrhoids, and varicose veins, and is also used internally for digestive disorders and gastric or duodenal ulcers (Šavikin et al., 2013)). In Suva Planina Mt, pot marigold flowers are used internally to support blood circulation, and as an antidiarrheal, while external applications serve to treat burns, skin complaints, varicose veins, and hemorrhoids (Jarić et al., 2015). In Bulgaria, pot marigold is used as a prophylactic and anti-inflammatory for nerve issues, stomach disorders, ulcers, wounds, and blood detoxification (Koleva et al., 2015). In South Kosovo and Metohija, pot marigold serves as an antibacterial and antifungal

Table 1. A comparative review of the traditional use of pot marigold (*Calendula officinalis* L.) across the Balkan Peninsula,

Group of disorder	Indication	Form of use	E / I	Region	Reference
Au	Bone ache	Ointment	E	Adriatic Islands – Croatia	Łuczaj et al., (2021)
	Bone pain	Ointment	E	Pčinja district	Živković et al., (2020)
	Carcinomas	Infusion	I	Rujan Mt	Simić et al., (2024)
	Hepatitis	Infusion	I	Pešter Plateau	Pieroni et al., (2011)
	Pain in the legs	Ointment	E	Bosnia and Herzegovina	Šarić Kundalić et al., (2010)
	Rheumatic pain	Extract in alcohol / ointment	E	Pirot District	Marković et al., (2024)
	Swelling of the leg	Ointment	E	Kopaonik Mt	Jarić et al., (2007)
		Extract in "rakija"		Adriatic Islands – Croatia	Łuczaj et al., (2021)
Ca	Blood vessels	Infusion	I	Svrljig region	Matejić et al., (2020)
	Detoxification of the blood	Infusion	I	Stara planina Mt	Jarić et al., (2024)
	Circulation	Infusion	I	Suva Planina Mt	Jarić et al., (2007)
				Pirot District	Marković et al., (2024)
	Good for blood	Infusion	I	Adriatic Islands – Croatia	Łuczaj et al., (2021)
				Zlatibor District	Šavikin et al., (2013)
	Hemorrhoids	Ointment / tincture	E	Suva Planina Mt	Jarić et al., (2015)
				Svrljig region	Matejić et al., (2020)
				Pirot District	Marković et al., (2024)
				Rujan Mt	Simić et al., (2024)
	Painful veins	Ointment	E	Kopaonik Mt	Jarić et al., (2007)
				Bosnia and Herzegovina	Šarić Kundalić et al., (2010)
	Varicose veins	Ointment / tincture	E	Zlatibor District	Šavikin et al., (2013)
				Suva Planina Mt	Jarić et al., (2015)
				Stara planina Mt	Jarić et al., (2024)
	Vein inflammation	Ointment	E	Adriatic Islands – Croatia	Łuczaj et al., (2021)
	Vein problems	Ointment	E	Pčinja district	Živković et al., (2020)
Dm	Abscesses	Ointment	E	Konjuh Mt	Saric-Kundalic et al., (2016)
				Kopaonik Mt	Jarić et al., (2007)
				Zlatibor District	Šavikin et al., (2013)
				Suva Planina Mt	Jarić et al., (2015)
				South Kosovo and Metohija	Mustafa et al., (2015)
	Burns	Ointment	E	Svrljig region	Matejić et al., (2020)
				Timok region	Matejić et al., (2020)
				Pčinja district	Živković et al., (2020)
				Kuršumlija	Đelić et al., (2021)
				Rujan Mt	Simić et al., (2024)
	Bruises	Ointment	E	Timok region	Matejić et al., (2020)
	Cuts	Ointment	E	Timok region	Matejić et al., (2020)
	Frostbite	Ointment	E	Kopaonik Mt	Jarić et al., (2007)
	Fungous ailments of the feet	Ointment	E	Kopaonik Mt	Jarić et al., (2007)
	Lacerations	Ointment	E	Štrpce region	Mustafa et al., (2020)
	Rashes	Ointment	E	Konjuh Mt	Saric-Kundalic et al., (2016)
	Skin complaints / diseases	Ointment	E	Zlatibor District	Šavikin et al., (2013)
				Suva Planina Mt	Jarić et al., (2015)
				Svrljig region	Matejić et al., (2020)
				Pčinja District	Živković et al., (2020)
	Skin infections	Ointment	E	Štrpce region	Mustafa et al., (2020)
	Skin injuries (in mixtures)	Balm	E	Bosnia and Herzegovina	Šarić Kundalić et al., (2010)
	Sunburns	Ointment	E	South Kosovo and Metohija	Mustafa et al., (2015)
	Ulcers	Ointment	E	Konjuh Mt	Saric-Kundalic et al., (2016)
				Kopaonik Mt	Jarić et al., (2007)
				Zlatibor District	Šavikin et al., (2013)
				South Kosovo and Metohija	Mustafa et al., (2015)
	Wounds	Ointment	E	Konjuh Mt	Saric-Kundalic et al., (2016)
				Timok region	Matejić et al., (2020)
				Pčinja District	Živković et al., (2020)
				Kuršumlija	Đelić et al., (2021)
				Adriatic Islands – Croatia	Łuczaj et al., (2021)
				Rujan Mt	Simić et al., (2024)
Dg	Antidiarrheal	Infusion	I	Suva Planina Mt	Jarić et al., (2015)

	Abdominal pain	Infusion	I	Timok region	Matejić et al., (2020)
	Light purgative	Infusion	I	Deliblato Sands	Popović et al., (2012)
	Liver diseases / complaints	Infusion	I	Svrljig region Pčinja District	Matejić et al., (2020) Živković et al., (2020)
	Digestive disorders / complaints	Infusion / tincture	I	Zlatibor District Pčinja District	Šavikin et al., (2013) Živković et al., (2020)
	For stomach	Infusion	E	Pirot District	Marković et al., (2024)
	Gastric and duodenal ulcer	Infusion / tincture	I	Zlatibor District	Šavikin et al., (2013)
	Gastrointestinal system disorders	Infusion	I	Konjuh Mt	Saric-Kundalic et al., (2016)
	Stomach diseases / troubles	Infusion / extract in alcohol	I	Pirot Disrict	Marković et al., (2024)
	Stomach pain / stomachache	Infusion	I	Timok region Rujan Mt	Matejić et al., (2020) Simić et al., (2024)
Dp	Liver cleanse	Infusion	I	Pirot Disrict	Marković et al., (2024)
	Blood purification	Infusion	I	Pirot Disrict	Marković et al., (2024)
Nr	Mental illnesses	Infusion	I	Konjuh Mt	Saric-Kundalic et al., (2016)
	Sedation	Infusion	I	Pirot Disrict	Marković et al., (2024)
Pr	Disease prevention (coffee substitute)	Infusion	I	Pirot Disrict	Marković et al., (2024)
	Improvement of the immune system	Infusion	I	Pirot Disrict	Marković et al., (2024)
Rp	Ovarian cysts	Infusion	I	Pirot Disrict Rujan Mt	Marković et al., (2024) Simić et al., (2024)
	Emmenagogue	Infusion	I	Deliblato Sands	Popović et al., (2012)
	Gynecological problems	Infusion	I	Pirot Disrict	Marković et al., (2024)
	Increased vaginal secretions	Infusion	I	Bosnia and Herzegovina Konjuh Mt	Šarić Kundalić et al., (2010) Saric-Kundalic et al., (2016)
	Inflammation of the ovaries	Infusion	I	Rujan Mt	Simić et al., (2024)
	Menstrual cycle disorders	Infusion	I	Rujan Mt	Simić et al., (2024)
	Painful menstruation	Infusion	I	Timok region	Matejić et al., (2020)
Rs	Dry cough	Infusion	I	Rujan Mt	Simić et al., (2024)
Ur	Diuretic	Infusion	I	Deliblato Sands	Popović et al., (2012)
	Urogenital system inflammations	Infusion	I	Konjuh Mt	Saric-Kundalic et al., (2016)
Vr	Against shoulder pain	Ointment	E	Pirot Disrict	Marković et al., (2024)
	Antibacterial	Infusion	I	South Kosovo and Metohija	Mustafa et al., (2015)
	Antifungal	Infusion	I	South Kosovo and Metohija	Mustafa et al., (2015)
	Bone fractures	Ointment	E	Konjuh Mt	Saric-Kundalic et al., (2016)
	Chills	Infusion	I	Rujan Mt	Simić et al., (2024)
	For better eyesight	Infusion	I	Pirot Disrict	Marković et al., (2024)
	In nutrition*	Fresh leaf	I	Pirot Disrict	Marković et al., (2024)
	Vermifuge	Infusion	I	Kopaonik Mt	Jarić et al., (2007)
	Weakness / tiredness	Infusion	I	Stara planina Mt	Jarić et al., (2024)

* The only known use of the leaf (all other uses are of flowers).
E / I – mode of administration: E – external, I – internal.
Group of disorders: Au - Autoimmune diseases, Cd – cardiovascular, Dm – dermatology, Dg – digestive, Dp – depurative, Nr – neurological conditions, Pr – preventive, Rp – reproductive system disorders, Rs – respiratory diseases, Ur – urinary system disorders; Vr – various.

treatment and is used to treat burns and skin wounds (Mustafa et al., 2015). Around Konjuh Mt in Bosnia and Herzegovina, pot marigold is used internally for urogenital and gastrointestinal issues, mental health, and externally for wounds, bone fractures, rashes, ulcers, and abscesses (Saric-Kundalic et al., 2016). In Svrljig, southeastern Serbia, it is applied externally for burns and skin issues, while internal uses include liver and vascular health (Matejić et al., 2020). In the Timok region, in eastern Serbia, pot marigold is used externally for burns, bruises, and wounds, and internally for abdominal and menstrual pain (Matejić et al., 2020). In Štrpce (Kosovo and Metohija), it treats lacerations and skin infections (Mustafa et al., 2020).

In Pčinja District, southeastern Serbia, pot marigold treats burns, wounds, skin issues, vein problems, and bone pain externally, and is used internally for digestive and liver ailments (Živković et al., 2020). In Kuršumlija, pot marigold is applied to heal burns and wounds (Đelić et al., 2021). On Croatia's Adriatic Islands, pot marigold flowers are used in infusions for blood health and as a cream for vein inflammation, wounds, and aching bones. It is also used in a homemade alcohol-based drink (rakija) for leg massages (Łuczaj et al., 2021).

In Stara Planina Mt, southeastern Serbia, pot marigold infusions are used internally for blood detoxification and to alleviate weakness, while ointments are used externally to treats varicose veins (Jarić et al., 2024). In Pirot District, ethnobotanical studies across 157 villages reveal widespread external use of pot marigold in ointments for cracked skin, shoulder pain, burns, hemorrhoids, skin conditions, and varicose veins, as well as internal use in infusions for blood purification, ovarian cysts, immunity, liver cleansing, and digestive issues (Marković et al., 2024). Pot marigold leaves are also used fresh in nutrition.

Lastly, around Rujan Mt, southeastern Serbia, pot marigold is used internally for carcinoma, hemorrhoids, abdominal pain, ovarian cysts, menstrual disorders, and dry cough, and applied internally for hemorrhoids, burns, and wounds (Simić et al., 2024).

3.1. The form of pot marigold used in the Balkans

Pot marigold cream, or ointment, is the primary form of external application cited in the studies. This preparation involves an oil extract of pot marigold flowers (*Calendulae flos*), which are macerated in homemade lard, olive or sunflower oil. Traditionally, homemade lard is washed multiple times (usually nine) until it becomes white. The pot marigold flowers are then simmered with the lard to create the ointment. First, 50 grams of dried pot marigold flowers and 500 grams of warm, unsalted lard are measured out and combined. Their mixture is fried for 5 minutes, and, after 24h, reheated and strained (Filipović and Ugrenović, 2015). For the preparation of oily macerate, the authors describe the following procedure: 200 grams of pot marigold flowers are poured over with one liter of olive or sunflower oil, immersed, subsequently strained, and ready to use after one month. According to the same authors, fresh pot marigold flowers can also be mixed with coconut oil. This preparation mixture needs to be warmed slightly, and then left to cool. If the mixture is too thick, a little more of another base oil can be added to reduce the density.
Pot marigold balm, less frequently mentioned in Balkan ethnobotanical studies, is a more fluid or liquid preparation, used to heal or soothe the skin. Balms known as "mehlems" are specific to Bosnia and Herzegovina, where they are traditionally prepared from freshly chopped flowers of pot marigold mixed with warmed resins from the *Abies* species and olive oil as additives (Šarić-Kundalić et al., 2010).
Tinctures (alcohol-based extracts) are the least common for external use and are often prepared by macerating the homemade alcoholic beverage, rakija (Łuczaj et al., 2021). Hundreds of dried pot marigold flowers are placed in a liter of medicinal ethanol or rakija (Janaćković et al., 2022), and stored in a cool, dark place for a period of 2–4 weeks. The preparation method is simple and effective, aligning with the findings of scientific research, which supports pot marigold's use in the treatment of a wide range of skin conditions and the promotion of tissue repair (Dinda et al., 2015; Patil et al., 2022). The mentioned references provided highlight the active compounds in *C. officinalis* and their therapeutic applications, validating the efficacy of its tincture both in traditional and modern herbal medicine.
Internally, pot marigold flowers are used as an infusion (tea). To prepare, 1–2 teaspoons of dried flowers are steeped in one cup of boiling water for five to ten minutes. The recommended dose is two to three cups of unsweetened tea, which is bitter in taste, per day (Filipović and Ugrenović, 2015; Kemper, 1999). Pharmacological studies (Bertges et al., 2006; Dilucia et al., 2023) provide scientific support for the common uses of pot marigold infusion, noted in ethnobotanical research, offering both traditional and modern perspectives on its medicinal value.
Additionally, fresh pot marigold leaves are sometimes consumed in salads across the Balkans (Marković et al., 2024), as they are rich in vitamins and minerals, with a taste and composition similar to dandelion (*Taraxacum officinale* L.) leaves.

3.2. Comparison with traditional uses in other regions

Pot marigold flowers are traditionally used not only as an infusion, tincture, or ointment, but also as a food dye and spice (Ercetin et al., 2012), especially in regions the species is native to: Spain, Portugal, Italy, Malta, Greece, Turkey, and northern parts of Africa (Ercetin et al., 2012). The people of Greece use pot marigold orally in the form of a decoction against thrombophlebitis, stomach ulcers, liver disorders, and topically for the treatment of wounds and eczema (Hanlidou et al., 2004), as do the subjects of the above-mentioned ethnobotanical studies of Bosnia and Herzegovina, Bulgaria, Croatia, and Serbia.
In Slovenia, the population uses pot marigold internally in the form of an infusion for the treatment of intestinal problems, stomach pain, insomnia, and to treat increased vaginal secretion. A pot marigold ointment with lard is used externally for the treatment of burns, sunburns, bruises, and eczema, as well as against contusions in painful areas of knees, shoulders, and joints (Lumpert and Kreft, 2017). Similar uses were reported in our research from other Balkan countries.
Cenk (2022) has recorded that in Turkey, pot marigold was used as an antipyretic for the treatment of wounds and burns, against mental illnesses, cardiovascular diseases, gastrointestinal ulcers, dysmenorrhea, and for cancer prevention—uses similar to those observed in Balkan countries. The same author mentioned that pot marigold is used in the treatment of eczema and psoriasis among the population of Turkey, which has not been noted in ethnobotanical studies of the Balkans. Ugulu and Aydin (2011) mentioned the use of pot marigold against skin cancer, burns, and wounds in Turkey—applications similar to those recorded in Balkan countries.
The people of France usually use pot marigold flowers as a tea to lower temperature and perspiration (Sharrif Moghaddasi, 2012). Similar uses have been recorded in southeastern Serbia (Jarić et al., 2024; Simić et al., 2024).
Among the people of England, fresh juice from pot marigold was traditionally used against jaundice, constipation, as well as to shortening the duration of menstrual bleeding, while the decoction of flowers was used for the treatment of measles and smallpox (Abdelwahab et al., 2022). These uses were not mentioned in the Balkans.
The Indian population used the flowers of *C. officinalis* for the treatment of wounds, ulcers, frostbite, skin injuries, and to purify blood (Abdelwahab et al., 2022), which were uses mentioned in Balkan countries.
The people of the Balkan Peninsula use the fresh leaves of pot marigold as a salad (Marković et al., 2024), while in North America the common practice is to eat it as a soup (Sharrif Moghaddasi, 2012).

3.3. Unique uses of pot marigold in the Balkans relative to global practices

Scientific existing ethnobotanical papers from the Balkans on the traditional uses of *C. officinalis* could potentially reveal new uses or unique regional applications that may not be widely documented in other parts of the world. Ethnobotanical studies focus on the ways in which plants have been used by local populations throughout history, and the Balkans, with their rich cultural diversity and long history of traditional medicine, may hold specific uses of pot marigold that are not commonly recognized in broader scientific or global contexts.

Pot marigold has long been used to treat a range of skin conditions, but its specific uses can vary from region to region. In some parts of the Balkans, pot marigold may be applied to conditions that are specific to the local environment or climate, such as the treatment of sunburns in South Kosovo and Metohija (Mustafa et al., 2015) (Table 1).
There may be local uses of *C. officinalis* in treating respiratory issues in the Balkans. Pot marigold has been used as a tea for soothing dry coughs, or other chest-related conditions, particularly in rural or mountainous areas, where herbal knowledge is passed down through generations, as noted by Simić at al. (2024) in areas surrounding Rujan Mt (Table 1).
While *C. officinalis* is not widely known as a culinary herb globally, it may be used traditionally as food in the Balkans, in

ways that are not documented in other regions. In the Pirot District (southeastern Serbia), pot marigold leaves may be added to salads (Marković et al., 2024), while in other areas of Serbia the flowers are used as a spice in soups, broths and meals (Filipović and Ugrenović, 2015) for both flavoring and medicinal purposes, such as aiding digestion or reducing inflammation. Filipović and Ugrenović (2015) also mentioned fresh pot marigold juice, which can be prepared with fresh pot marigold flowers and stems mixed up in a blender with apple and carrot.

In some Balkan rural areas, *C. officinalis* might be used for the treatment of animal health issues, particularly in livestock. Pot marigold flowers in the form of water extract have been used in the Pirot District (southeastern Serbia) for the treatment of colds in animals such as cattle, horses, or sheep, or infections, such as swine and sheep erysipelas on the hoof (Marković et al., 2021). Šubarević et al. (2015) mentioned the antiseptic and soothing use of pot marigold salve with lard for wounds. The same author mentioned the preparation procedure for this formulation. Melt the lard and marigold petals on low heat. When everything is well combined, remove it from the heat and let it stand for 24 hours, then melt it again on low heat and strain. Once cooled down, it can be used for coating the skin of livestock. The same use was mentioned by Simić et al. (2024) at Rujan Mt. Improvement of blood count of cow, goat, and pig in the form of pot marigold infusion, taken internally, was also mentioned at Rujan Mt by the same authors. The treatment of diarrhea in ruminants with an infusion of pot marigold was mentioned by Davidović et al. (2012; 2011). It is possible that the mentioned uses of pot marigold may not be widely documented outside of the region, because traditional veterinary practices are often localized.

Finally, pot marigold has symbolic significance in some Balkan traditions and is often linked to festivals and rituals. For example, it may be used in traditional Balkan celebrations, such as those around the summer solstice or harvest, as a protection or fertility symbol. In the traditional culture of Serbia, pot marigold flowers are picked on St. George's Day, put in water together with colored Easter eggs, and this water is then used for face washing (Dajić Stevanović et al., 2014). In the folk practices of Bulgaria, the flowers of pot marigold may be used in spiritual protection rituals or to bring good luck and prosperity, in the form of amulets, i.e., wearable charms (Nedelcheva and Draganov, 2014), which may not be commonly known in global ethnobotanical studies.

4. POT MARIGOLD'S ACTIVE COMPOUNDS AND THEIR LINK TO ITS EFFECTS AND APPLICATIONS

Pot marigold is rich in a variety of bioactive compounds, including carotenoids, flavonoids, triterpenoids, glycosides, saponins, polysaccharides, steroids, sterols, quinones, essential oils, and amino acids (Ashwlayan et al., 2018; Ercetin et al., 2012; Patil et al., 2022).

The antioxidant compounds in pot marigold, notably flavonoids and carotenoids, help protect the skin from oxidative stress and contribute to the healing of various skin conditions (Bernatoniene et al., 2011). Lutein and beta-carotene, the most plentiful carotenoids in pot marigold flowers, influence wound healing (Dhingra et al., 2022), and cell rejuvenation (Ullah and Hamza, 2023). Glycosides from pot marigold show anti-inflammatory activities (Ullah and Hamza, 2023). The essential oil was found to be high in alpha-cadinol, which demonstrated antioxidative properties against different skin complaints (Dhingra et al., 2022).

Saponins isolated from pot marigold flowers have demonstrated antimutagenic properties (Prabhu Venkatesh et al., 2023), and the cytotoxic effects of pot marigold suggest its potential as a future cancer treatment (Patil et al., 2022). Additionally, the phenolic compounds in pot marigold tea may enhance its antitumor activity, particularly against melanoma cells (Matić et al., 2013). Carotenoid lutein increases tumor latency (Cruceriu et al., 2020). Calenduloside F 6′-O-n-butyl ester is effective against melanoma, leukemia and colon cancer (Ullah and Hamza, 2023).

5. PRECAUTIONS FOR USE

Pot marigold products are not considered safe for eye application due to the risk of irritation and potential infection. Allergic reactions, such as contact dermatitis, may occur, as in the case of other plant species from the family Asteraceae (Ercetin et al., 2012). Side effects include nausea, vomiting, and anorexia (Ingersoll, 2015). Pot marigold products should not be used alongside sedatives, nor administered to pregnant women, children, or nursing mothers, as toxicological data remains limited (Kemper, 1999).

Studies on the hydroalcoholic extract of *Calendula officinalis* L. indicate no toxicity in rats; however, signs of kidney and liver strain suggest potential hepatotoxic effects (Silva et al., 2007). Continued chemical and pharmacological research is necessary to establish the safe medicinal use of this plant species.

6. ALIGNMENT BETWEEN TRADITIONAL USES AND MODERN PHARMACOLOGICAL RESEARCH

Recent scientific findings mainly confirm mentioned traditional uses in treating conditions. There are alignments in the healing effects of pot marigold against skin diseases in traditional medicine, mentioned in this study, especially for the treatment of wounds of any type (Đelić et al., 2021; Jarić et al., 2007; Łuczaj et al., 2021; Šavikin et al., 2013; Simić et al., 2024; Živković et al., 2020) and pharmacological studies (Bedi and Shenefelt, 2002; Fronza et al., 2009; Givol et al., 2019; Leach, 2008). Injuries and inflammation of the skin are treated with marigold both traditionally, in mixtures with other plants species (Mustafa et al., 2020; Šarić-Kundalić et al., 2010), and through modern pharmacology, with *Calendula officinalis* listed as an ingredient of the multicomponent medication "Traumeel" (Müller-Löbnitz and Göthel, 2011).

Pot marigold is traditionally used to treat digestive disorders and gastric or duodenal ulcers (Šavikin et al., 2013), and these findings are consistent with studies in modern pharmacology (Bertges et al., 2006; Ingersoll, 2015; Ullah and Hamza, 2023). The use of pot marigold tea as an antidiarrheal agent was mentioned in an ethnobotanical study by Jarić et al., (2007) as well as in the pharmacological research of Ashwlayan et al,. (2018).

C. officinalis is being used in the traditional medicine of Balkan countries to treat hemorrhoids (Jarić et al., 2015; Marković et al., 2024; Matejić et al., 2020; Šavikin et al., 2013; Simić et al., 2024) and blood vessels (Matejić et al., 2020). These uses are in accordance with the pharmacological research of Sharrif Moghaddasi (2012) and Dhingra et al., (2022), which noted constricting effects to vessels that can terminate hemorrhoid bleeding. In addition, pot marigold provided cardiological protection by reducing myocardial infarct size (Ullah and Hamza, 2023).

C. officinalis has been also mentioned as an anticancer agent in the ethnomedicine of Rujan Mt (Simić et al., 2024), as well as in a pharmacological study by Ashwlayan et al., (2018). Pot marigold extracts show effects on different tumor cell lines derived from leukemias, melanomas, breast, cervix, colon, prostate, pancreas and lung (Ukiya et al., 2006; Ullah and Hamza, 2023).

The use of pot marigold against menstrual problems was mentioned in some ethnobotanical studies from the Balkans (Marković et al., 2024; Matejić et al., 2020; Simić et al., 2024) as well as in pharmacology (Ingersoll, 2015).

Pot marigold is traditionally used in the form of tea for the treatment of mental illnesses (Ercetin et al., 2012; Saric-Kundalic et al., 2016) have studied antioxidant activity of pot marigold extracts from Turkey that can provide neuroprotective effects against oxidative damage at the time of appearance of mental illness, such as Alzheimer's disease or Down syndrome. In addition, Jasoria et al., (2024) has also noted that pot marigold flowers are useful against neurodegeneration connected with oxidative stress, which can cause Alzheimer's disease. Furthermore, an infusion of flowers, taken internally, is beneficial as a sedative in the ethnomedicine of Pirot District (Marković et al., 2024) as well as in modern pharmacology (Ashwlayan et al., 2018).

7. CONCLUSION

The traditional uses of pot marigold on the Balkan Peninsula highlight nature's potential to address many health concerns. However, further research is necessary to validate these applications and support the development of new medicinal products, particularly for dermatological uses.

Some pot marigold uses, such as those for specific respiratory conditions, in veterinary care, or rituals, may not be found in other parts of the world. Therefore, further ethnobotanical studies from this region could uncover valuable new insights into the diverse roles of pot marigold in local traditions and health practices.

ACKNOWLEDGMENTS

The present study is part of the project: "Ethno-pharmacological study of the region of Southeastern Serbia", O-02-17, supported by the Serbian Academy of Sciences and Arts, as well as within the Agreement on the implementation and financing of scientific research work of scientific research organizations in 2024, financed by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (No. 451-03-66/2024-03/200027, 451-03-66/2024-03/200003).

This research was also supported by the Minister of Science of the Republic of Poland under the Program "Regional Initiative of Excellence," Contract No. RID/SP0010/2024/1, concluded between The State Treasury, The Minister of Science and the University of Rzeszów.

CONFLICT OF INTEREST

The authors declare that they have no financial and commercial conflicts of interest.

REFERENCES

Abdelwahab, S. I., Taha, M. M. E., Taha, S. M. E., and Alsayegh, A. A. (2022): Fifty-year of Global Research in *Calendula Officinalis* L. (1971–2021): A Bibliometric Study, *Clinical Complementary Medicine and Pharmacology*, **2**(4), 100059. https://doi.org/10.1016/j.ccmp.2022.100059

Ashwlayan, V. D., Kumar, A., Verma, M., Garg, V. K., and Gupta, S. (2018): Therapeutic Potential of *Calendula officinalis*, *Pharmacy & Pharmacology International Journal*, **6**(2). https://doi.org/10.15406/ppij.2018.06.00171

Bedi, M. K., and Shenefelt, P. D. (2002): Herbal Therapy in Dermatology, *Archives of Dermatology*, **138**(2). https://doi.org/10.1001/archderm.138.2.232

Bernatoniene, J., Masteikova, R., Davalgiene, J., Peciura, R., Gauryliene, R., Bernatoniene, R., Majiene, D., Lazauskas, R., Civinskiene, G., Velziene, S., Muselik, J., and Chalupova, Z. (2011): Topical application of *Calendula officinalis* (L.): Formulation and evaluation of hydrophilic cream with antioxidant activity, *Journal of Medicinal Plants Research*, **5**(6), 868–877.

Bertges, L. C., Gonçalves Felga, Â. M., Piccinini Teixeira, J. B., Magalhães Girardin Pimentel, C. F., and Neves, P. O. (2006): Effect of *Calendula officinalis* infusion on indomethacin-induced gastric lesions in Wistar rats, *Revista Cubana de Plantas Medicinales*, retrieved from internet: http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S1028-47962006000200007, **11**(2).

Cenk Paşa (2022): Compilation on the medicinal uses *Calendula officinalis* and *Calendula arvensis* species distributed in the flora of Turkey, *GSC Biological and Pharmaceutical Sciences*, **21**(3), 048–052. https://doi.org/10.30574/gscbps.2022.21.3.0451

Cruceriu, D., Diaconeasa, Z., Socaci, S., Socaciu, C., Rakosy-Tican, E., and Balacescu, O. (2020): Biochemical profile, selective cytotoxicity and molecular effects of *Calendula officinalis* extracts on breast cancer cell lines, *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, **48**(1), 24–39. https://doi.org/10.15835/nbha48111778

Dajić Stevanović, Z., Petrović, M., and Aćić, S. (2014): Ethnobotanical Knowledge and Traditional Use of Plants in Serbia in Relation to Sustainable Rural Development, 229–252 *in* A. Pieroni and C. L. Quave, eds., *Ethnobotany and Biocultural Diversities in the Balkans*, Springer New York, New York, NY. https://doi.org/10.1007/978-1-4939-1492-0_12

Davidović, V., Joksimović-Todorović, M., Maksimović, Z., Hristov, S., Stanković, B., and Relić, R. (2011): A review of plants used in ethnoveterinary medicine, *Macedonian Journal of Animal Science*, **1**(2), 377–382. https://doi.org/10.54865/mjas112377d

Davidovic, V., Todorovic, J., Stojanovic, B., and Relic, R. (2012): Plant usage in protecting the farm animal health, *Biotechnology in Animal Husbandry*, **28**(1), 87–98. https://doi.org/10.2298/BAH1201087D

Đelić, G., Simović, G., Stanković, M., Zlatić, N., Todorović, M., and Pavlović, M. (2021): Traditional use of plants in Kuršumlija, *Etnobotanika*, **1**(1), 33–55. https://doi.org/10.46793/EtnBot21.33DJ

Dhingra, G., Dhakad, P., and Tanwar, S. (2022): Review on phytochemical constituents and pharmacological activities of plant *Calendula officinalis* Linn, *Biological Sciences*, **02**(02). https://doi.org/10.55006/biolsciences.2022.2205

Dilucia, F., Rutigliano, M., Libutti, A., Quinto, M., Spadaccino, G., Liberatore, M. T., Lauriola, M., Di Luccia, A., and La Gatta, B. (2023): Effect of a Novel Pretreatment Before Freeze-Drying Process on the Antioxidant Activity and Polyphenol Content of *Malva sylvestris* L., *Calendula officinalis* L., and *Asparagus officinalis* L. Infusions, *Food and Bioprocess Technology*, **16**(10), 2113–2125. https://doi.org/10.1007/s11947-023-03035-y

Dinda, M., Dasgupta, U., Singh, N., Bhattacharyya, D., and Karmakar, P. (2015): PI3K-Mediated Proliferation of Fibroblasts by *Calendula officinalis* Tincture: Implication in Wound Healing, *Phytotherapy Research*, **29**(4), 607–616. https://doi.org/10.1002/ptr.5293

Džamić, A. M., and Matejić, J. S. (2017): Aromatic Plants from Western Balkans: A Potential Source of Bioactive Natural Compounds *in* H. A. El-Shemy, ed., *Active Ingredients from Aromatic and Medicinal Plants*, InTech. https://doi.org/10.5772/67039

Ercetin, T., Senol, F. S., Erdogan Orhan, I., and Toker, G. (2012): Comparative assessment of antioxidant and cholinesterase inhibitory properties of the marigold extracts from *Calendula arvensis* L. and *Calendula officinalis* L., *Industrial Crops and Products*, **36**(1), 203–208. https://doi.org/10.1016/j.indcrop.2011.09.007

Filipović, V., and Ugrenović, V. (2015): Pot marigold-one of the more demanded medicinal plants, J. Subić, B. Kuzman, and A. J. Vasile, eds., *Thematic proceedings, International Scientific Conference „Sustainable agriculture and rural development in terms of the Republic of Serbia strategic goals realization within the Danube region" - Regional specificitie*, 296–313.

Fronza, M., Heinzmann, B., Hamburger, M., Laufer, S., and Merfort, I. (2009): Determination of the wound healing effect of Calendula extracts using the scratch assay with 3T3 fibroblasts, *Journal of Ethnopharmacology*, **126**(3), 463–467. https://doi.org/10.1016/j.jep.2009.09.014

Givol, O., Kornhaber, R., Visentin, D., Cleary, M., Haik, J., and Harats, M. (2019): A systematic review of *Calendula officinalis* extract for wound healing, *Wound Repair and Regeneration*, **27**(5), 548–561. https://doi.org/10.1111/wrr.12737

Gostuški, R. (1973): *Lečenje lekovitim biljem*, Narodna knjiga, Beograd.
Hanlidou, E., Karousou, R., Kleftoyanni, V., and Kokkini, S. (2004): The herbal market of Thessaloniki (N Greece) and its relation to the ethnobotanical tradition, *Journal of Ethnopharmacology*, **91**(2–3), 281–299. https://doi.org/10.1016/j.jep.2004.01.007
Ingersoll, K. (2015): Diseases of the gastrointestinal tract: The pharmacy professional's role in treatment management, *Cancer*, **4**(7), 23–27.
Janaćković, P., Gavrilović, M., Miletić, M., Radulović, M., Kolašinac, S., and Stevanović, Z. D. (2022): Small regions as key sources of traditional knowledge: a quantitative ethnobotanical survey in the central Balkans, *Journal of Ethnobiology and Ethnomedicine*, **18**(1), 70. https://doi.org/10.1186/s13002-022-00566-0
Jarić, S., Kostić, O., Miletić, Z., Marković, M., Sekulić, D., Mitrović, M., and Pavlović, P. (2024): Ethnobotanical and ethnomedicinal research into medicinal plants in the Mt Stara Planina region (southeastern Serbia, Western Balkans), *Journal of Ethnobiology and Ethnomedicine*, **20**(1), 7. https://doi.org/10.1186/s13002-024-00647-2
Jarić, S., Mačukanović-Jocić, M., Djurdjević, L., Mitrović, M., Kostić, O., Karadžić, B., and Pavlović, P. (2015): An ethnobotanical survey of traditionally used plants on Suva planina mountain (southeastern Serbia), *Journal of Ethnopharmacology*, **175**, 93–108. https://doi.org/10.1016/j.jep.2015.09.002
Jarić, S., Popović, Z., Mačukanović-Jocić, M., Djurdjević, L., Mijatović, M., Karadžić, B., Mitrović, M., and Pavlović, P. (2007): An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia), *Journal of Ethnopharmacology*, **111**(1), 160–175. https://doi.org/10.1016/j.jep.2006.11.007
Jasoria, Y., Agrawal, M., Kumar, S., Chaudhary, H., Sahu, K. K., Singhal, M., Arora, S., Chandolia, P., Saha, S., Singh, K., Mahour, S., Akram, W., and Jain, D. (2024): In-vivo evaluation of neuroprotective effect of Chinese plant calendula officinalis Linn. Flower Extract against Aluminium chloride-induced Alzheimer's in Wistar rats, *Pharmacological Research - Modern Chinese Medicine*, **12**, 100458. https://doi.org/10.1016/j.prmcm.2024.100458
Kemper, K. J. (1999): Calendula (Calendula officinalis), *Longwood Herbal Task Force*, **1**.
Koleva, V., Dragoeva, A., Nanova, Z., Koynova, T., and Dashev, G. (2015): An ethnobotanical study on current status of some medicinal plants used in Bulgaria, *International Journal of Current Microbiology and Applied Sciences*, **4**(4), 297–305.
Leach, M. J. (2008): Calendula officinalis and wound healing: a systematic review, *Wounds: A Compendium of Clinical Research and Practice*, **20**(8), 236–243.
Łuczaj, Ł., Jug-Dujaković, M., Dolina, K., Jeričević, M., and Vitasović-Kosić, I. (2021): Insular Pharmacopoeias: Ethnobotanical Characteristics of Medicinal Plants Used on the Adriatic Islands, *Frontiers in Pharmacology*, **12**, 623070. https://doi.org/10.3389/fphar.2021.623070
Lumpert, M., and Kreft, S. (2017): Folk use of medicinal plants in Karst and Gorjanci, Slovenia, *Journal of Ethnobiology and Ethnomedicine*, **13**(1), 16. https://doi.org/10.1186/s13002-017-0144-0
Marković, M., Nikolić, B., Rakonjac, L., and Jovanović, S. (2024): *Ethnobotany*, Institute of Forestry, Belgrade.
Marković, M., Pljevljakušić, D., Matejić, J., Nikolić, B., Smiljić, M., Đelić, G., Papović, O., Đokić, M., and Stankov-Jovanović, V. (2022): The plants traditionally used for the treatment of respiratory infections in the Balkan Peninsula (Southeast Europe), *Lekovite Sirovine*, (42), 68–88. https://doi.org/10.5937/leksir2242068M
Marković, M., Pljevljakušić, D., Matejić, J., Nikolić, B., Zlatković, B., Rakonjac, L. B., Djokić, M. M., Papović, O. M., and Stankov Jovanović, V. P. (2024): Traditional uses of medicinal plants in Pirot District (southeastern Serbia), *Genetic Resources and Crop Evolution*, **71**(3), 1201–1220. https://doi.org/10.1007/s10722-023-01685-7
Marković, M. S., Pljevljakušić, D. S., Nikolić, B. M., Miladinović, D. L., Djokić, M. M., Rakonjac, L. B., and Stankov Jovanović, V. P. (2021): Ethnoveterinary knowledge in Pirot County (Serbia), *South African Journal of Botany*, **137**, 278–289. https://doi.org/10.1016/j.sajb.2020.10.025
Matejić, J. S., Stefanović, N., Ivković, M., Živanović, N., Marin, P. D., and Džamić, A. M. (2020): Traditional uses of autochthonous medicinal and ritual plants and other remedies for health in Eastern and South-Eastern Serbia, *Journal of Ethnopharmacology*, **261**, 113186. https://doi.org/10.1016/j.jep.2020.113186
Matić, I. Z., Juranić, Z., Šavikin, K., Zdunić, G., Nađvinski, N., and Gođevac, D. (2013): Chamomile and Marigold Tea: Chemical Characterization and Evaluation of Anticancer Activity, *Phytotherapy Research*, **27**(6), 852–858. https://doi.org/10.1002/ptr.4807
Müller-Löbnitz, C., and Göthel, D. (2011): Review of the clinical efficacy of the multicomponent combination medication Traumeel and its components, *Alternative Therapies in Health & Medicine*, S18.
Mustafa, B., Hajdari, A., Pieroni, A., Pulaj, B., Koro, X., and Quave, C. L. (2015): A cross-cultural comparison of folk plant uses among Albanians, Bosniaks, Gorani and Turks living in south Kosovo, *Journal of Ethnobiology and Ethnomedicine*, **11**(1), 39. https://doi.org/10.1186/s13002-015-0023-5
Mustafa, B., Hajdari, A., Pulaj, B., Quave, C. L., and Pieroni, A. (2020): Medical and food ethnobotany among Albanians and Serbs living in the Shtërpcë/Štrpce area, South Kosovo, *Journal of Herbal Medicine*, **22**, 100344. https://doi.org/10.1016/j.hermed.2020.100344
Nedelcheva, A., and Draganov, S. (2014): Bulgarian Medical Ethnobotany: The Power of Plants in Pragmatic and Poetic Frames, 45–65 *in* A. Pieroni and C. L. Quave, eds., *Ethnobotany and Biocultural Diversities in the Balkans*, Springer New York, New York, NY. https://doi.org/10.1007/978-1-4939-1492-0_4
Patil, K., Sanjay, C., Doggalli, N., Devi, K. R., and Harshitha, N. (2022): A Review of Calendula OfficinalisMagic in Science, *Journal of Clinical and Diagnostic Research*. https://doi.org/10.7860/JCDR/2022/52195.16024
Pavlović, M. M., and Marković, M. S. (2024): Plants in natural cosmetics, *Etnobotanika*, **4**, 101–118. https://doi.org/10.46793/EtnBot24.101P
Pieroni, A., Giusti, M. E., and Quave, C. L. (2011): Cross-Cultural Ethnobiology in the Western Balkans: Medical Ethnobotany and Ethnozoology Among Albanians and Serbs in the Pešter Plateau, Sandžak, South-Western Serbia, *Human Ecology*, **39**(3), 333–349. https://doi.org/10.1007/s10745-011-9401-3
Popović, Z., Smiljanić, M., Matić, R., Kostić, M., and Bojović, N. P. (2012): Phytotherapeutical plants from the Deliblato Sands (Serbia): Traditional pharmacopoeia and implications for conservation, *Indian Journal of Traditional Knowledge*, **11**(3), 385–400.
Prabhu Venkatesh, D., S, G., Ramani, P., S, R., and Ramalingam, K. (2023): *In Vitro* Evaluation of Antioxidant and Anti-inflammatory Potentials of Herbal Formulation Containing Marigold Flower (*Calendula officinalis* L.) Tea, *Cureus*. https://doi.org/10.7759/cureus.43308
Ramos, A., Edreira, A., Vizoso, A., Betancourt, J., López, M., and Décalo, M. (1998): Genotoxicity of an extract of Calendula officinalis L., *Journal of Ethnopharmacology*, **61**(1), 49–55. https://doi.org/10.1016/S0378-8741(98)00017-8
Sarić, M. (Ed.) (1989): *Lekovite biljke SR srbije*, Srpska akademija nauka i umetnosti, Odeljenje prirodno-matematičkih nauka, Beograd.
Šarić-Kundalić, B., Dobeš, C., Klatte-Asselmeyer, V., and Saukel, J. (2010): Ethnobotanical study on medicinal use of wild and cultivated plants in middle, south and west Bosnia and Herzegovina, *Journal of Ethnopharmacology*, **131**(1), 33–55. https://doi.org/10.1016/j.jep.2010.05.061
Saric-Kundalic, B., Mazic, M., Djerzic, S., and Kerleta-Tuzovic, V. (2016): Ethnobotanical study on medicinal use of wild and cultivated plants on Konjuh Mountain, North-East Bosnia and Herzegovina, *Technics Technologies Education Management*, **11**, 208–222.
Šavikin, K., Zdunić, G., Menković, N., Živković, J., Ćujić, N., Tereščenko, M., and Bigović, D. (2013): Ethnobotanical study on traditional use of medicinal plants in South-Western Serbia, Zlatibor district, *Journal of Ethnopharmacology*, **146**(3), 803–810. https://doi.org/10.1016/j.jep.2013.02.006
Schultz, V., Hänsel, R., and Tyler, E. V. (2001): *Rational phytotherapy: a physician's guide to herbal medicine* (4th ed.), Springer-Werlag Berlin, 1–264.
Sharrif Moghaddasi, M. (2012): Pot marigold (*Calendula officinalis*) medicinal usage and cultivation, *Scientific Research and Essays*, **7**(14). https://doi.org/10.5897/SRE11.630
Silva, E. J. R., Gonçalves, E. S., Aguiar, F., Evêncio, L. B., Lyra, M. M. A., Coelho, M. C. O. C., Fraga, M. D. C. C. A., and Wanderley, A. G. (2007): Toxicological studies on hydroalcohol extract of *Calendula officinalis* L., *Phytotherapy Research*, **21**(4), 332–336. https://doi.org/10.1002/ptr.2009
Simić, M. N., Joković, N. M., Matejić, J. S., Zlatković, B. K., Djokić, M. M., Stankov Jovanović, V. P., and Marković, M. S. (2024): Traditional uses of plants in human and ethnoveterinary medicine on Mt. Rujan (southeastern Serbia), *Genetic Resources and Crop Evolution*, **71**(6), 3061–3081. https://doi.org/10.1007/s10722-023-01821-3
Šubarević, N., Stevanović, O., and Petrujkić, B. (2015): Use of phytotherapy as a form of ethnoveterinary medicine in the area of Stara

planina mountain in Serbia, *Acta Medico-Historica Adriatica*, **13**(1), 75–94.

Tucakov, J. (1990): *Lečenje biljem: fitoterapija* (5th ed.), Rad, Beograd.

Ugulu, I., and Aydin, H. (2011): Research on students' traditional knowledge about medicinal plants: Case study of high schools in izmir, turkey, *Journal of Applied Pharmaceutical Science*, **1**, 43–46.

Ukiya, M., Akihisa, T., Yasukawa, K., Tokuda, H., Suzuki, T., and Kimura, Y. (2006): Anti-Inflammatory, Anti-Tumor-Promoting, and Cytotoxic Activities of Constituents of Marigold (*Calendula officinalis*) Flowers, *Journal of Natural Products*, **69**(12), 1692–1696. https://doi.org/10.1021/np068016b

Ullah, M. A., and Hamza, A. (2023): Calendula (*Calendula officinalis*) marigold as medicinal plant, *Clinic*, **1**(302), 520–2644.

Živković, J., Ilić, M., Šavikin, K., Zdunić, G., Ilić, A., and Stojković, D. (2020): Traditional Use of Medicinal Plants in South-Eastern Serbia (Pčinja District): Ethnopharmacological Investigation on the Current Status and Comparison With Half a Century Old Data, *Frontiers in Pharmacology*, **11**, 1020. https://doi.org/10.3389/fphar.2020.01020

Breathe deeply again

DIETARY SUPPLEMENT

Made from honey and

Marshmallow Thyme Cowslip

Natural support with honey and medicinal herbs

- To relieve a dry cough
- To facilitate expectoration

Altiprim® & Altiprim P®

Oral solutions for adults and children to support throat and respiratory health

moć bilja

Institute for Medicinal Plants Research
"Dr. Josif Pančić"

www.institutjosifpancic.rs