December 2023 | Volume 43 | 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"

Paeonia tenuifolia L.

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. 43 (2023)

Aims and scope: The journal Lekovite Sirovine (transl. Natural Medicinal Materials) is dedicated to all areas of Medicinal Plants research, Cultivation, Ethnopharmacology, Fitoterapia, 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 Education, Science and Technological Development of Serbia supported hard-copy printing cost of this journal.

PRODUCT INFORMATION
Printed at JP Službeni glasnik, Belgrade
Belgrade, Serbia
Printed in 50 copies
ISSN 0455-6224
December, 2023

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Research Articles

Review Articles

Liposomes as a carrier for 4–hydroxycoumarin: characterisation, stability and antioxidant potential

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Received: October 31, 2023
Accepted: November 8, 2023
Published on-line: December 29, 2023
Published: December 25, 2023

The aims of the present research were to develop and characterize 4-hydroxycoumarin-loaded liposomes *via* the determination of encapsulation efficiency, antioxidant capacity, vesicle size, polydispersity index (PDI), zeta potential, conductivity, mobility, density, surface tension, viscosity, and 60-day storage stability. The encapsulation efficiency of 4-hydroxycoumarin in liposomal particles was 96.7 ± 1.2%. The ABTS and DPPH radical scavenging capacity of the prepared liposomes was 89.76 ± 0.56% and 93.18 ± 0.23%, respectively, whereas cupric ion-reducing antioxidant capacity amounted to 0.367 ± 0.003 mmol Trolox equivalent (TE)/L. The density of liposomes with 4-hydroxycoumarin was 1.007 ± 0.002 g/cm^3, surface tension was 22.7 ± 0.2 mN/m, and viscosity was 14.3 ± 0.2 mPa·s. Vesicle size and PDI of 4-hydroxycoumarin-loaded liposomes were changed from 1286.3 ± 73.8 nm to 2077.3 ± 63.2 nm and from 0.409 ± 0.050 to 0.676 ± 0.064, respectively, during the 60-day stability study. The zeta potential of the obtained liposomes was changed from -16.73 ± 0.47 mV to -10.31 ± 0.42 mV, while mobility varied from -1.311 ± 0.036 μmcm/Vs to -0.806 ± 0.031 μmcm/Vs. The conductivity did not change during 60 days and amounted to approximately 0.020 mS/cm. Overall, due to the high encapsulation efficiency and antioxidant capacity, the obtained results qualify liposomes to be used as 4-hydroxycoumarin carriers for future examination of its biological activities and potential application in medicine and pharmaceutical products. However, future experiments should include the optimization of the liposomal composition with the aim of improving 4-hydroxycoumarin-loaded liposome stability.

Keywords: 4-hydroxycoumarin; antioxidant potential; liposomes; particle size; zeta potential

https://doi.org/10.61652/leksir2343e128J

1. INTRODUCTION

4-hydroxycoumarin, as an important compound of secondary metabolites of the plant kingdom, is a known oral anticoagulant, *i.e.*, the inhibitor of the synthesis of vitamin K-dependent coagulation factors and employed for this purpose for a long time (Anderes and Nand, 2014; Kılıç, 2022). 4-hydroxycoumarin also represents an important compound of various synthetic and natural products with several biological activities, such as anticoagulant, insecticidal, anthelminthic, anti-inflammatory, hypnotic, antifungal, phytoalexin, and HIV protease inhibition effects (Kirkiacharian et al., 2008; Kontogiorgis and Hadjipavlou-Litina, 2005; Lee et al., 2006; Li et al., 2014). These specific advantages of 4-hydroxycoumarin have stimulated its considerable interest in the scientific field, and different 4-hydroxycoumarin derivatives have been synthesized (Kontogiorgis and Hadjipavlou-Litina, 2005; Lee et al., 2006; Li et al., 2014). However, since 4-hydroxycoumarin and its derivatives are soluble in organic solvents and practically insoluble in water, their bioavailability is very low. Additionally, the mentioned compounds are particularly susceptible to interactions with other drugs that can compete with them for plasma protein binding, altering their metabolism in the liver and excretion through the kidneys, resulting in the inhibition or stimulation of the synthesis of the clotting factors that can cause the complications of the diseases (Polifka and Habermann, 2015). With the aim to overcome the mentioned disadvantages, providing controlled release of the bioactive

compounds, as well as increasing their effective concentration in the target place, 4-hydroxycoumarin and its derivatives can be encapsulated into numerous carriers.

Liposomal particles are widely employed as carriers for delivering bioactive and non-active compounds, such as proteins, enzymes, polyphenols, vitamins, aromas, and antioxidants in food, pharmaceutical, and cosmetic formulations (Jovanović et al., 2019; Reza Mozafari et al., 2008; Taylor et al., 2005). Liposomes, as biocompatible micro- or nano-sized lipid vesicles, are characterized by one or more phospholipid bilayers, and compared to lipid monolayers, liposomal particles show better fluidity and mobility through the native plasma membrane due to three-dimensional and spherical structure. The main advantages of liposomes, among other encapsulation procedures, are their ability to encapsulate hydrophilic, amphiphilic, and lipophilic compounds, non-toxicity, and biodegradability (Desai and Jin Park, 2005; Jovanović et al., 2019). The liposomal bilayer also provides higher bioavailability of drugs, proteins, nutraceuticals, and polyphenols (Jash et al., 2021; Lee, 2020; Shade, 2016).

Therefore, in the present study, 4-hydroxycoumarin-loaded liposomes were developed and characterized in terms of encapsulation efficiency, vesicle size, polydispersity index (PDI), zeta potential, conductivity, mobility, density, surface tension, viscosity, and 60-day storage stability, as well as antioxidant capacity.

2. MATERIALS AND METHODS

2.1. Plant material and reagents

Distilled water was purified through a Simplicity UV® water purification system (Merck Millipore, Merck KGaA, Germany). 4-hydroxycoumarin, dimethyl sulfoxide (DMSO), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, copper(II)-chloride, ammonium acetate, neocuproine, methanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or Trolox, and 2,2-diphenyl-1-picrylhydrazyl or DPPH (Sigma-Aldrich, Germany), Phospholipon 90 G (unsaturated diacyl-phosphatidylcholine) (Lipoid GmbH, Germany), ethanol (Fisher Science, UK), and potassium persulfate (Centrohem, Serbia).

2.2. Liposomal preparation

4-hydroxycoumarin-loaded liposomes were prepared using the proliposome method (Jovanović et al., 2022). Phospholipon (2 g), 4-hydroxycoumarin solution in DMSO (66.67 mg/mL, 3 mL), and 5 mL of ethanol were stirred at 50 °C. After cooling to room temperature, ultrapure water (15 mL) was added in small portions; the mixture was stirred at 800 rpm for 2 h. The samples were stored at 4 °C.

2.3. Determination of encapsulation efficiency

Encapsulation efficiency (EE) of 4-hydroxycoumarin in liposomes was determined using the indirect method. EE was calculated as shown in Equation (1):

$$\mathrm{EE}\ [\%] = (\mathrm{mi} - \mathrm{m\,sup})/\mathrm{mi}\ \times 100 \qquad \textbf{(1)}$$

where m_i is the initial content of 4-hydroxycoumarin in DMSO solution (66.67 mg/mL) used for the preparation of liposomes, and m_{sup} is the content of 4-hydroxycoumarin determined in the supernatant using direct UV-Vis spectroscopy. Non-encapsulated 4-hydroxycoumarin was removed from liposome dispersions by centrifugation at 17,500 rpm and 4 °C for 45 min in Thermo Scientific Sorval WX Ultra series ultracentrifuge (ThermoScientific, USA).

2.4. Antioxidant potential of 4-hydroxycoumarin-loaded liposomes

2.4.1. ABTS method

The ABTS was performed in the following way (Re et al., 1999): (1) ABTS solution (5 mL) and potassium persulfate solution (88 μL) were mixed and left to react for 16 h at 4 °C, (2) The obtained mixture was diluted with ethanol (an absorbance of ~0.700 at 734 nm), and (3) $ABTS^{\bullet+}$ solution (2 mL) was mixed with 20 μL of 4-hydroxycoumarin-loaded liposomes. The absorbance was measured after 6 min of incubation, and the ABTS radical scavenging potential (as a percentage of neutralization) of 4-hydroxycoumarin-loaded liposomes was calculated using the following Equation (2):

$$\%neutralization = 100 - ((100 \times Ax)/A0) \qquad \textbf{(2)}$$

where A_x was the absorbance of $ABTS^{\bullet+}$ solution and the sample (the liposomes or 4-hydroxycoumarin solution), while A_0 was the absorbance of $ABTS^{\bullet+}$ solution. The ABTS antioxidant potential of 4-hydroxycoumarin solution in DMSO (11.25 mg/mL, the same concentration as in the liposomal sample) was measured as well.

2.4.2. DPPH method

The DPPH radical scavenging potential of 4-hydroxycoumarin-loaded liposomes was determined using the DPPH antioxidant method (Batinić et al., 2022). The liposomal suspension (200 μL) was added in 2.8 mL of ethanol $DPPH^{\bullet}$ radical solution (an absorbance of ~0.800 at 517 nm). After 20 min of incubation, the absorbance was read and the percentage of the inhibition of $DPPH^{\bullet}$ radicals was calculated using the following Equation (3):

$$\%inhibition = 100 - ((100 \times Ax)/A0) \qquad \textbf{(3)}$$

where A_x was the absorbance of the $DPPH^{\bullet}$ solution and the sample (the liposomes or 4-hydroxycoumarin solution), while A_0 was the absorbance of the $DPPH^{\bullet}$ radical solution. The DPPH antioxidant capacity of 4-hydroxycoumarin solution in DMSO (11.25 mg/mL, the same concentration as in the liposomes) was also measured.

2.4.3. CUPRAC method

The cupric ion-reducing antioxidant capacity was measured according to the assay described by (Apak et al., 2009). The solution of cupric (II) ion (10^{-2} mol/mL) was prepared by dissolving 0.0853 g of the copper (II)-chloride dihydrate into 250 mL of distilled water. Ammonium-acetate buffer solution (1 mol/mL) was prepared by dissolving 19.27 g of ammonium acetate in 250 mL of distilled water. The fresh solution of neocuproine was prepared by dissolving 0.078 g of neocuproine in 50 mL of methanol (7.5×10^{-3} mol/mL). Each reaction solution consisted of 0.8 mL of 4-hydroxycoumarin-loaded liposomes, 1 mL of copper (II)-chloride solution, 1.2 mL of ammonium-acetate buffer solution, and 1 mL of neocuproine solution. The absorbance was measured at 450 nm after incubation for 30 min in the dark place. Trolox was used as a standard for the calibration curve. The results were expressed as mmol of Trolox equivalents per L (mmol TE/L). Additionally, the cupric ion-reducing antioxidant capacity of 4-hydroxycoumarin solution in DMSO (11.25 mg/mL, the same concentration as in the liposomes) was determined.

All absorbance readings are performed using the UV Spectrophotometer UV-1800 (Shimadzu, Japan). Every spectrophotometric measurement was done in triplicate.

2.5. Photon correlation spectroscopy

The size, PDI, zeta potential, conductivity, and mobility of 4-hydroxycoumarin-loaded liposomes were determined *via* photon correlation spectroscopy (PCS) in Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., UK). The sample was 500-fold diluted and measured three times at room temperature. Additionally, the stability study,*i.e.*, the measurement of the mentioned parameters was repeated on the 30thand 60th days.

2.6. Density, surface tension, and viscosity analyses

The density and surface tension of 4-hydroxycoumarin-loaded liposomes were determined using silicon crystal as the immersion body and Wilhelmy plate, respectively, in Force Tensiometer K20 (Kruss, Hamburg, Germany). Each sample (20 mL) was examined three times at 25 °C.

The viscosity of 4-hydroxycoumarin-loaded liposomes was examined using Rotavisc *lo-vi* device equipment with VOL-C-RTD chamber, VOLS-1 adapter, and spindle (IKA, Germany). Each sample (6.7 mL) was examined three times at 25 °C.

2.7. Statistical analysis

The statistical analysis was performed by using the analysis of variance (one-way ANOVA) followed by Duncan's *post hoc* test, within the statistical software STATISTICA 7.0. The differences were considered statistically significant at $p<0.05$, n=3.

3. RESULTS AND DISCUSSION

In the present research, 4-hydroxycoumarin-loaded liposomes were developed employing the proliposome technique. Encapsulation efficiency, antioxidant capacity, particle size, PDI, zeta potential, conductivity, mobility, density, surface tension, and viscosity of prepared liposomes were determined. In addition, a 60-day stability study was performed. The results of encapsulation efficiency, antioxidant capacity, density, surface tension, and viscosity are presented in Table 1, while the data from the stability study are shown graphically in Figure 1.

In order to determine the efficiency of 4-hydroxycoumarin encapsulation into phospholipid liposomes, the concentration of 4-hydroxycoumarin in supernatant was quantified using direct spectrophotometric analysis. The liposomes were able to encapsulate 4-hydroxycoumarin in a very high yield (96.7%). Our results are in agreement with the literature data (Batinić et al., 2020; Jovanović et al., 2019).

The antioxidant capacity of 4-hydroxycoumarin-loaded liposomes and 4-hydroxycoumarin solution (the same concentration as in the case of the liposomes) was measured using three antioxidant assays, ABTS, DPPH, and CUPRAC methods.

As can be seen from Table 1, the ABTS radical neutralization capacity of the liposomes was 89.76 ± 0.56%, while this value was 90.17 ± 0.10% for the solution of 4-hydroxycoumarin. Therefore, there was no statistically significant difference between the antioxidant capacity of liposomes and solution. The DPPH antioxidant potential of the liposomes and solution was 93.18 ± 0.23% and 89.82 ± 0.92%, respectively (Table 1). It can be noticed that 4-hydroxycoumarin-loaded liposomes showed significantly better antioxidant capacity in the neutralization of free DPPH radicals in comparison to the solution, probably due to the addition of the synthetic antioxidants in the commercial mixture of phospholipids employed for the liposome preparation. Additionally, there was no statistically significant difference between the cupric ion-reducing antioxidant capacity of the liposomes and solution with 4-hydroxycoumarin (0.367 ± 0.003 mmol TE/L and 0.374 ± 0.005 mmol TE/L, respectively, Table 1). The antioxidant potential of natural

Table 1. Encapsulation efficiency (EE), antioxidant capacity, density, surface tension, and viscosity of 4-hydroxycoumarin-loaded liposomes, and antioxidant capacity of 4-hydroxycoumarin solution.

Sample	EE (%)	ABTS activity (%)	DPPH activity (%)	CUPRAC (mmol TE/L)	density (g/mL)	surface tension (mN/m)	viscosity (mPa•s)
liposomes	96.7±1.2	89.76±0.56^{a}	93.18±0.23^{a}	0.367±0.003^{a}	1.007±0.002	22.7±0.2	14.3±0.2
solution	n.a.*	90.17±0.10^{a}	89.82±0.92^{b}	0.374±0.005^{a}	/	/	/

*n.a., non-applicable; TE, Trolox equivalent; CUPRAC, cupric ion-reducing antioxidant capacity; values with the same letter in each column showed no statistically significant difference ($p>0.05$; n=3; analysis of variance, Duncan's *post hoc* test).

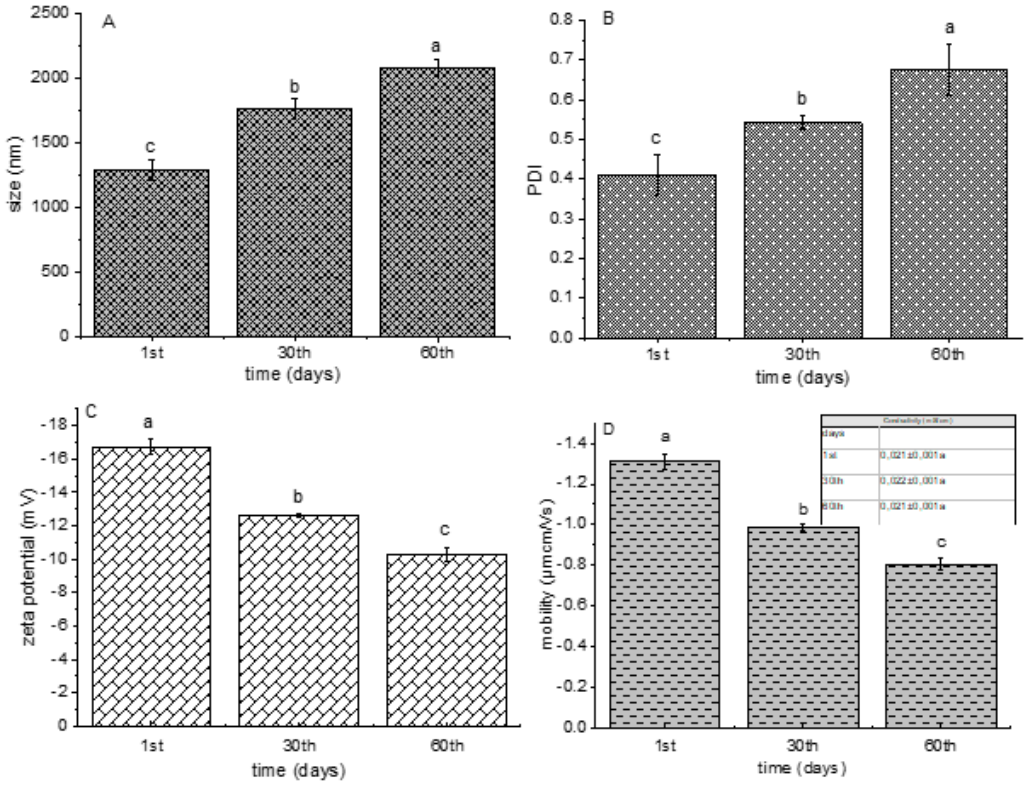

Fig. 1. Particle size (A), polydispersity index (B), zeta potential (C), and conductivity (as a table) and mobility (D) of 4–hydroxycoumarin–loaded liposomes during 60 days of storage at 4 °C; different letters above the bars showed statistically significant difference ($p < 0.05$; n=3; analysis of variance, Duncan's *post hoc* test).

coumarins and their derivatives has been the subject of intense study for at least two decades and proven in several tests (Ozalp et al., 2020; Todorov et al., 2023). The presented results showed that 4-hydroxycoumarin, although encapsulated into phospholipid liposomal particles retains its antioxidant properties.

Physical characteristics of 4-hydroxycoumarin-loaded liposomes, such as density, surface tension, and viscosity were also measured, and the results are presented in Table 1. The density was 1.007 ± 0.002 g/cm^3, surface tension was 22.7 ± 0.2 mN/m, and viscosity was 14.3 ± 0.2 mPa·s. The obtained values of the density and surface tension are in accordance with the data obtained for the liposomes with encapsulated compounds of plant origin (Jovanović et al., 2023). Additionally, (Jovanović et al., 2022) study has reported that the liposomes with plant-origin compounds showed a viscosity of approximately 14 mPa·s.

As can be seen from Figure 1A, the particle size of 4-hydroxycoumarin-loaded liposomes was 1286.3 ± 73.5 nm on the 1st day. The obtained value is in agreement with the literature data, where phospholipid liposomes with encapsulated resveratrol or silibinin (the compounds of plant origin) possessed similar diameter (Isailović et al., 2013; Maheshwari et al., 2011). According to the literature, different factors, such as lipid type, the method for the liposomal formulation, and the physicochemical properties of the encapsulated components significantly influenced the vesicle size of the liposomes (Isailović et al., 2013; Jovanović et al., 2019). The measurements of the vesicle size were repeated on the 30th and 60th days and it can be concluded that there were significant changes in the diameter (1758.0 ± 77.6 nm and 2077.3 ± 63.2 nm, respectively, Figure 1A). The increase in the size was expected since the sample showed a relatively low value of zeta potential, particularly after the 30th and 60th days (described below, Figure 1C).

PDI values, as a measure of the particle size distribution in the suspension, were determined in 4-hydroxycoumarin-loaded liposomes suspension on the 1st, 30th, and 60th days as well, and the data are shown in Figure 1B. PDI value was 0.409 ± 0.050 on the 1st day indicating the existence of a moderately dispersed distribution (Ardani et al., 2017). The method employed for the preparation of the liposomes significantly influences the uniformity of the liposomal population (Isailović et al., 2013; Jovanović et al., 2019). PDI values significantly increased during the 60-day storage study, as in the case of liposome diameter (0.542 ± 0.019 on the 30th day and 0.676 ± 0.064 on the 60th day, Figure 1B).

The zeta potential, as a measure of the stability of 4-hydroxycoumarin-loaded liposomes, was monitored during 60 days of storage at 4 °C and the results are presented in Figure 1C. The zeta potential was -16.73 ± 0.47 mV on the 1st day, which indicates the presence of a moderately stable liposomal system. Phosphatidylcholines, as neutral lipids in the water surrounding, can be reoriented causing the presence of a surface charge, *i.e.*, zeta potential. Namely, the negative value of zeta potential is related to the exposure of the phosphate group lying in an outer plane concerning the choline groups (Jovanović et al., 2019). However, after the 30th and 60th days, a statistically significant drop can be noticed in the absolute values of zeta potential (-12.63 ± 0.06 mV and -10.31 ± 0.42 mV, respectively, Figure 1C). Therefore, the results of zeta potential prove that liposomal vesicles with 4-hydroxycoumarin were not stable during the 60 days of storage at 4 °C. The absence of stability was also confirmed by the significant changes in the particle size and PDI values (Figures 1A and 1B), *i.e.*, fusion or fission of the liposomes probably occurred.

The conductivity of the liposomal suspension is affected by the exposed charge of the phospholipid components and correlates to a volume of liposome entrapment. The measured conductivity of 4-hydroxycoumarin-loaded liposomes was

0.021 ± 0.001 mS/cm on the 1st day (table in Figure 1D), as in the case of plain phospholipid liposomes described in a previous study (Jovanović et al., 2022). Considering that there were no changes in the conductivity values of the liposomes with 4-hydroxycoumarin during the 60 days of storage, it can be concluded that there was no leakage of the encapsulated compound from the liposomal vesicles. Hence, the increase in conductivity values during the storage of liposomes is usually related to the leakage of the encapsulated components. The mobility of liposomal particles represents a function of size, zeta potential, and lipid composition (Duffy et al., 2001). The mentioned parameter was measured on the 1st, 30th, and 60th days and the results are shown in the graph of Figure 1D. The mobility significantly decreased during storage (from -1.31 ± 0.03 μmcm/Vs and -0.806 ± 0.03 μmcm/Vs), as in the case of zeta potential. The decrease in mobility can be explained by the increase in vesicle size.

4. CONCLUSION

In the present research, 4-hydroxycoumarin-loaded liposomes were developed and characterized in terms of encapsulation efficiency, antioxidant potential, particle size, PDI, zeta potential, conductivity, mobility, density, surface tension, viscosity, and storage stability. The liposomal population showed a very high level of encapsulation capacity, as well as the ABTS and DPPH radical scavenging and cupric ion-reduction antioxidant potential. The values of the density, surface tension, and viscosity meet the criteria for the product that can be further used. However, size, PDI, zeta potential, and mobility significantly changed during the 60-day storage study indicating the existence of an unstable liposomal system. On the other hand, the conductivity of the liposomes with 4-hydroxycoumarin did not vary, thus, it can be concluded that there was no leakage of the encapsulated compound. Therefore, future research should include the optimization of the lipid composition with the aim of improving 4-hydroxycoumarin-loaded liposome stability and investigation of other biological effects *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

The authors acknowledge their gratitude to the Ministry of Science, Technological Development, and Innovation of Serbia, contract numbers 451-03-47/2023-01/200019, 451-03-47/2023-01/200287, 451-03-47/2023-01/200378, 451-03-47/2023-01/200252 and 451-03-47/2023-01/200135.

CONFLICT OF INTEREST

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

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Microwawe–assisted extraction of antioxidant compounds from *Vaccinium Myrtillus* leaves

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Received: November 9, 2023
Accepted: November 19, 2023
Published on-line: December 10, 2023
Published: December 25, 2023

***Vaccinium myrtillus* L. (bilberry, Ericaceae), a perennial, wild, and small deciduous shrub that grows in the mountains and forests of Europe. The leaf extracts are widely used in traditional medicine due to their astringent, antiseptic, antioxidant, anti-inflammatory, hypolipidemic, and hypoglycemic activities. Microwave-assisted extraction provides various benefits, including reducing solvent consumption and extraction time and increasing extraction yield. In the present study, bilberry extracts were prepared using dried leaves, pure ethanol, or an ethanol-water mixture, and different temperatures in a microwave reactor (60, 100, and 160 °C). The extracts were examined in terms of total polyphenol content (TPC) and antioxidant activity. The TPC of the 96% ethanol extracts rose with the increase of the extraction temperature; the extract obtained at 60 °C (37.2 ± 0.5 mg gallic acid equivalents (GAE)/g of plant material)<the extract at 100 °C (46.6 ± 0.3 GAE/g)<the extract at 160 °C (55.1 ± 0.5 mg/g). The TPC of the 50% ethanol extract prepared at 60 °C was significantly lower (54.9 ± 1.0 mg GAE/g) in comparison to the extracts obtained at 100 and 160 °C (58.3 ± 1.0 and 58.0 ± 1.5 mg GAE/g, respectively). ABTS antioxidant capacity was higher in the 50% ethanol extracts compared to 96% ethanol parallels, while in the DPPH assay, there was no statistically significant difference between the 50 and 96% ethanol extracts. Due to higher TPC and anti-ABTS activity and reduced consumption of organic solvent, a 50% ethanol extract of *V. myrtillus* was favored, while the optimal temperature was 60 °C for the extract with the highest antioxidant capacity and 100 °C for the extract with the highest amount of polyphenols.**

Keywords: Antioxidant activity; microwave-assisted extraction; polyphenols; *Vaccinium myrtillus* leaves

https://doi.org/10.61652/leksir2343e135E

1. INTRODUCTION

Vaccinium myrtillus L. (bilberry) represents a perennial, wild, and small deciduous shrub growing in the mountains and forests of Europe and belongs to the family of Ericaceae. The plant possesses significant economic importance due to the use of its fruit and leaf in numerous food, functional food, pharmaceutical, cosmetic, and health-care products (Vrancheva et al., 2021). Bilberry contains anthocyanins, phenolic acids, fatty acids, stilbenes, iridoid glycosides, dietary fibers, vitamins, and minerals (Jensen et al., 2002; Riihinen et al., 2008). The extracts prepared using bilberry leaves showed astringent, antioxidant, antibacterial, anti-inflammatory, hypolipidemic, and hypoglycemic effects (Jensen et al., 2002; Riihinen et al., 2008; Vrancheva et al., 2021).

In the present study, a microwave-assisted procedure was employed for the extraction of polyphenols from *V. myrtillus* leaves, as an innovative method for extracting valuable components from various plant materials, due to its higher extraction and polyphenol yields, shorter extraction time, lower amount of extraction medium, higher selectivity, and better quality of plant extracts, compared to traditional technologies (Ballard et al., 2010; Jovanović et al., 2021; Zhang et al., 2011).

The aims of the study were the preparation of *V. myrtillus* leaf extracts using different extraction mediums and temperatures in the microwave reactor, and the chemical characterization of

the extracts *via* determination of the total polyphenol content (TPC), as well as measurement of their radical scavenging potential.

2. MATERIALS AND METHODS

2.1. Plant material and reagents

V. myrtillus leaves were purchased from the Institute for Medicinal Plants Research "Dr Josif Pančić", Pančevo, Serbia. The plant material was then ground into a very fine powder using a non-metallic electric grinder (particle size of ~0.3 mm). The plant material was kept in zipper storage bags in a dry and dark place until future extraction.

Distilled water was purified through a Simplicity UV® water purification system (Merck Millipore, Merck KGaA, Germany). Ethanol was obtained from Fisher Science (UK). For chemical spectrophotometric assays and/or for investigations of antioxidant activity following chemicals were used: sodium carbonate (Fisher Science, UK), Folin-Ciocalteu reagent and gallic acid (Merck, Germany), potassium persulfate (Centrohem, Serbia), 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or Trolox, and 2,2-diphenyl-1-picrylhydrazyl or DPPH (Sigma-Aldrich, Germany).

2.2. Microwave-assisted extraction

MAE was carried out using a microwave reactor, Monowave 300 (Anton Paar, Austria). A defined amount of *V. myrtillus* leaves (0.66 g) was mixed with 20 mL of the extraction medium (50% or 96% ethanol), in a closed reactor vial, using a magnetic stirring bar at the speed of 600 rpm for 2 min. MAE was performed using three different temperatures (60, 100, and 160 °C).

All extracts were filtered through a cellulose filter paper (fine pore, 0.45 μm) and stored at 4 °C until further analyses.

2.3. Polyphenol content of the extracts

The TPC values were determined using the modified Folin-Ciocalteu procedure (Skotti et al., 2014). The extract (100 μL) was added to distilled water (5 mL) and mixed with Folin-Ciocalteu reagent (500 μL). Subsequently, a 20% sodium carbonate solution (1.5 mL) was added, and the volume was made up to 10 mL with distilled water. The mixtures were left in the dark at room temperature for 100 min. The absorbance was measured at 765 nm against a blank (all reagents except the extract). Gallic acid was used as a standard for the calibration curve. The TPC was expressed as milligrams of gallic acid equivalents per g of plant material (mg GAE/g).

2.4. Antioxidant potential of the extracts

2.4.1. ABTS assay

The ABTS assay is based on the reduction of $ABTS^{\bullet+}$ free radicals by antioxidant compounds from the sample (Re et al., 1999). A mixture of ABTS solution (5 mL) and potassium persulfate solution (88 μL) was left to react for 16 h in a refrigerator. The $ABTS^{\bullet+}$ working solution was diluted using ethanol (an absorbance of ~0.700 at 734 nm). $ABTS^{\bullet+}$ solution (2 mL) was mixed with diluted liquid extract (1:9, 20 μL). After 6 min of incubation, the absorbance was read and the ABTS radical scavenging activity of the extract was calculated using the following Equation 1:

$$A = A_0 - A_x \quad \textbf{(1)}$$

where A_0 was the absorbance of $ABTS^{\bullet+}$ solution, whereas A_x was the absorbance of $ABTS^{\bullet+}$ solution and the extract. Trolox was used as a standard for the calibration curve. The scavenging capacity was expressed as μmol Trolox equivalents per g of plant material (μmol TE/g).

2.4.2. DPPH assay

The antioxidant activity of the samples was determined *via* hydrogen donating or radical scavenging ability using the stable $DPPH^{\bullet}$ radicals (Batinić et al., 2022). Various concentrations of liquid extract (200 μL) were mixed with 2.8 mL of ethanol $DPPH^{\bullet}$ radical solution (an absorbance of ~0.800 at 517 nm). The absorbance was recorded after 20 min of incubation and the percentage of inhibition was calculated using the following Equation 2:

$$\%inhibition = (A_0 - A_x)x100/A_0 \quad \textbf{(2)}$$

where A_0 was the absorbance of the control, and A_x was the absorbance of $DPPH^{\bullet}$ solution and extract. The results were expressed as IC_{50} (mg/mL) which represented the concentration of the extract required to scavenge 50% of $DPPH^{\bullet}$ radicals.

All absorbance readings are performed using the UV Spectrophotometer UV-1800 (Shimadzu, Japan). Every spectrophotometric measurement was performed in triplicates.

2.5. Statistical analysis

The statistical analysis was performed by using the analysis of variance (one-way ANOVA) followed by Duncan's *post hoc* test, within the statistical software STATISTICA 7.0. The differences were considered statistically significant at $p<0.05$, n=3.

3. RESULTS AND DISCUSSION

In the present study, microwave extraction from *V. myrtillus* leaves was performed by investigating factors of interest: solvent type and extraction temperature. The extraction was performed using a microwave reactor (a modern procedure that requires an expensive device but provides faster kinetic). The results of polyphenol yield are presented in Table 1, while the data of ABTS and DPPH radical scavenging potential are shown in Figures 1A and 1B.

Table 1. Total polyphenol content (TPC) in *Vaccinium myrtillus* leaf extracts prepared using microwave-assisted extraction.

Solvent type	Extraction temperature (°C)	TPC (mg GAE*/g)
96% ethanol	60	37.2±0.5d
	100	46.6±0.3c
	160	55.1±0.5b
50% ethanol	60	54.9±1.0b
	100	58.3±1.0a
	160	58.0±1.5a

* GAE, gallic acid equivalents; values with the same letter showed no statistically significant difference ($p>0.05$; n=3; analysis of variance, Duncan's *post hoc* test).

As can be seen from Table 1, the polyphenol yield of the absolute ethanol *V. myrtillus* leaf extracts exponentially rose with the increase of the extraction temperature (from 37.2±0.5 mg gallic acid equivalents (GAE)/g of plant material at 60 °C to 55.1±0.5 mg GAE/g at 160 °C). The polyphenol concentration of the 50% ethanol extract prepared at 60 °C was significantly lower (54.9±1.0 mg GAE/g) compared to the samples prepared at 100 and 160 °C (58.3±1.0 and 58.0±1.5 mg GAE/g, respectively). The obtained data are in agreement with the literature, where the TPC of ethanol wild thyme extracts significantly increased with the increase of the temperature in the microwave reactor (Jovanović et al., 2022). Namely, in

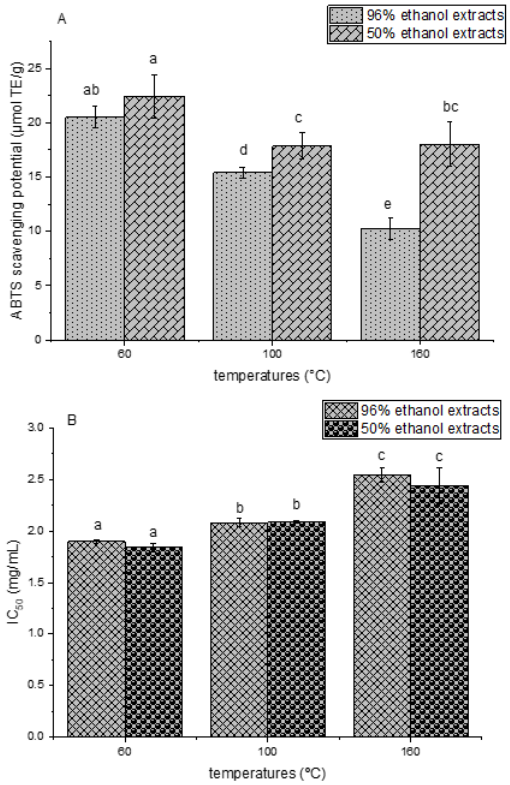

Fig. 1. (A) ABTS and (B) DPPH radical scavenging potential of *Vaccinium myrtillus* leaf extracts prepared using microwave-assisted extraction; TE, Trolox equivalents; IC_{50}, the concentration of the extract required to scavenge 50% of DPPH$^{\bullet}$ radicals; the same letter above the bars showed no statistically significant difference ($p>0.05$; n=3; analysis of variance, Duncan's *post hoc* test).

microwave-assisted extraction, there is a rapid delivery of energy to the extraction medium and plant material resulting in the efficient and homogeneous heating of the whole sample. Furthermore, water in the plant cells and vacuoles absorbs microwave energy causing interior superheating and degradation of plant cells and tissues, consequently resulting in the enhancement of the extraction and polyphenol yields (Ballard et al., 2010; Jovanović et al., 2022; Zhang et al., 2011). The temperature of microwave-assisted extraction should be investigated and optimized, as microwave irradiation can cause enzymatic degradation and oxidation of polyphenol compounds, particularly at higher temperatures (Wang and Weller, 2006). Regarding the comparison of the extracts prepared using different extraction solvents, it can be noticed that 50% ethanol extracts showed significantly higher values of the TPC at all levels than the extract with absolute ethanol. According to the literature, ethanol-water mixtures have been usually employed for the extraction of polyphenol compounds from different plant sources, because ethanol provides a decrease in the dielectric constant of the extraction medium enabling easier separation of the solvent molecules, while water allows the efficient wetting of plant solid (Costa et al., 2012; Jovanović et al., 2021; Pompeu et al., 2009). However, in 50% ethanol extracts, there was no statistically significant difference between the samples obtained at 100 and 160 °C which can be explained by the saturation of the extraction medium (Gao et al., 2007). Hence, steady state was achieved already at 100 °C in the case of 50% ethanol extracts due to better solubility of the polyphenolic compounds in ethanol-water surrounding, while in pure ethanol extract, the temperature in the microwave reactor had to be 160 °C for the highest polyphenol yield.

The ABTS and DPPH radical scavenging ability of ethanol *V. myrtillus* leaf extracts did not correlate with the polyphenol yield and significantly decreased with the increase of the extraction temperature in the case of both extraction mediums (Figure 1). It can be explained by the fact that other components, apart from polyphenols, can have an important role in the overall antioxidant capacity of the extracts (plant pigments, vitamins, free organic acids, sugars, etc.) (Petrovic et al., 2019; Özyürek et al., 2011). However, the mentioned compounds can be significantly degraded under higher temperatures. Additionally, flavonoids, as a subgroup of polyphenols, represent potent antioxidants (Carocho and Ferreira, 2013), as well as a higher sensitivity to high temperature, particularly during microwave-assisted extraction (Liazid et al., 2007). On the other hand, the Folin-Ciocalteu method is not a selective method and can quantify the content of proteins apart from the polyphenols (Sánchez-Rangel et al., 2013). However, plant proteins do not show significant radical scavenging potential that can explain the absence of the correlation between the TPC determined in the Folin-Cioacalteu assay and the antioxidant capacity of the extracts. Nevertheless, ABTS antioxidant activity was higher in the 50% ethanol samples in comparison to 96% ethanol parallels, as in the case of TPC. In the DPPH assay, there was no statistically significant difference between the 50 and 96% ethanol extracts. Probably, both solvents can extract the compounds responsible for the neutralization of free DPPH radicals.

4. CONCLUSION

The aim of the present study was the optimization of microwave-assisted extraction *via* varying the extraction solvent and temperature, measurement of polyphenol yield and ABTS and DPPH radical scavenging effects of bilberry leaf extracts. Polyphenol concentration of the extracts rose with the increase of the extraction temperature, while the highest polyphenol yield was achieved employing 50% ethanol, as an extraction medium compared to the absolute ethanol. Nevertheless, the ABTS and DPPH radical scavenging capacity of the extracts did not correlate with the polyphenol content at all levels. Namely, the antioxidant capacity decreased with the increase of the temperature in the microwave reactor, whereas 50% ethanol extracts showed higher ABTS radical scavenging capacity in comparison to 96% ethanol parallels. Therefore, the extraction temperature should be chosen depending on the future application of bilberry extracts, while due to higher polyphenol yield and anti-ABTS effect and reduced consumption of organic solvent, a 50% ethanol *V. myrtillus* extract was favored. The present research was an initial step in the preparation of bilberry extracts in microwave-assisted extraction which can be potentially implemented in food, pharmaceutical, and cosmetic formulations.

ACKNOWLEDGMENTS

The authors acknowledge their gratitude to the Ministry of Science, Technological Development, and Innovation of Serbia, contract numbers 451-03-47/2023-01/200019, 451-03-47/2023-01/200003, 451-03-47/2023-01/200026, and 451-03-47/2023-01/200135.

CONFLICT OF INTEREST

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

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Antioxidant, antibacterial and enzyme inhibitory activity of the leaf extracts of *Paeonia daurica* Andrews wild growing in Serbia

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Received: November 20, 2023
Accepted: December 2, 2023
Published on-line: December 20, 2023
Published: December 25, 2023

This study was designed to investigate the impact of different extraction methods (ultrasound-assisted extraction, maceration, and microwave-assisted extraction) on the content of polyphenols and biological activities of leaf extract of *Paeonia daurica* subsp. *daurica* collected in Južni Kučaj (Serbia). The total polyphenol and flavonoid content were achieved by the UV-Vis spectroscopy. FTIR analysis was conducted to investigate structural properties of the extracts. *In vitro* methods (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) - ABTS and 2,2-diphenyl-1-picrylhydrazyl – DPPH assays) were carried out to measure the antioxidant activity of the extracts, while their antibacterial activity was tested against three Gram-positive and three Gram-negative bacterial strains. Analyzing the results of antioxidant activity, the highest anti-DPPH potential was achieved in the extracts of LPD obtained by maceration. The extraction method proven to produce the most effective antibacterial agents is maceration. Moreover, inhibitory activity of extracts against acetyl–and butyrylcholinesterase, α-amylase, α-glucosidase, and tyrosinase was evaluated in the enzyme inhibition assays. Overall, the results of this research indicated the possible use utilization of leaf extracts of *Paeonia daurica* subsp. *daurica* Andrews in food technology and pharmacy.

Keywords: *P. daurica* subsp.*daurica* Andrews; total polyphenol content; structural analysis; biological activities

https://doi.org/10.61652/leksir2343e159B

1. INTRODUCTION

The genus *Paeonia* L. (Paeoniaceae) includes a number of herbaceous peony species, and among them, five taxa of *Paeonia* were found in Serbia (Čutović et al., 2022), including *Paeonia daurica* subsp. *daurica* Andrews. In addition to being decorative, herbaceous peonies are considered important sources of crude drugs in traditional Chinese and Persian medicine (Tosun et al., 2011). In particular, their leaves, when used as a powder or macerate, have been used in cardiovascular, cerebrovascular, brain, kidney, and uterus disorders (Tahmasebi et al., 2021).

Paeonia daurica subsp. *daurica* Andrews is a perennial plant that grows on all continents of the northern hemisphere, including the territories of countries of the Middle (Turkey, Iran) and Far East (China, Japan) (Güner et al., 2000). Its roots are thin and resemble carrots, the leaves are made up of nine leaflets, and each stalk bears on flower, supported by one or two leafy bracts. There are several subspecies which differ in the size and shape of the leaflets, the hairiness of the carpels and the leaflets, and the color of the petals (white, pale yellow, pink, and red). *P. daurica* subsp. *daurica* is found in Serbia. Despite the use of *P. daurica* in disease prevention, there are only two reports on the chemical analysis of its root extracts (Monsef-Esfahani et al., 2023; Tahmasebi et al., 2021) and none on its leaf extracts, particularly on *P. daurica* subsp. *daurica.*

The flowers and roots of some members of genus *Paeonia* proved to be source of many beneficial components, includ-

Fig. 1. Full blooming *Paeonia daurica* subsp. *daurica* Andrews, at locality Južni Kučaj (May 2022).

ing anthocyanins, flavonoids, tannins, stilbenes, triterpenoids, steroids, and alkaloids (Li et al., 2021). Even though chemical profiling of *P. daurica* is still very poor and refers only to the essential oil of its flower, leaves and stems (Tosun et al., 2011), its use in folk medicine for various disorders (epilepsy, rheumatism, cough, whooping cough, asthma, tuberculosis, gastroenteritis, colic, diarrhoea, diabetes, etc.) is well documented (Demirboğa et al., 2021; Ugulu et al., 2009).

Therefore, the aim of this study was to evaluate antioxidant, antibacterial, and anti- enzymatic activities of the leaf extract of *P. daurica* using indirect *in vitro* tests, as well as to determine total phenolic and flavonoid contents and FTIR spectra. The antioxidant activity was conducted using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays, the antibacterial activity was evaluated against three Gram-positive and three Gram-negative bacteria, while anti-enzymatic activity was performed against five enzymes associated with neurological, endocrinological, and skin diseases. Also, in the present study, ultrasound- (UAE) and microwave-assisted extractions (MAE) were used as highly selective and reproducible extraction techniques, in comparison to a conventional method such as maceration (M) known for its economic efficiency, in order to determine the most satisfactory method for polyphenol extraction from the leaves of *P. daurica.*

2. MATERIALS AND METHODS

2.1. Plant material

The leaves of *P. daurica* subsp. *daurica* Andrews (LPD – Figure 1) were collected in May 2022, from Južni Kučaj, the mountain in Eastern Serbia (630-870 m a. s. l.). The collecting of leaves was conducted with the permission of the Government of Republic of Serbia (No. 353-01-1467/2021-04, May 26, 2022). The voucher specimen (2-1107) of this plant species was deposited in the herbarium BUNS of the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Serbia, where the identify confirmation was conducted.

Prior to extraction, plant material was shade dried at 21 °C, for 21 days. Dried leaves were grounded by a universal mill (M 20, IKA®-Werke, GmbH & Co. KG, Germany), and obtained particles were separated using a sieve of 0.5 mm.

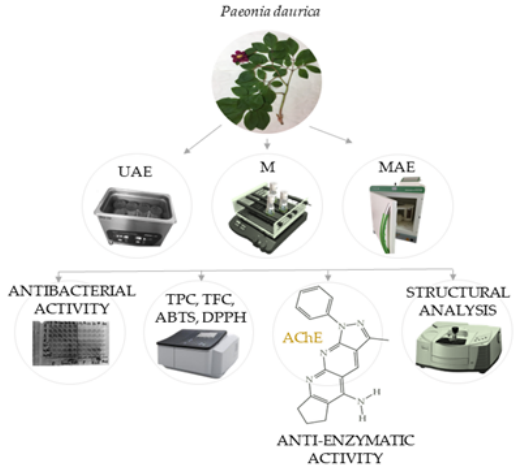

Fig. 2. The schematic view of the experiment by which *P. daurica* subsp. *daurica* Andrews leaf extracts were analyzed.

2.2. Standards and reagents

Ethyl alcohol (96%, v/v, Zorka Pharma, Serbia) and deionized water were used as solvents. Deionized water was obtained using a Simplicity® UV water purification system (Merck Millipore, Germany). Folin-Ciocalteu's phenol reagent (2N), gallic acid (97.5-102.5%), catechin monohydrate (≥98%), potassium hexacyanoferrate (III) (≥99%), aluminum chloride (III) (98%), sodium nitrate (≥99%), sodium carbonate (≥99.5%), sodium hydroxide (95%), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, 2,2-diphenyl-1-picrylhydrazyl or DPPH, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or Trolox, 5,5′-Dithiobis(2-nitrobenzoic acid), acetylcholinesterase from Electrophorus electricus (electric eel), Type-VI-S, EC 3.1.1.7, butyrylcholinesterase from equine serum, EC 3.1.1.8, acetylthiocholine iodide (AS, >99%), butyrylthiocholine chloride, kojic acid (AS, >99%), α-amylase solution (ex-porcine pancreas, EC 3.2.1.1), acarbose (>95%), *p*-iodonitrotetrazoliumviolet (>95%), α-glucosidase solution (from *Saccharomyces cerevisiae*, EC 3.2.1.20), Lugol reagents (diluted iodine-potassium iodide solution), and formic acid (98-100 %, HPLC grade), were obtained by Sigma Aldrich, Germany. Potassium persulfate (≥99%) was purchased from Centrohem, Serbia.

2.3. Preparation of the extracts

The extracts of LPD were prepared using UAE, M, and MAE; each extraction protocol is described below in the individual section. The schematic view of the experiment is presented in Figure 2.

2.3.1. Ultrasound-assisted extraction (UAE)

UAE was performed using the digital ultrasound bath (DU-32, ARGO LAB, Italy) with a frequency of 35 kHz at room temperature (25 ± 5 °C). The time of the extraction was 15 min, the concentration of ethyl alcohol was 50% while the solid-to-solvent ratio was 1:10. After extraction, the extract was filtered through the cellulose acetate filter (0.2 μm) and stored at 4 °C until further analysis.

2.3.2. Maceration (M)

Maceration was carried out on the tube roller mixer (Stuart SRT6, Germany) at room temperature (25 ± 5 °C). The duration of extraction was 15 min, solid-to-solvent ratio was 1:10, temperature of 100 °C, while the concentration of ethyl alcohol was 50%. After maceration, the extract was filtered through the cellulose acetate filter (0.2 μm) and stored at 4 °C until further analysis.

2.3.3. Microwave-assisted extraction (MAE)

MAE was carried out using a microwave device (Milestone ETHOS X, Italy), equipped with a 2.45 GHz reactor and two 2 magnetrons achieving a maximum operative power of 1.8 kW. All tests were conducted at the normal atmospheric pressure in SR-15 rotor segment containing a high-density polypropylene mold with a modified poly(tetrafluoroethylene) vessel, cover and stirrer bar. The time of extraction was 2 min, solid-to-solvent ratio was 1:10, temperature of extraction was 100 °C, while the concentration of ethyl alcohol was 50%. After extraction, the extract was filtered through the cellulose acetate filter (0.2 μm) and stored at 4 °C until further analysis.

2.4. Total polyphenol content (TPC)

TPC was determined using the modified Folin-Ciocalteu method (Čutović et al., 2022). The extract (0.02 mL) was added to deionized water (1.5 mL) and mixed with Folin-Ciocalteu phenol reagent (0.1 mL). Subsequently, sodium carbonate solution (0.3 mL, 20%, w/v) was added and mixture was made up to 2 mL with deionized water. Then, the mixture was left in the dark at room temperature for 120 min. The absorbance was read at 765 nm against blank solution (all reagents except the extract) on the UV-Vis scanning spectrophotometer (UV/Vis 1800, Shimadzu, Japan). Gallic acid (GA) was used as a standard for construction of the calibration curve. The TPC was expressed as milligrams of gallic acid equivalents per milliliter of extract (mg GAE/mL).

2.5. Total flavonoid content (TFC)

The modified aluminum chloride colorimetric method of Čutović et al. (2022) was used to estimate the TFC in extract of LPD. In short, 0.25 mL of extract and 0.75 mL of sodium nitrite (5%, w/v) were mixed with 1.25 mL of deionized water. The solution was incubated in dark for 6 min at room temperature (25 ± 5 °C). Then, the mixture was treated with 0.15 mL of aluminum chloride (10%, w/v) and 0.5 mL of sodium hydroxide (1 mol/L) before being topped off to a volume of 3 mL. The mixture was vortexed and kept in the dark for 30 min. The absorbance was read at 425 nm against blank solution (all reagents except the extract). Catechin hydrate (CA) was used as a standard for construction of the calibration curve. The TFC was expressed as milligrams of catechin hydrate equivalents per milliliter of extract (mg CAE/mL).

2.6. Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of extracts of LPD were performed to identify the present characteristic functional groups. The spectra were recorded using IR-spectrometer (Nicolet^{TM} iS^{TM}, USA) in the wavenumber range between 4000-500 cm^{-1}, and resolution of 4 cm^{-1} (25 ± 5 °C). The analysis was performed on dry extracts, previously prepared using a Rotary vacuum evaporator (Rotavapor Heidolph 4001-efficient, Heidolph Instruments, Germany) under pressure of 0.5 bar at 25 ± 5 °C. Test was performed by fixing a small amount of dry extract (20 mg) to the metal chassis of the IR-spectrometer equipped with diamond crystal. FTIR spectra were processed using OMNIC^{TM} software (Thermo Fischer Scientific, USA). The graphical view of results was generated by ORIGIN^{TM} software (Origin 9.0., OriginLab, USA).

2.7. Antioxidant activity

2.7.1. ABTS assay

The ABTS assay is based on the reduction of ABTS$^{\bullet+}$ free radicals by antioxidants from the extract. The analytical protocol was defined by Čutović et al. (2022). The ABTS$^{\bullet+}$ solution (7.8 x 10^{-3} mol/L) was prepared by dissolving 0.02 g ABTS in 5 mL of deionized water and then adding 0.088 mL of potassium persulfate (2.45 x 10^{-3} mol/L). Prior to its use, the ABTS$^{\bullet+}$ solution was stored for 16 h at 4 °C to complete the reaction and to activate the radicals. Following activation, the ABTS$^{\bullet+}$ solution was diluted by ethyl alcohol yielding an absorbance of 0.70 ± 0.02 at 734 nm. The main solution was prepared by mixing 2.8 mL of ABTS$^{\bullet+}$ solution with 0.2 mL of extract, while the blank was prepared by mixing of the same amount of mL of ABTS$^{\bullet+}$ solution with 0.2 mL of ethyl alcohol (50%, v/v). After 30 min of incubation, the absorbance was read and the radical scavenging activity (SA_{ABTS}) was calculated according to the Equation 1:

$$SA_{ABTS} = \frac{A_{co} - A_{sa}}{A_{co}} x100\,(\%) \quad \textbf{(1)}$$

where A_{co} represents the absorbance of ABTS$^{\bullet+}$ solution, while A_{sa} is the absorbance of ABTS$^{\bullet+}$ solution and the extract. Trolox was used as a standard for the calibration curve. The SA_{ABTS} was expressed as μmol Trolox equivalents per mL of extract (μmol TE/mL).

2.7.2. DPPH assay

The antioxidant activity of the extract was determined *via* hydrogen donating or radical scavenging ability using the stable DPPH$^{\bullet}$ radicals (Čutović et al., 2022). The DPPH solution was made by dissolving 0.252 mg of DPPH in 9 mL of ethyl alcohol. The main solution was prepared by mixing 2.8 mL of DPPH$^{\bullet}$ solution with 0.2 mL of extract, while the blank was ethyl alcohol. The absorbance readings were taken after 30 min against the blank at 517 nm. The scavenging radical activity (SA_{DPPH}) was calculated according the Equation 2:

$$SA_{DPPH} = \frac{A_{co} - A_{sa}}{A_{co}} x100\,(\%) \quad \textbf{(2)}$$

where A_{co} represents the absorbance of DPPH$^{\bullet}$ solution, while A_{sa} is the absorbance of DPPH$^{\bullet}$ solution and the extract. The results were expressed as the concentration of extract required to neutralize 50% of DPPH$^{\bullet}$ (IC_{50}, mg/mL).

2.8. Antibacterial activity

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of extract of LPD were determined by microdilution method. All of the extracts of LPD were dissolved in ethyl alcohol (30%, v/v) to prepare the extract stock solution with the concentration of 10 mg/mL. The MIC and MBC values were evaluated by serial sub-cultivations of 0.01 mL of extract into microtiter plates containing 0.1 mL of broth per well and further incubation for 24 h at 37 °C; the values were detected following the addition of 0.04 mL of *p*-iodonitrotetrazolium violet (0.2 mg/mL) and incubation at 37 °C for 30 min, as previously described by Nikolić et al. (2014). The lowest concentration of extract of LPD without any visible growth of microbial strains was taken as MIC value, while the MBC shows the lowest extract concentration at which the initial inoculum was killed by 99.5%. The antibacterial activity was examined against three Gram-positive (*Listeria monocytogenes* NCTC 7973, *Staphylococcus aureus* ATCC 11632, and *Bacillus cereus* human isolate) and three Gram-negative (*Salmonella* typhimurium ATC 13311, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922) bacterial strains. The bacterial strains were gained from the Collection of the Department of Plant Physiology, University of Belgrade, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. Fresh overnight cultures of all bacterial strains were adjusted with sterile saline to a concentration of 1.0 x 10^{6} CFU (colony-forming unit) per well.

2.9. Determination of enzyme inhibitory effects

For the determination of anti-enzymatic activity, all of the dry extracts of LPD were dissolved in ethyl alcohol (30%, v/v) in order to prepare the stock solution with concentration of 10 mg/mL. The inhibitory effects of extracts of LPD were investigated against cholinesterases (acetylcholinesterase and butyrylcholinesterase (associated with Parkinson's and Alzheimer's diseases)), amylase and glucosidase (both related with diabetes mellitus type 2) and tyrosinase (associated with skin melanoma).

2.9.1. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymatic assays

The AChE and BChE inhibitory activities of the extracts of LPD was accessed following instructions given by Sut et al. (2019). In short, 0.025 mL of extract of LPD (1 mg/mL) was combined with 0.125 mL of 5,5'-dithiobis (2-nitrobenzoic acid) and 0.025 mL of AChE, or BChE in tris-hydrochloride buffer solution (1 mol/L, pH 8.0) in a 96-well microplate and incubated for 30 min at 25 °C. The absorbances were read at 405 nm. Standard inhibitor for cholinesterase's was galantamine. Results were quantified by subtracting the absorbance of the control probe from that of the test solution and expressed as milligrams of galantamine equivalents per gram of extract (mg GALAE/g). A control was prepared by adding the test solution to all reagents without an enzyme.

2.9.2. Enzymatic assay of α-amylase and α- glucosidase

The α-amylase enzymatic assay was performed as carried out following the instructions given by (Sut et al., 2019). In brief, 0.05 mL of extract of LPD (1 mg/mL) was mixed with the α-amylase solution (*ex*-porcine pancreas) in phosphate buffer (6×10^{-3} mol/L) in a 96-well microplate and incubated for 10 min at 37 °C. The reaction was initiated by adding of 0.05 mL of starch (0.05%, w/v). A control was made by adding the test solution to all reagents without enzyme. Then, the mixture was thermostated for 10 min at 37 °C, after which 0.025 mL of hydrochloric acid (1 mol/L) and 0.1 mL of iodine-potassium iodide (Lugol's reagent) were added to end the process. The absorbances were read at 630 nm. Also, the absorbance of the control was subtracted from that of the extract and the α-amylase inhibitory activity was expressed as millimoles of acarbose equivalents per gram of extract (mmol ACAE/g). On the other hand, the α-glucosidase inhibitory activity was carried out as follows: 0.05 mL of the extract of LPD (10 mg/mL) was mixed with a 0.05 mL of glutathione and 0.05 mL of α-glucosidase solution in phosphate buffer (1 mol/L, pH 6.8) in 96-well microplate, and incubated for 15 min at 37 °C. A blank was prepared by adding the test solution to all reagents without adding enzyme. Then, the reaction was ended by adding 0.05 mL of disodium carbonate (0.2 mol/L). The absorbances were read at 400 nm, while the α-glucosidase inhibitory activity was expressed as millimoles of acarbose equivalents per gram of extract (mmol ACAE/g).

2.9.3. Enzymatic assay of tyrosinase

Tyrosinase inhibitory activity was conducted following the experimental protocol provided by Mocan et al. (2017). Specifically, 0.025 mL of the extract of LPD (1 mg/mL) was mixed with 0.04 mL of tyrosinase and 0.01 mL of phosphate buffer (1 mol/L, pH 6.8) in a 96-well microplate and incubated for 15 min at 25 °C. Then, the reaction was initiated with the addition of 0.04 mL of L-3,4-dihydroxyphenylalanine. A control was prepared by adding the test solution to all reagents without enzyme. The absorbances of the sample and control were recorded at 492 nm after 10 min of incubation at 25 °C. Tyrosinase inhibitory activity was calculated by subtracting the absorbance of the control from that of the test solution and expressed as milligrams of kojic acid equivalents per gram of extract (mg KAE/g).

Table 1. Total polyphenol (TPC) and total flavonoid contents (TFC) of the leaf extracts of *P. daurica* subsp. *daurica* Andrews (LPD) collected from Južni Kučaj.

Extraction method	TPC [mg GAE/mL]	TFC [mg CAE/mL]
UAE	1968.88 ± 112.34 [b]	1062.67 ± 87.76 [a]
M	2151.38 ± 134.11 [a]	1031.00 ± 69.99 [a]
MAE	2141.38 ± 105.97 [a]	1096.00 ± 98.97 [a]

GAE: gallic acid equivalent; CAE: catechin equivalent; UAE: ultrasound-assisted extraction; M: maceration; MAE: microwave-assisted extraction; values with the same letter in each showed no statistically significant difference ($p > 0.05$, n = 3, one-way ANOVA, analysis of variance, Duncan's *post hoc* test).

2.10. Statistical analysis

The statistical analysis was conducted by using the analysis of variance (one-way ANOVA) followed by Duncan's *post hoc* test, within the statistical software STATISTICA 7.0. The differences were considered statistically significant at $p < 0.05$, n=3.

3. RESULTS AND DISCUSSION

The extracts of LPD, obtained by UAE, M, and MAE were analyzed via TPC, TFC, ABTS- and DPPH radical scavenging activity. The TPC and TFC, with GA and CA as the referent standards, are shown in Table 1, while the results of ABTS and DPPH radical scavenging activities are shown in Table 2. All extracts were analyzed in term of structural properties (FTIR), as well as the antibacterial and anti-enzymatic activities.

3.1. Total polyphenol and flavonoid contents of the extracts

TPC was significantly higher in the extracts obtained by M and MAE compared with those obtained by UAE, while the results obtained by M and MAE were similar to each other (Table 1). Although the literature showed the advantages of UAE, including high extraction yield, quality of extract, better kinetics, lower price, simplicity, and employment of a wide range of mediums (Lee et al., 2013), the results from this research suggest that the UAE method (performed in an ultrasound bath) provided lower values of TPC compared to the M and MAE procedures. The potential explanation could be that the excessive amount of plant material (a ratio of 1:10) causes the drastic increase in viscosity and inhibits the expansion of ultrasound waves, as well as the diffusion of polyphenol compounds (Vuleta et al., 2012). The results of TPC are in accordance with the previously published papers, where higher temperatures (implemented during MAE) had a strong influence on the polyphenols extractability in water-ethyl alcohol extracts of medicinal plants (Dent et al., 2013; Miron et al., 2011). This phenomenon could be explained by the fact that high temperature during MAE decreases the viscosity of the solvent, assisting its penetration through the plant tissue which causes the more intensive dissolution of bioactive molecules and accelerates mass transfer. In addition, some reports suggest that increment of solvent temperature (which is the case during MAE) could decrease the surface tension and consequently enhance the wetting and swelling of plant material resulting in more efficient extraction (Vergara-Salinas et al., 2012). Moreover, some findings suggest that temperatures were the dominant factor in maximizing the values of TPC in numerous plant extracts (Hossain et al., 2011). The results of TFC show different trend. Namely, the highest TFC was observed in extracts of LPD obtained by MAE, followed by UAE and M, but differences were not significant.

These results are in agreement with those published earlier by other researchers (Jovanović et al., 2022; Nayak et al., 2015). In fact, the high efficiency of MAE is based on the following phenomenon - that rapid heating of the solid content and solvent creates a high vapor pressure of free aqueous molecules in the plant material, which disrupts the cell wall and accelerates the release of the content into the surrounding extraction medium. On the other hand, during UAE, solid and liquid phases were vibrated and accelerated, the intramolecular forces are not able to hold the structure of molecule in the intact state, so the formed bubbles can produce mechanical effect which results in cell disruption of biological membranes of plant tissue (Batinić et al., 2022). However, M also provided a higher flavonoid yield confirming that flavonoids are thermosensitive compounds that can be degraded by microwaves and ultrasound waves.

3.2. FTIR study

The FTIR analysis was used to identify the presence of specific functional groups of LPD obtained by the UAE, M, and MAE methods. The FTIR spectrum of extracts of LPD is shown in the Figure 3. As can be seen from the adsorbent spectrum, the modest adsorption at 3330 cm^{-1} occurs as a result of the O-H stretching from both hydroxyl and phenolic groups (Lee et al., 2009). The adsorption at 2966 and 2764 cm^{-1} is due to the C–H stretching vibrations originating from $=CH_2/R-O-CH_3/-CH_3$ groups (Lee et al., 2009; Oancea et al., 2021). A band at 1737 cm^{-1} indicates the presence of C=O vibration originating from carboxylic and ester functionalities (Lazzari et al., 2018). The broad band at 1640 cm^{-1} is mainly attributed to the C–H bending vibrations of the aromatic structure (Geng et al., 2016). A peak around 1480 cm^{-1} originated from the C-H stretching vibration and O−C−H *in plane* bending and has been associated with the phenyl core of phenolic acids. The band at 1360 cm^{-1} originated from the O−H bending modes in gallic acid derivatives (Oancea et al., 2021). Vibration at 1230 cm^{-1} is attributed to the C−O stretching modes of aromatic alcohols, while the strong absorption at 1064 cm^{-1} occurs as a result of C−O−C asymmetric stretching vibrations derived from primary alcohols in gallic acid derivatives (Lazzari et al., 2018).

3.3. Determination of antioxidant activity

The antioxidant activity of extracts of LPD was examined *via* analyzing $ABTS^{\bullet+}$ scavenging potential and through the ability of tested extract to donate ^1H, using the stable $DPPH^{\bullet}$ (Čutović et al., 2022).

According to the results from the ABTS assay, the highest antioxidant activity was achieved in the extract of LPD obtained using MAE method, followed by M and UAE, but the differences were not significant (Table 2). In global, the antioxidant activity has arisen from the content of some representatives of polyphenols, such as gallic acid, quercetin, isorhamnetin, and similar (Miraj et al., 2016). As mentioned, the extract of MAE was the most potent probably due to the strong interaction of microwaves with molecules of the solvent, resulting in increase of internal temperature and pressure within plant material, facilitating the breakage of the cell wall and delivering bioactive molecules in the extraction medium.

The results of DPPH radical scavenging activity differ from those obtained by ABTS (Table 2). The highest antioxidant activity (the lowest IC_{50}) was found in the extract of LPD obtained by M, followed by MAE and UAE. This result is partially in agreement with previously published paper of Jovanović, Đorđević, Zdunić, Pljevljakušić, Šavikin, Gođevac and Bugarski (2017). Namely, the high temperatures during MAE favor the degradation of polyphenols (Burns et al., 1999). Moreover, in some literature reports it can be found that extended time of sonication could also damage extracted natural antioxidants and degrade extracts quality, due to the generation of reactive oxygen species by ultrasound waves (Jovanovic, Petrovic, Đordjevic, Zdunic, Savikin and Bugarski, 2017).

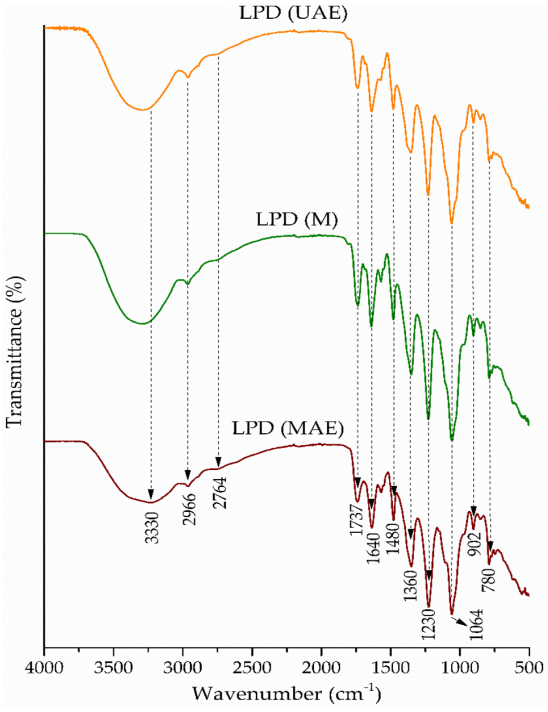

Fig. 3. FTIR spectra of the extracts of the leaves of *P. daurica* subsp. *daurica* Andrews (LPD) from Južni Kučaj obtained by ultrasound-assisted extraction (UAE), maceration (M), and microwave-assisted extraction (MAE).

3.4. Determination of antibacterial activity

The results of the antibacterial activity evaluated by the microdilution method are summarized in Table 3. The three different extracts (UAE, M, and MAE) of LPD originating from Južni Kučaj were all tested for their antibacterial activities against six bacterial strains. The extracts of LPD were assessed as a potential source of antibacterial agents intended for application in the human GI against different pathogens. The extracts of LPD had the highest antibacterial activity against *P. aeruginosa*, while other extracts did not differ visibly in term of inhibition of bacterial growth. The extraction procedure proven to produce the most potent antibacterial agents is M, followed by UAE and MAE. In fact, according to Fick's second law of diffusion, the quantity of extracted polyphenols, the carriers of antibacterial activity, will be proportionally enhanced due to the extension of the extract time (Jovanovic, Petrovic, Đordjevic, Zdunic, Savikin and Bugarski, 2017). Previous investigations on this theme have shown that the leaves of *P. daurica* have strong antibacterial effects against some Gram-positive and Gram-negative bacteria, such as *S. aureus*, *L. monocytogenes*, *P. aeruginosa* and *E. coli* (Tosun et al., 2011). It can also be found that the alcoholic extract of different organs of *P. daurica* subsp. *tomentosa* has strong antibacterial potential against *S. aureus* and *E. coli* (Mahdavi Fikejvar et al., 2018).

3.5. Determination of anti-enzymatic activity

3.5.1. The inhibitory activities of AChE and BChE

One of the most widely employed therapeutic agents for treating the symptoms of Alzheimer's (AD) and Parkinson's diseases (PD) are cholinesterase inhibitors (Sut et al., 2019). Specif-

Table 2. Antioxidant activity of leaf extract of *P. daurica* subsp. *daurica* Andrews (Južni Kučaj) determined by ABTS and DPPH scavenging radical assays.

Extraction method	ABTS [μmol TE/mL]	DPPH IC_{50} [mg/mL]
UAE	0.2628 ± 0.09 [a]	0.051 ± 0.01 [b]
M	0.2631 ± 0.12 [a]	0.046 ± 0.02 [a]
MAE	0.2634 ± 0.09 [a]	0.048 ± 0.02 [ab]

TE: Trolox equivalent; DPPH IC_{50}: the concentration of the extract required to neutralize 50% of DPPH$^{\bullet}$; UAE: ultrasound-assisted extraction; M: maceration; MAE: microwave-assisted extraction; values with the same letter in each showed no statistically significant difference ($p > 0.05$, n = 3, one-way ANOVA, analysis of variance, Duncan's *post hoc* test).

Table 3. Antibacterial activity of the leaf extracts of *P. daurica* subsp. *daurica* Andrews (Južni Kučaj) (mg/mL).

Extraction method	*Salmonella* typhimurium		*Listeria monocytogenes*		*Bacillus cereus*		*Pseudomonas aeruginosa*		*Staphylococcus aureus*		*Escherichia coli*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
UAE	0.5	1	0.5	1	0.5	1	0.5	1	1	2	0.5	1
M	0.5	1	0.5	1	0.5	1	0.25	0.5	0.5	1	0.5	1
MAE	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	1	2

UAE: Ultrasound-assisted extraction; M: Maceration; MAE: Microwave-assisted extraction; MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration.

Table 4. The anti-enzymatic activity of the leaf extracts of *P. daurica* subsp. *daurica* Andrews (Južni Kučaj).

Extraction method	AChE inhibition	BChE inhibition	Amylase inhibition	Glucosidase inhibition	Tyrosinase inhibition
	[mg GALAE/g]		[mmol ACAE/g]		[mg KAE/g]
UAE	1.89 ± 0.01 [a]	0.96 ± 0.05 [b]	0.25 ± 0.00 [a]	1.07 ± 0.00 [a]	51.00 ± 1.18 [a]
M	1.88 ± 0.09 [a]	0.84 ± 0.04 [a]	0.28 ± 0.02 [b]	1.09 ± 0.00 [b]	50.08 ± 0.83 [a]
MAE	1.89 ± 0.02 [a]	0.97 ± 0.08 [b]	0.33 ± 0.00 [c]	1.09 ± 0.00 [b]	53.92 ± 0.53 [b]

GALAE: galantamine equivalents; ACAE: acarbose equivalents; KAE: kojic acid equivalents; AChE: acetylcholinesterase; BChE: butyrylcholinesterase; Values with the same letter in each column showed no statistically significant difference ($p > 0.05$; n = 3, one-way ANOVA, analysis of variance, Duncan's *post hoc* test).

ically, the neurological theories indicate that acetylcholine, a neurotransmitter that appears to play a crucial role in memory, is deficient in the neocortex or hippocampal regions of the brain and is responsible for at least some of the cognitive impairment observed in AD and PD patients. This hypothesis is especially gaining importance in the late AD and PD stages when levels of AChE have declined by up to 85% so that the BChE, becomes main cholinesterase in the brain (Loizzo et al., 2008). Results from this study suggest that an extract of LPD can be selected as the most promising candidate for treating the previously mentioned neurodegenerative disorders. As can be seen from Table 4., all tested extracts of LPD had the same effect on the AChE, while the extract of LPD obtained by MAE had the greatest effect on the BChE. The substantial inhibitory effect observed in the tested extracts can be attributed to the noncovalent interactions between paeoniflorin from LPD and macromolecular receptors presented in both enzymes (Montanari et al., 2019).

3.5.2. The inhibitory activities against α-amylase and α-glucosidase

Hyperglycemia represents a risk factor in the development of diabetes mellitus type 2 (DM), so the control of glucose levels in serum is crucial in the early treatment of this disease and in further decreasing of the incidence of cardiovascular and cerebrovascular complications (Inzucchi, 2002). Salivary amylase and intestinal glycosidase are the key enzymes in the digestion of dietary carbohydrates. Their inhibitors are effective in retarding glucose absorption and suppression of hyperglycemia (Ramkumar et al., 2009). Also, acarbose (a commercially available drug) inhibits α-glycosidase in the epithelium of the small intestine, which directly influences the decrease of postprandial hyperglycemia and improves the impaired metabolism of glucose without promoting the secretion of insulin (Ramkumar et al., 2009). In this study, noticeable α-amylase and α-glucosidase inhibitory activity of LPD obtained by the MAE method was observed. Previous reports already suggested individual polyphenols (ellagic acid) or classes of polyphenols (anthocyanins) in the herbal extracts responsible for the inhibition of activities of α-amylase and α-glucosidase (Mai et al., 2007; Matsui et al., 2001). However, to create novel medications for the management of DM and its complications, the isolation of specific active principles is required.

3.5.3. The inhibitory activity against tyrosinase

Tyrosinase is a key enzyme in synthesis of melanin and the major therapeutic target to slow/mitigate the unusual darkening of the skin, *i.e.*, cutaneous hyperpigmentation (Sut et al., 2019). Regarding tyrosinase inhibitory potential, the best activity was achieved in extracts of LPD obtained by MAE, at 53.92 mg KAE/g. This anti-enzymatic potential might be associated with the higher concentration of polyphenols in the extracts of LPD. To support this claim, Kim and Uyama (2005) found that some phenolic compounds, such as kaempferol and chlorogenic acid could chelate Cu^{2+} in the flexible active sites of

tyrosinase, causing the inhibition of the enzyme. Also, results from this study corroborate earlier reports that some phenolic and flavonoid compounds can inhibit tyrosinase activity (Abdul Karim et al., 2014; Zamani and Gazali, 2015).

4. CONCLUSION

This study was the first attempt to evaluate the biological activities of leaf extracts of *P. daurica* originating from Serbia which are obtained by different extraction methods. As the research showed, the activities of the extracts varied depending on the employed methods of extraction. Namely, the maximal TPC was detected when M and MAE were used as extraction techniques, while the maximal TFC was obtained when MAE was employed. Considering the results of antioxidant activity, the highest anti-DPPH potential was achieved in the extracts of LPD obtained by M. The analysis of antibacterial activity suggests that the highest potential to inhibit bacterial growth was achieved in the extracts obtained by M, against *P. aeruginosa.* The research also showed that extracts of LPD, obtained by MAE had the greatest effect on the both AChE and BChE enzymes. Furthermore, the extracts of LPD obtained by MAE showed similar effects on both enzymes associated with the onset of diabetes mellitus type 2. Also, the highest tyrosinase inhibitory activity was achieved in the extracts obtained by MAE. Therefore, the M and MAE proved to be the most favorable extraction procedures used to produce extracts of LPD with wide a range of therapeutic activities related to human health. Finally, the extracts of LPD could be used as effective functional ingredients/supplements in food and pharmaceutical products, as they possess prominent antioxidant, antibacterial, and anti-enzymatic activities.

ACKNOWLEDGMENTS

The authors acknowledge their gratitude to the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Grants 451-03-1202/2021-09/3; 451-03-47/2023-01/200003; 451-03-47/2023-01/200007; 451-03-47/2023-01/200019).

CONFLICT OF INTEREST

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

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Apple cider vinegar vs. ethanol as an extraction solvent in the ultrasound-assisted extraction of elderberry fruits

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Received: November 16, 2023
Accepted: December 7, 2023
Published on-line: December 20, 2023
Published: December 25, 2023

Traditional plant extraction methods commonly employ hazardous solvents that threaten human health. Considering these, the study sought to explore the efficacy of seven distinct types of apple cider vinegar (ACV) as an alternative solvent for isolating polyphenols from dry elderberries as a raw material. In the pursuit of identifying the most suitable solvent, a conventional solid-liquid extraction was executed, with a comparative assessment involving three different ethanol solution solvents (30%, 50%, and 70%, (v/v)). Subsequently, once the optimal solvent was determined, an ultrasound-assisted extraction was carried out. The optimization process encompassed a range of sonication amplitudes (20-100%) and extraction durations spanning from 120 to 360 s. The extracts' characterization, including extraction yield and total phenolic content, was performed to ascertain the most favorable extraction parameters. The utilization of ACV in conjunction with a sonication amplitude of 100% and an extraction duration of 360 s yielded the highest TPC, amounting to 84.44 mg GAE/g dry extract (DE). In contrast, the lowest ethanol concentration (30%) produced the highest TPC, reaching 101.70 mg GAE/g DE when a sonication amplitude of 100% and an extraction time of 120 s were applied.

Keywords: apple cider vinegar; elderberry fruit; ultrasound-assisted extraction; phenolic compounds; anthocyanins

https://doi.org/10.61652/leksir2343e163M

1. INTRODUCTION

In recent years, the use of dietary supplements, as a means of preventing various health complications, has seen a remarkable increase. A key challenge for the dietary supplement industry is to improve production capacity while adhering to the highest principles of protecting human health and the environment. In the number of dietary supplements herbal extracts are the primary bioactive constituents of the product. They are produced using different extraction techniques and solvents. A forward-looking approach in herbal extract production suggests replacing the conventional, often hazardous solvents used in the manufacturing processes, with environmentally friendly alternatives. The increased market demand for these kinds of supplements and extracts has led the scientific community to explore an expanded repertoire of solvents suitable for the precise isolation of the various bioactive constituents including polyphenolic compounds. This endeavor includes the development of innovative extraction techniques specifically designed to achieve these goals. Among the solvents that have been favored in the past for the commercial production of extracts with a high concentration of phenolic compounds, water, and ethanol in varying concentrations are the most recommended. In addition to these established solutions, the potential of other solvents for the isolation of compounds, including polyphenols, is currently being explored. These include glycerol (Kowalska et al., 2021), methanol (Domínguez et al., 2020; Manizabayo et al., 2019), citric acid (Pliszka, 2017), acetone (Zhou et al., 2020), deep eutectic solutions (Perna et al., 2020; Vladić et al., 2023) and others. Apple cider vinegar (ACV) is obtained by the dual process of acetate and alcoholic fermentation of apples or concentrated apple juice, as explained by Joshi and Sharma (2009). ACV serves as a reservoir of bioactive compounds and vital nutrients, including polyphenols, organic acids, sugars,

melanoidins and other components, as confirmed by Ho et al. (2017), Xia et al. (2020) and Budak (2021). The quality and sensory properties of ACV are closely related to its production method. As Chen et al. (2016) explained, the composition and concentrations of the ingredients of ACV depend on the selection of the raw materials, the production technology used and the specific processes. The various ingredients of ACV play a central role in the treatment and prevention of diseases due to their recognized anti-inflammatory and antibacterial properties, as stated by Hindi (2013). In addition, these ingredients have effects on weight management, obesity prevention, and regulation of blood lipid levels (Hadi et al., 2021). There are two main methods for the production of ACV: the traditional method and the industrial (commercial) method. The traditional method, although time-consuming, involves the sequential alcoholic and acetic acid fermentation of apple juice with natural yeasts and acetic acid bacteria (AAB). In modern times, ACV is mainly produced by submerged cultivation, similar to the production of wine vinegar (Joshi and Sharma, 2009).

Elderberry (*Sambucus nigra* L.), a small tree or wild deciduous shrub, has a remarkable range of medicinal properties. Extensive literature highlights its diverse therapeutic uses, including the treatment of respiratory and rheumatic conditions, as well as its use as a diuretic and laxative (Hawkins et al., 2019). Elderberry fruits are particularly rich in secondary metabolites consisting mainly of polyphenolic compounds, including flavonols, phenolic acids, proanthocyanidins, and anthocyanins, which are responsible for the characteristic purple color of the fruit. These compounds have been well described by Domínguez et al. (2020). The main anthocyanins identified in elderberries are derivatives of cyanidin, with cyanidin-3-glucoside and cyanidin-3-sambubioside predominating alongside traces of cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-rutinoside, pelargonidin-3-glucoside, pelargonidin-3-sambubioside and delphinidin-3-rutinoside. Elderberries also contain important flavonoids, namely quercetin, kaempferol, isoquercetin, and rutin, which contribute to their therapeutic efficacy, as shown in the study by Avula et al. (2022). In addition, elderberries contain chlorogenic acid, derivatives of caffeic acid, and traces of p-coumaric acid and ellagic acid (Terzić et al., 2022).

As already documented in the scientific literature, the quality of herbal extracts depends on several crucial factors, including the choice of extraction agent, the quality of the plant material under consideration, and the extraction methodology employed (Chemat et al., 2017; Sulejmanović et al., 2024). The ultrasound-assisted extraction (UAE) stands out as a superior alternative to conventional techniques that offer significant advantages in terms of energy efficiency, time efficiency, solvent consumption, and ease of subsequent solvent removal. The application of ultrasound in extraction processes is characterized by its simplicity, adaptability, feasibility on an industrial scale and lower investment requirements, especially compared to high-pressure techniques (Tiwari, 2015).

In view of these considerations, the main objective of this research project was to evaluate the suitability of ACV as an alternative solvent for the extraction of bioactive compounds from elderberry fruits using an advanced and environmentally friendly UAE method. Given the lack of scientific data in this field, a comparative analysis was performed between the extraction of elderberry fruit with ACV and ethanol, a conventional and widely used solvent. In the first phase, a solid-liquid extraction (SLE) was performed with ground elderberries using different concentrations of ethanol (30%, 50%, and 70% (v/v)) and ACV (seven different ACVs sourced from different manufacturers and produced according to different protocols). After identifying the most suitable extraction solvents, the influence of UAE parameters on the extraction of polyphenols from elderberries was investigated. The studies included a range of extraction durations from 120 to 360 s and investigated the effects of three different sonication amplitudes, ranging from 20% to 100%. The optimization of the process conditions for the production of elderberry fruit extracts was achieved by a comparative evaluation of the extraction yield. In this context, this study investigates the feasibility of using ACV as an alternative solvent for the isolation of phenolic compounds from elderberries. The underlying premise of this investigation was whether we can utilize the existing commercial liquid natural product, of the proven health benefits, to selectively isolate the bioactive compounds, such are phenolics, and thus increase the value of the resulting extracts. To evaluate the potential of ACV as a solvent for the extraction of polyphenols, a case study was conducted using elderberry fruit as a starting material for extraction and then subjected to a comprehensive analysis.

2. MATERIALS AND METHODS

2.1. Herbal material and chemicals

Commercially available dried berries of *Sambucus nigra* L. originating from the Institute "Dr. Josif Pančić" in Belgrade, Serbia formed the primary plant material for extraction in this study. Prior to extraction, these dried elderberries were mechanically crushed in a blender. The average particle size of the resulting material was determined using a sieve set (CISA Cedaceria Industrial, Spain). The milled material was subsequently stored in a controlled environment, *i.e.*, in a dark and dry place at room temperature, to prepare it for subsequent analysis.
Various ACVs from different manufacturers were used for the study, including Aroma, Body Guard, Ekofarm, and Status. In addition, ethanol with a concentration of 96% was obtained from the manufacturer Centrochem, Šabac, Serbia. The chemical reagents Folin–Ciocalteu reagent and 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals used in this study were in accordance with analytical standards.

2.2. Conventional solid-liquid extraction

The initial phase of this study, SLE was used to investigate the range of solvents available for the extraction of phenolic compounds from elderberries. During the SLE procedure, 5 g of the plant material was subjected to the extraction with 50 mL (1:10) of various solvents. These solvents included aqueous ethanol solutions at concentrations of 30%, 50%, and 70% (v/v) ethanol and undiluted ACVs from different manufacturers, each prepared according to different protocols. BodyGuard's ACVs, referred to as ACV 1, ACV 2, and ACV 3, and Aroma's ACVs, referred to as ACV 5 and ACV 6, were obtained by traditional manufacturing processes. In contrast, the ACVs from Status and Ekofarm, designated ACV 4 and ACV 7 respectively, were produced using accelerated industrial processes. The extraction processes were carried out at a controlled temperature condition of 25 °C over a period of 24 h, without mechanical agitation. After the extraction process, the obtained extracts were filtered through filter paper under vacuum conditions. After filtration, the extracts were safely bottled and stored at 4 °C until they were subjected to subsequent analytical tests. The extraction yield (EY) was expressed as the mass of dry extract (g) per g of dry plant material, *i.e.*, the percentage (%).

2.3. Ultrasound-assisted extraction

The UAEs were performed with different sonication amplitudes, namely 20%, 60% and 100%, over different time in-

tervals of 120, 240, and 360 s. These extractions were performed with a specific drug-solvent ratio of 1:10, whereby 10 g of the plant material was mixed with 100 mL of the intended solvent. Based on the results of the SLE, water-diluted ethanol (30% ethanol, v/v) and ACV 4 were selected as optimal solvents for the UAE. Throughout the extraction process, changes in temperature and energy consumption were closely monitored. The UAE procedures were performed using an ultrasonic probe system from Hielscher Ultrasonic GmbH (Teltow, Germany), supplemented with magnetic stirrer to enable continuous mixing. After completion of the extraction phase, the resulting extracts were filtered through filter paper under vacuum conditions. Subsequently, these extracts were systematically collected in storage vessels and kept at a controlled temperature of 4 °C until the time of analysis.

2.4. Total phenolic, and anthocyanins contents

Total phenolic content (TPC) was determined by the Folin-Ciocalteu method as described by Singleton and Rossi (1965). Gallic acid was used to generate a standard curve. The absorbance of the samples was quantified at a wavelength of 750 nm using a Jenway 6300 spectrophotometer (Cambridge, UK). The TPC values were subsequently expressed in milligrams of gallic acid equivalent (GAE) per gram of dry extract (DE).
Quantification of total monomeric anthocyanin content (TAC) was performed using the pH differential method according to the protocol described by Lee et al. (2005). Potassium chloride (0.025M) at a pH of 1.0 and sodium acetate (0.4M) at a pH of 4.5 were used to prepare the required buffer solutions. The absorbance of the samples was measured at two different wavelengths, 520 nm and 700 nm. The TAC values were expressed in milligrams of cyanidin-3-glycoside (C3G) per liter of extract.

2.5. Antioxidant activity

The evaluation of radical scavenging activity in the samples was performed using the DPPH assay, a method previously described by Espin et al. in 2000. The absorbance at a specific wavelength, exactly 515 nm, was precisely quantified. The results were then expressed as radical scavenging capacity (RSC) in percent (%) and inhibitory concentration (IC_{50}), which indicates the concentration at which 50% of the radicals are inhibited.

2.6. Statistical analysis

The analyses were performed carefully and according to strict scientific standards in triplicate. The resulting data were presented as mean values with the corresponding standard deviations (SD). This approach ensures the robustness and reliability of the results obtained. To determine meaningful differences between the results, we performed a one-way ANOVA analysis followed by a Tukey test. Significance was recognized when $p < 0.05$, n=3.

3. RESULTS AND DISCUSSION

3.1. Characterization of ACVs

The potential of ACV as an alternative solvent was explored by Mohammed et al. (2021). Their study involved the extraction of bioactive compounds from roselle, green tea, and clove with ACV to increase the antimicrobial activity of these extracts. The study yielded promising results suggesting that ACV, which is also rich in phenolic components, can contribute significantly to the bioactivity of the resulting plant extracts. According to scientific findings, the phenolic compounds in ACV mainly include gallic acid, catechin, epicatechin, chlorogenic acid and *p*-coumaric acid (Budak, 2021; Tripathi and Mazumder, 2020). To gain a deeper insight into the subsequent extraction process and to evaluate the role of ACV in shaping the properties and bioactivity of the final extract, the ACV was subjected to further characterization. This included the evaluation of dry weight (DW), TPC and antioxidant activity. At the same time, the pH values of the ACV were monitored and values between 2.66 and 3.65 were consistently observed. These pH values are consistent with the results already published (Budak et al., 2011). However, it is noteworthy that different pH ranges for ACV are reported in the literature such as 3.18-3.83 (Ousaaid et al., 2021; Ozturk et al., 2015).
Table 1 shows the differences in DW and TPC observed between the different ACVs, a variation that is probably due to the different production methods and raw materials used. ACV 5 had the highest DW at 20.73 mg/mL, while ACV 2 had the lowest DW at 7.69 mg/mL. The ACV 1-3 are vinegars produced using the same process and by the same manufacturer, but from a different batch. This observation underlines that despite identical production processes, the quality of the ACV differed and depended on the quality of the raw materials. Consequently, these differences in raw materials and processing had a recognizable influence on the properties of the final ACVs, as shown by the significant differences in DW value between the studied ACVs (Table 1). In the study by Karadag et al. (2020), traditional ACV production resulted in an ACV of 9.5% DW. A comparison of the results of Karadag et al. (2020) with the DW content of ACV 5 (20.73%), which was also produced by traditional methods, showed that the DW of ACV 5 was more than twice as high, although both were produced by the same method. Furthermore, the analysis revealed a significant difference in TPC in the ACV analyzed. The highest total TPC was detected in ACV 2 with 60.32 mg GAE/g DE, while ACV 7 had the lowest TPC with 2.64 mg GAE/g DE. In contrast to present study, Sengun et al. (2019) found TPC values of 1.02 and 0.988 mg GAE/mL for grape and apple cider vinegar, respectively, which is approximately 60 times lower than the current results for the ACVs studied.

3.2. Characterization of extracts obtained by SLE

The SLE serves as a valuable benchmark for evaluating the feasibility of new solvents and the efficacy of alternative techniques (Naviglio et al., 2019; Živković et al., 2022). This approach is characterized by the fact that a significant volume of solvent is required and it takes a longer time for solvent to permeate the raw material and facilitate the extraction of the target compounds. In this study, SLE was strategically used as a tool to identify the most suitable solvent to optimize the extraction of berry constituents and obtain high-quality extracts in the further advanced extraction process. Besides, to get an assumption on the scaled process feasibility, the subtletyof the separation process, where the obtained extract was separated from the residues, was also observed in the selection of a suitable solvent. The qualitative evaluation of the extracts obtained in the SLE, considering parameters such as EY, TPC, and antioxidant activity, led to the selection of the two most promising solvents for further studies within the UAE.
In the experiments where ethanol was used for the extraction of elderberry fruit, the EY varied between 18.83% and 37.07%. The highest EY (37.07%) was obtained with a 30% ethanolsolution. Accordingly, the TPC values also reflect this trend, with the 30% ethanol solution proving to be the most effective. In the same extract, produced using 30% ethanol, aquantified TPC of 82.89 mg GAE/g DE was obtained, while the lowest concentration was observed in the extract obtained with 70% ethanol as extraction solvent (Table 2). Duymuş et al. (2014) reported that the TPC values, ranging from 49.17 to 89.74 mg

Table 1. Basic values and the contents measured in the studied and applied ACVs.

	pH	Dry weight (mg/mL)	TPC (mg GAE/g DE)	IC_{50} (μg/mL)
ACV 1	3.43	12.64 ± 0.68^{c}	29.54 ± 3.04^{c}	42.54 ± 0.83^{f}
ACV 2	3.65	7.69 ± 0.65^{d}	60.32 ± 2.23^{a}	39.73 ± 0.74^{f}
ACV 3	3.28	13.52 ± 1.03^{bc}	52.49 ± 1.48^{b}	82.62 ± 1.76^{e}
ACV 4	3.1	16.17 ± 1.04^{b}	7.22 ± 0.45^{d}	226.16 ± 10.86^{c}
ACV 5	3.1	20.73 ± 2.05^{a}	5.74 ± 0.40^{d}	251.69 ± 5.21^{b}
ACV 6	3.1	15.71 ± 0.32^{b}	8.44 ± 1.36^{d}	160.86 ± 3.91^{d}
ACV 7	2.66	20.63 ± 0.29^{a}	2.64 ± 0.64^{e}	931.76 ± 14.20^{a}

Unequal letters within a column indicate a significant difference between the samples with a significance level of $p < 0.05$, n=3. ACVs, apple cider vinegars; TPC, total phenolic content; IC_{50}, inhibitory concentration at which 50% of the radicals are inhibited.

GAE/g DE, are to be expected in the dried elderberry fruit extracts where the extraction was carried out through SLE using various solvents, including water, 70% ethanol, and methanol. It is worth mentioning that our previous study also confirmed the suitability of the 30% ethanol solution for the extraction of phenolic compounds from elderberry pomace (Mutavski et al., 2022).

Also, the elderberry fruit ethanolic extract obtained with 30% ethanol as solvent showed the highest antioxidant activity (IC_{50} value of 32.54 μg/mL). This result is consistent with the highest TPC observed in the same extract. Oniszczuk et al. (2019) determined IC_{50} values of 3.17, 3.68, and 3.79 in elderberry extracts obtained during 15, 30, and 60 min of UAE with 80% ethanol, respectively. Interestingly, in this study an increase in the extraction time led to a decrease in the antioxidant activity of the extracts, which could be due to the time degradation of the bioactive compounds under the set process conditions. Some studies have reported the opposite results (Domínguez et al., 2020). By varying the concentrations of ethanol in the extraction solvent, they found out that 50% ethanol solution was the most effective for extracting the phenolic compounds from the elderberries. These discrepancies may be due to different experimental conditions and methods used in their study compared to the present investigation.

The EYs of the extracts obtained using ACVs as extraction solvents were between 42.56% and 57.37%. Application of ACV 2 resulted in the lowest yield, while the highest was obtained using ACV 5. However, the extract obtained using ACV 2 as solvent resulted in the highest TPC with 75.39 mg GAE/g DE, while the lowest TPC value was measured in the extract obtained by ACV 7. It is important to emphasize that the phenolic compounds of ACV extracts exceed that of pure ACVs. According to the obtained results this was especially case in the extracts obtained by application of ACVs 4 and 5 (ACVs obtained from traditional production) and ACVs 6 and 7 (obtained from industrial production), where TPC was around 10 times higher in the extracts than in the pure ACVs. This indicates that these ACVs are efficient extraction solvents for elderberries' phenolics. The increase was also noticed in the application of ACVs 1, 2, and 3, but it was not so conspicuous. The antioxidant activity of the elderberry ACV extracts ranged from IC_{50} 51.61 μg/mL to 69.82 μg/mL. The antioxidant activity generally was also multiplied in the case of ACVs extracts in comparison to the activities of pure ACVs (except in the case of ACVs 1-3), what is also confirming the assumption that some ACVs are the efficient systems for the elderberries' phenolics isolation.

In the ACV elderberry extracts production, problems occur during the filtration of the extracts obtained by the ACV 1-3, such as the clogging of the pores of the filter paper and the inability to separate the liquid medium. Therefore, they were not considered as alternative solvents for further application in the UAE. It can be assumed that due to this, their application on the industrial scale would likely be associated with significant technical complications. Therefore, ACV 4, with highest TPC value of 61.42 mg GAE/g DE, was identified as an optimal solvent for producing elderberry fruit extract with an alternative solventand for further exploration of its potential in the context of UAE (Table 3).

3.3. Characterization of extracts obtained by UAE

The UAE is well established in the chemical and food industry due to its remarkable impact on the acceleration of various processes. It is based on the principle of acoustic cavitation, a phenomenon that breaks down the cell walls within the plant matrix, facilitating the extraction of bioactive compounds (Kumar et al., 2021; Razola-Díaz et al., 2022).

The EY values for black elderberry fruit extracts obtained with UAE using 30% ethanol as extraction solvent ranged from 21.64% to 47.28%. The EY values increased continuously with higher sonication amplitude and longer extraction time, as shown in Table 3. Considering the TPC, there was no clear pattern showing the impact of prolonged time or increased amplitude on the TPC. The highest TPC was obtained in the ethanol extract sonicated for 120 s at an amplitude of 100%, with 101.70 mg GAE/g DE, while the ethanol extract with the lowest TPC was obtained in sonication for 120 s at an amplitude of 60%, resulting in a TPC of 85.49 mg GAE/g DE (Table 3). It is noteworthy that the elderberry ethanolic extract obtained by the UAE showed a similar or some higher TPC value to that of the SLE. To assess the superiority of one technique over the other, the extraction times must be compared. It is obvious that UAE, which required less than 360 s, was a much more efficient method compared to SLE, which required a significantly longer extraction (24 h), in the process where the same raw material and same extraction solvent were used. This underlines the considerable time-saving advantages that the UAE brings.

The present study also included the evaluation of the role of 30% ethanol in the isolation of TAC from elderberry fruit in relation to UAE. Table summarizes the effects of different sonication amplitudes and extraction durations on TAC extraction. The optimum TAC from elderberry fruits was achieved with an extraction duration of 120 s and a sonication amplitude of 60%, resulting in a considerable amount of 30.36 mg C3G per liter (L). Same as in the case of TPC no clear pattern showing the impact of prolonged extraction time or increased amplitude on the TAC was not noticed.

Table 2. EY of SLE, and TPC in ethanol and ACV extracts of elderberry fruit.

Solvent	EY (%)	TPC (mg GAE/g DE)	IC_{50} (μg/mL)
30% ethanol	37.07 ±1.50^{f}	82.89 ± 4.23^{a}	32.54 ± 1.71^{c}
50% ethanol	31.11 ± 0.72^{g}	76.60 ± 4.52^{ab}	34.04 ± 2.55^{c}
70% ethanol	18.83 ± 0.68^{h}	48.18 ± 4.40^{g}	56.42 ± 3.12^{b}
ACV 1	53.48 ± 1.96^{ab}	66.36 ± 2.70^{cd}	69.82 ± 4.95^{a}
ACV 2	42.56 ± 1.57^{e}	75.39 ± 4.75^{b}	51.61 ± 3.62^{b}
ACV 3	44.93 ± 1.13^{de}	67.72 ± 1.37^{c}	53.64 ± 4.95^{b}
ACV 4	51.69 ± 1.40^{bc}	61.42 ± 1.96^{cde}	55.71 ± 3.91^{b}
ACV 5	57.37 ± 2.32^{a}	55.05 ± 3.40^{ef}	55.42 ± 5.42^{b}
ACV 6	49.54 ± 1.86^{bc}	60.27 ± 2.82^{def}	54.75 ± 4.95^{b}
ACV 7	48.18 ± 1.55^{cd}	53.83 ± 1.48^{fg}	60.72 ± 3.42^{ab}

Unequal letters within a column indicate a significant difference between the samples with a significance level of $p < 0.05$, n=3. EY, extraction yield; SLE, solid-liquid extraction; TPC, total phenolic content; ACV, apple cider vinegar.

In the case of UAE where ACV 4 was applied as extraction solvent, the EY values ranged from 39.55% to 50.55% and also showed an upward trend with increasing amplitude and time, except for the sample with 60% amplitude and 240 s, but with no significant difference.

Employing ACV as a solvent in the UAE process, variations in TPC were observed depending on the amplitude and duration of sonication. The most remarkable TPC was achieved under a sonication amplitude of 100% and an extraction duration of 360 s, resulting in a remarkable value of 84.52 mg GAE/g DE. The lowest TPC was measured in the ACV extract resulting from the sonication amplitude of 60% at an extraction time of 120 s (see Table 3).

Significantly, the use of ACV 4 as a solvent in the SLE resulted in a TPC of 61.42 mg GAE/g DE, but the use of UAE not only resulted in the extracts with higher TPC values but also did so in the significantly shorter time span of 360 s compared to the 24 h long SLE.

Extraction using ACV 4 as solvent yielded a peak TAC concentration of 8.65 mg C3G/L from elderberry fruit using UAE for 360 s at 100% amplitude. Here the TAC was impacted by the prolonged sonication time and by more intensive amplitude. Interestingly, for ethanolic extracts, this was not the case. However, the use of ultrasound with an amplitude of 60% showed a striking positive correlation between amplitude and TAC. Overall, ethanol can be considered a more efficient solvent for the extraction of anthocyanins from elderberry fruit, especially at a sonication amplitude of 100% and an extraction time of 360 s, which resulted in an approximately 2.5–fold higher TAC if ethanol is applied as extraction solvent.

3.4. Monitoring parameter changes during the UAE

The dynamics of temperature change over time is an important process parameter in the UAE. This interplay of temperature and time is of immense importance as it profoundly affects the extraction process. In certain cases, temperature can be a limiting factor in the extraction process, mainly due to the presence of heat-sensitive compounds and, accordingly, the boiling point of the solvent used. Oancea (2021) conducted an insightful study on the influence of temperature on the degradation of anthocyanins in elderberry fruit. The results of the study showed that an aqueous extract exposed to a temperature of 70 °C retained about 80% of its anthocyanin content during a heating period of 3 h. In view of these potential degradation processes and depending on the compounds to be isolated, strict temperature monitoring is essential throughout the extraction process.

The relationship between extraction temperature and extraction time for elderberry extracts obtained with ethanol is shown in Figure 1, while Figure 2 shows the same correlation for elderberry extracts obtained with ACV. It is noteworthy that at a sonication amplitude of 20%, an initial temperature of 27 °C was measured, which increased slightly to 32.5 °C after 360 s of extraction. It is unlikely that this slight increase in temperature, especially in the lower temperature range, negatively affects the extracted polyphenolic compounds (Antony and Farid, 2022). Conversely, it could be assumed that this slight increase in temperature could even have a positive effect on the recovery of these compounds. At a sonication amplitude of 60%, a more pronounced increase in process temperature over time can be seen, and this increase is significant at an amplitude of 100%. Indeed, at this amplitude, the temperature starts at 30 °C and rises to 65.5 °C for 360 s. Obviously, in this scenario, the temperature variations during the extraction process are significant. This could either have a positive effect on the recovery of the compounds if the target compounds are thermostable, or a detrimental effect if they are susceptible to degradation at these high temperatures.

Figure 2 shows that the process temperature increases during the UAE with ACV as an extraction solvent. At a sonication amplitude of 20%, the process temperature was the lowest, 25.3 °C after 60 s and 29 °C at the end of the extraction process, after 360 s. It is important to note a gradual increase in temperature with time, but also with the amplitude of the extraction. Moreover, the temperature increases faster with time as the amplitude increases (Figure 2). At an amplitude of 100%, the temperature was 30.3 °C at the beginning of the extraction and 70 °C after 360 s.

Oancea et al. (2018) investigated the kinetics of anthocyanin degradation in elderberry fruit extracts. They concluded that conversions of anthocyanin compounds occur at temperatures above 100 °C . This could indicate that the highest temperature reached in this process had no negative effects on elderberry anthocyanins.

Energy consumption is an important process parameter in any production process and has a significant impact on the profitability and cost efficiency of the extraction process, especially when compared to alternative methods. Therefore, the

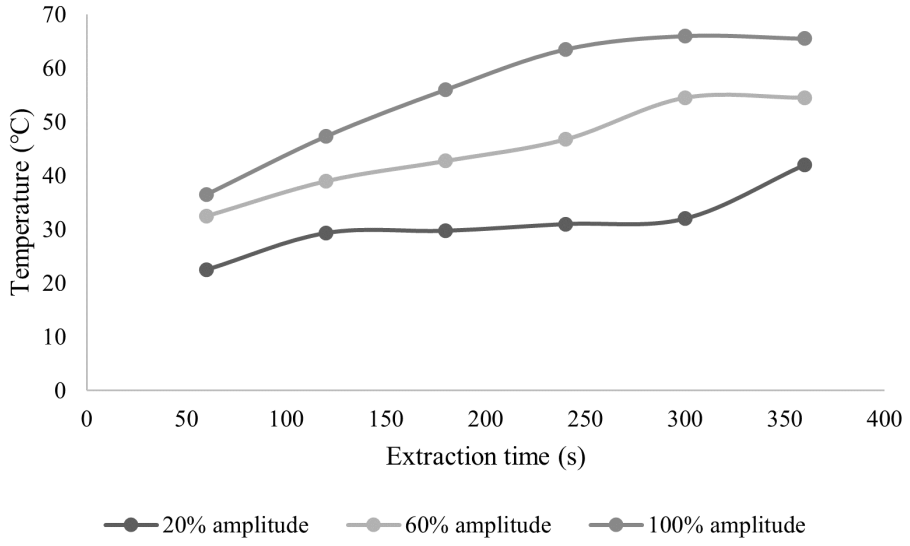

Fig. 1. Process temperature changes during elderberry ultrasound-assisted extraction (UAE), using ethanol as extraction solvent.

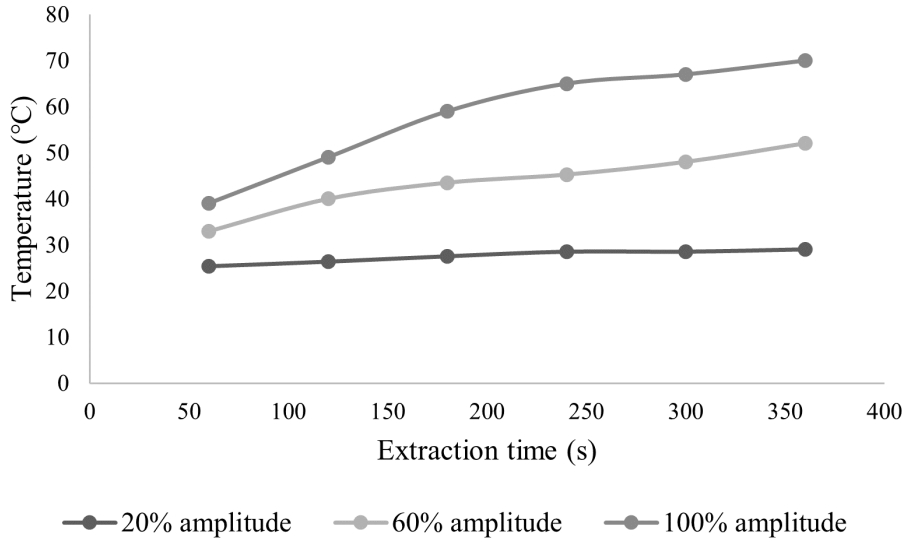

Fig. 2. Process temperature changes during elderberry ultrasound-assisted extraction (UAE), using apple cider vinegar (ACV) as extraction solvent.

Table 3. The EY, TPC and TAC of elderberry extracts obtained in UAE using 30% ethanol and selected ACV.

		30% ethanol			ACV 4		
A	**t (s)**	**EY (%)**	**TPC (mg GAE/g DE)**	**TAC (mg C3G/L)**	**EY (%)**	**TPC (mg GAE/g DE)**	**TAC (mg C3G/L)**
20%	120	21.64 ± 0.56^{f}	100.63 ± 4.28^{ab}	12.21 ± 0.37^{f}	39.55 ± 0.88^{e}	65.71 ± 2.26^{d}	7.05 ± 0.22^{c}
	240	24.12 ± 0.79^{ef}	98.36 ± 2.87^{abc}	15.17 ± 0.36^{de}	39.94 ± 1.73^{de}	69.33 ± 2.86^{bcd}	7.21 ± 0.29^{bc}
	360	27.70 ± 1.20^{e}	89.16 ± 3.40^{abc}	16.84 ± 0.47^{d}	43.78 ± 1.64^{cde}	66.54 ± 2.80^{cd}	7.82 ± 0.36^{abc}
60%	120	36.18 ± 1.04^{d}	85.49 ± 9.87^{c}	30.37 ± 0.63^{a}	44.58 ± 2.11^{be}	62.83 ± 5.29^{d}	7.88 ± 0.20^{abc}
	240	37.06 ± 0.93^{d}	95.17 ± 15.46^{abc}	11.69 ± 0.35^{f}	43.62 ± 0.98^{cde}	67.05 ± 3.42^{cd}	7.95 ± 0.22^{ab}
	360	41.74 ± 1.42^{bc}	90.60 ± 5.26^{abc}	24.36 ± 1.02^{b}	44.97 ± 2.18^{bcd}	67.99 ± 1.89^{cd}	8.42 ± 0.29^{a}
100%	120	38.88 ± 1.19^{cd}	101.70 ± 3.05^{a}	14.29 ± 0.49^{e}	46.06 ± 2.11^{abc}	72.76 ± 3.00^{bc}	7.18 ± 0.16^{bc}
	240	44.50 ± 1.43^{ab}	95.50 ± 5.08^{abc}	21.04 ± 1.03^{e}	49.34 ± 2.47^{ab}	75.39 ± 4.04^{b}	8.42 ± 0.41^{a}
	360	47.28 ± 2.28^{a}	88.33 ± 3.04^{bc}	22.15 ± 0.82^{e}	50.55 ± 1.02^{a}	84.44 ± 5.76^{a}	8.65 ± 0.30^{a}

Unequal letters within a column indicate a significant difference between the samples with a significance level of $p < 0.05$, n=3. EY, extraction yield; TPC, total phenolic content; TAC, total monomeric anthocyanin content; UAE, ultrasound-assisted extraction ACV, apple cider vinegar.

evaluation of energy consumption is a critical dimension in the context of extraction processes. The results of the study showed a remarkable correlation between energy utilization and certain process variables. The lowest energy consumption of 0.50 W/h was observed at an extraction process amplitude of 20% and an extraction time of 60 s. In contrast, the highest energy consumption of 11.31 W/h was observed at an amplitude of 100% and an extraction time of 360 s. These results corresponded to the expected trends and confirmed the fundamental interplay between sonication parameters and energy consumption in UAE.

The study highlights an important result: increasing the sonication amplitude to 100% does not increase the efficiency of extraction, but leads to a significant increase in energy consumption of 33.4%. This has remarkable implications, especially for future extraction processes on an industrial scale. In addition to monitoring extract quality, it is crucial to evaluate the economic aspects, aiming for a double objective: optimizing extraction efficiency for resource utilization and minimizing energy consumption. This approach is crucial for cost efficiency and sustainable practices in large-scale production. In industrial production, every unit of energy consumed has a measurable economic impact, so examining the results of energy consumption is essential.

In the case of the ACV application, the results showed an increase in energy consumption over time that is in line with the trend observed in ethanol production. The lowest energy consumption was found to be 0.50 W/h at sonication amplitude of 20% for 60 s, while the highest energy consumption was measured at 12.30 W/h at a sonication amplitude of 100% for 360 s. This observation is in line with the results of Krivošija et al. (2023), who conducted a study on UAE using orange peel dust.

Therefore, it is advisable to opt for a lower sonication amplitude corresponding to the reduced output power to mitigate the significant 37% increase in energy consumption. This measure is in line with sustainable practices and responsible use of resources.

4. CONCLUSION

The results of this study provide convincing evidence for the effectiveness of ACV as an extraction solvent, particularly in facilitating the extraction of polyphenolic compounds, but this efficiency depends on the inherent properties of the ACV used. The highest TPC of 101.70 mg GAE/g dry extract (DE) was achieved by using 30% ethanol with a sonication amplitude of 100% and an extraction time of 120 s. Conversely, the use of ACV 4 resulted in the highest TPC, which reached 84.44 mg GAE/g DE with a sonication amplitude of 100% and an extraction time of 120 s. Indeed, ethanol proves to be a more effective solvent than ACV in every aspect. However, this does not diminish the significance of ACV application. The results emphasize the versatile utility of ACV as a solvent for the extraction of bioactive compounds and represent an important extension in the field of environmentally friendly extraction methods. The use of ACV as a solvent not only paves the way for novel, sustainable extraction methods, but also responds to today's imperative to improve the environmental sustainability and efficiency of extraction processes in various scientific and industrial fields. Furthermore, the use of ACV (which in itself is a health-promoting, commercially available product) for the isolation of elderberry phenols enables the production of an elderberry product with strong quality characteristics that combine the health benefits of ACV and elderberry.

ACKNOWLEDGMENT

This work was supported by the Serbian Ministry of Education, Science, and Technological Development (451-03-47/2023-01/200134).

CONFLICT OF INTEREST

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

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Antioxidant activity of *Paeonia tenuifolia* L. petal extract-loaded liposomes

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Received: November 18, 2023
Accepted: December 7, 2023
Published on-line: December 22, 2023
Published: December 25, 2023

Steppe peony (*Paeonia tenuifolia* L., Paeoniaceae) is a perennial, herbaceous plant species which has been widely known for its medicinal use. This study is aimed at the development of *P. tenuifolia* petal dry methanolic extract-loaded liposomes and the determination of their antioxidant activity. The liposomes were prepared with three different phospholipid mixtures by the proliposome method. The antioxidant potential of the liposomes was assessed by the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methods. The ABTS radical scavenging activity ranged from 66.99 to 69.15%, whereas for the DPPH, the values varied from 85.12 to 90.11%. These results revealed promising antioxidant activities of *P. tenuifolia* petal extract-loaded liposomes with the potential to be used for the treatment of health disorders associated with oxidative stress, such as skin-related problems.

Keywords: peony; antioxidant potential; liposomes; phospholipids; encapsulation

https://doi.org/10.61652/leksir2343e167C

1. INTRODUCTION

Steppe peony (*Paeonia tenuifolia* L.), also known as the fern leaf peony, is a perennial, herbaceous plant species belonging to the *Paeonia* genus of the Paeoniaceae family (Li et al., 2021). On the basis of the phytochemical analyses of the petals of *P. tenuifolia*, a variety of biologically active compounds was identified, such as flavonoids, including anthocyanins and anthocyanidins, phenolic acids and terpenoids (Čutović, Batinić, Marković, Radanović, Marinković, Bugarski and Jovanović, 2022). Due to the presence of the mentioned components, steppe peony possesses a number of beneficial activities, such as antimicrobial, antibiofilm, and antioxidant properties, as well as the potential to enhance wound healing and inhibit the adhesion and invasion of the bacteria *Staphylococcus lugdunensis*, which is recognized as a skin pathogen (Čutović, Marković, Kostić, Gašić, Prijić, Ren, Lukić and Bugarski, 2022). On account of the fact that there is a limited bioavailability and instability of flavonoids, phenolic acids, and anthocyanins detected in the *P. tenuifolia* petal extracts (Fang and Bhandari, 2010), carriers for the extract should be prepared in order to protect the bioactive compounds and enable a more comfortable dermal application (de Vos et al., 2010). Plant extracts can be encapsulated into a matrix or membrane in the particulate form to achieve one or more of the previously listed desired effects (Armendáriz-Barragán et al., 2016). Encapsulation is intended to improve the stability of extracted compounds during processing, storage, or transportation. Its primary goal is to convert liquid active chemicals into solid forms in order to improve active compound management. When direct intake of an active substance affects human health, the use of pharmaceutical formulations containing them in an encapsulated form can be very advantageous. In addition, the encapsulation can also be used to improve the quality of the final product, isolate incompatible compounds, and provide the release of bioactives in a controlled way (El-Desoky et al., 2022).

Liposomes have been used as carriers for the delivery of various compounds, such as enzymes, polyphenols, drugs, proteins, vitamins, and antioxidants (Isailović et al., 2013; Jovanović et al., 2022). Liposomal preparation has several advantages over other kinds of encapsulation techniques, including liposome stability in products with a high water content and their capacity to encapsulate hydrophilic, amphiphilic, as well as lipophilic compounds (Jovanović et al., 2018). Additionally, after encapsulation, a controlled or sustained release profile can be achieved. Liposomes are also biodegradable and have a high affinity for cells (Lee et al., 1992). Further, the target impact of liposomal preparations has the power to modify the *in vivo* distribution of the loaded substance, therefore im-

proving the biological effects of some treatments (Mohammed et al., 2004). Also, liposomes are non-toxic and biodegradable delivery carriers that are often made from naturally occurring substances, thus novel formulations might be easily deployed (Isailović et al., 2013).

The proliposome method provides a greater agitation energy input, resulting in smaller and more homogenous liposomes (Isailović et al., 2013). This procedure may be the simplest way to produce liposomes (Šturm and Poklar Ulrih, 2021). The key disadvantage is that, while it generates far greater encapsulation efficacy, it is not as repeatable when producing lower quantities of liposomes. During the production process of proliposome method, the compounds can be mixed with ethanol (in the case of lipophilic substances) or in an aqueous solution (in the case of hydrophilic substances). The utilization of proliposome technology opens up the possibility of large-scale liposome production (Elhissi et al., 2006).

No previous research was focused on the encapsulation of *P. tenuifolia* petal extracts into liposomal particles. Therefore, the goal of this study was to create *P. tenuifolia* petal extract-loaded liposomes and evaluate their antioxidant capacity, in order to potentially protect the sensitive biologically active components, preferably making them suitable for use in a variety of pharmaceutical and cosmetic formulations.

2. MATERIALS AND METHODS

2.1. Plant material and reagents

Steppe peony (*P. tenuifolia*) fresh petals were collected on a warm, sunny day in May of 2023 from spontaneously growing plants in their natural habitat in Gulenovci (840 m a.s.l.), Serbia. The Ministry of Environmental Protection of the Republic of Serbia has granted the license for their wild collecting (No. 353-01-121/2023-04, issued on March 3, 2022). The petals were hand-picked from full-blooming flowers at random. Less than 10% of the blooming plants discovered in the region were chosen to collect one-third of the petals per flower. The gathered petals were shade-dried at room temperature before being extracted.

Distilled water was purified through a Simplicity UV® water purification system (Merck Millipore, Merck KGaA, Germany). 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), methanol, potassium-persulfate, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were bought from Sigma-Aldrich (Taufkirchen, Germany), Phospholipon 90 G (a commercial lipid mixture, sunflower phosphatidylcholine from non-genetically modified plants, $\geq$ 90%) from Lipoid GmbH (Germany), liquid phospholipid mixtures Phosal 75 SA (containing phosphatidylcholine in ethanol and safflower oil, content $\geq$ 72.0%) and Phosal 53 MCT (phosphatidylcholine in medium-chain triglyceride, content $\geq$ 53.0%) were from Lipoid Skopje (Macedonia), ethanol (Fisher Science, UK), and potassium persulfate (Centrohem, Serbia).

2.2. Extraction of Plant Material

The biologically active components were extracted from the petals using the maceration method, by employing the linear mechanical homogenizer (Roller mixer SRT6, Potsdam, Germany) at room temperature (25 $\pm$ 5 °C) for 24 h using analytical grade methanol as the extraction solvent, with a solid-to-liquid ratio of 1:20. The extracts were filtered using a qualitative laboratory filter paper. Before further analysis, the obtained extract was evaporated to a dry mass at 30 °C in a drying oven (Sanyo drying oven MOV-212, Eschborn, Germany), after which they were kept in the dark at 4 °C.

2.3. Preparation of liposomes

The liposomes containing *P. tenuifolia* petal extract were prepared by employing the proliposome technique (Jovanović et al., 2022). In short, the liposomes were prepared using three phospholipids Phosal SA 75, Phosal MCT 53, as well as Phospholipon. At 50 °C, the phospholipids (4 g), analytical grade ethanol (15 mL), deionized water (3 mL), and dried petal extract (0.4 g) were mixed together. After the mixture had cooled to room temperature, deionized water (20 mL) was added in small amounts and the mixture was stirred at 800 rpm for the duration of 1 h.

2.4. Antioxidant potential of Paeonia tenuifolia petal extract-loaded liposomes

2.4.1. ABTS method

The ABTS assay was performed employing a slightly modified procedure that was previously described by Čutović, Batinić, Marković, Radanović, Marinković, Bugarski and Jovanović (2022). The stock solution of the $ABTS^{\bullet+}$ radical (7.8 mmol/L) was prepared by dissolving 20 mg of $ABTS_{(s)}$ in 5 mL of deionized water, followed by the addition of 88 μL of $K_2S_2O_8$ solution with a concentration of 2.45 mmol/L (380 mg of potassium-persulfate$_{(s)}$ was dissolved in 10 mL of deionized water). The ABTS stock solution mixture was incubated for 16 to 20 h at 4 °C in the dark before use in order to activate the radical cation solution ($ABTS^{\bullet+}$). The obtained mixture was diluted with analytical grade ethanol to an absorbance value of approximately 0.700 at a wavelength of 734 nm. The precipitate obtained by the centrifugation of liposomes at 17500 rmp at 4 °C for 45 min was mixed with 200 μL of deionized water, in order to neutralize the amount of the supernatant drawn from the mixture. A sample (200 μL) was mixed with 2.8 mL of $ABTS^{\bullet+}$ solution and incubated for 30 min in the dark at room temperature. The measurements were performed in three parallel tries. The scavenging capacity was calculated as per Equation 1:

$$SC_{ABTS} = \frac{A_{cont} - A_{sample}}{A_{cont}} \cdot 100\% \quad \textbf{(1)}$$

where A_{sample} was the absorbance of $ABTS^{\bullet+}$ solution and the sample (the liposomes with *P. tenuifolia* petal extract), while A_{cont} was the absorbance of the blank solution.

2.4.2. DPPH method

The DPPH radical scavenging activity was assessed using method previously described by Blois (1958). The DPPH solution was prepared by combining 9 mL of ethanol with 0.252 mg of DPPH. The liposomal suspension (200 μL) was combined with 2.8 mL of this solution, and the mixture was then left to incubate in the dark, at room temperature for 30 min. The scavenging activity (SC_{DPPH}) was calculated using the following Equation (2) after the absorbance was measured at 517 nm:

$$SC_{DPPH} = \frac{A_{cont} - A_{sample}}{A_{cont}} \cdot 100\% \quad \textbf{(2)}$$

where A_{cont} represents the absorbance value of the blank solution, while A_{sample} is the absorbance of the liposomal suspension sample treated with the DPPH ion solution.

2.5. Statistical analysis

The statistical analysis was performed using the analysis of variance (one-way ANOVA) followed by Duncan's *post hoc* test, within the statistical software STATISTICA 7.0. The differences were considered statistically significant at $p < 0.05$, n=3.

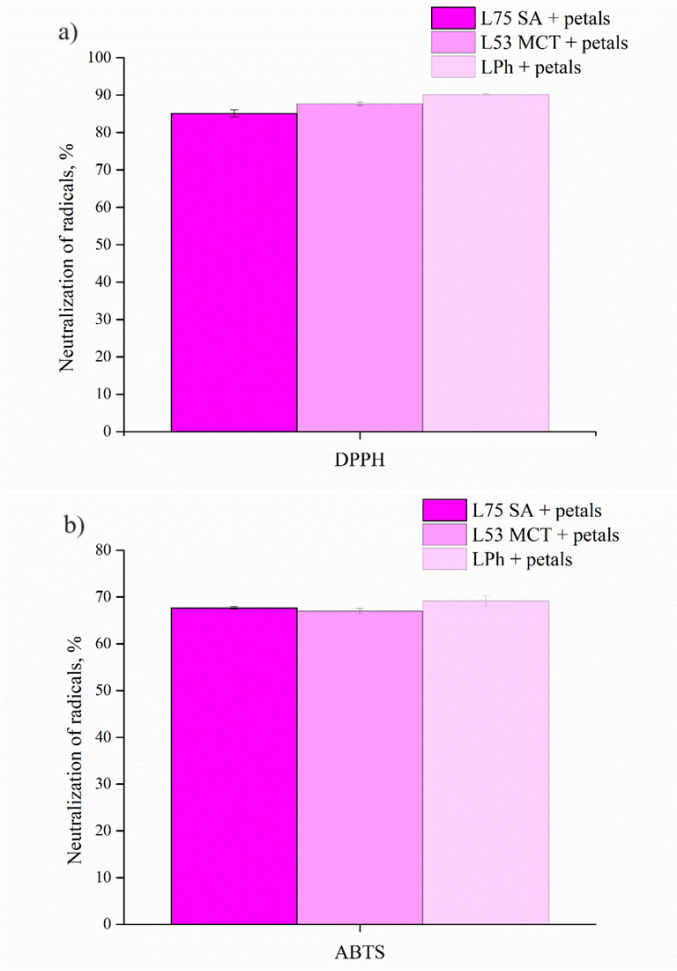

Fig. 1. Antioxidant potential of *Paeonia tenuifolia* petal extract-loaded liposomes: a) DPPH; b) ABTS.
*values with the same letter in each column showed no significant difference (p>0.05; n=3; analysis of variance, Duncan's *post hoc* test).

3. RESULTS AND DISCUSSION

In this study, the antioxidant potential of the *P. tenuifolia* petal dry methanolic extract-loaded liposomes, prepared using the proliposome method, was measured using two radical scavenging assays, ABTS and DPPH. The results of the antioxidant activity of the obtained liposomes are presented in Figure 1. L75 SA + petals – liposomes composed of Phosal 75 SA phospholipid mixture, and the petal extract, the L53 MCT + petals liposome contains Phosal 53 MCT and the petal extract, while LPh + petals, consists of Phospholipon and the petal extract. As can be seen from Figure 1, the ABTS radical neutralization capacity of the LPh + petals liposome was the highest, with 69.15 ± 1.14% of radical neutralization, followed by L75 SA + petals liposome at 67.68 ± 0.24%, which were similar to the L53 MCT + petals liposomes (66.99 ± 0.54%). The DPPH antioxidant potential of the liposomes followed a similar trend, with the highest level of DPPH ion neutralization achieved by LPh + petals liposomes (90.11 ± 0.3%), and L75 SA + petals being the least effective (85.12 ± 0.94%). The reason why LPh + petal liposomes showed the best ability to neutralize both $ABTS^+$ and DPPH ions could be attributed to difference in the composition of the lipid mixtures, as Phospholipon is a solid lipid mixture of sunflower phosphatidylcholine, whereas Phosal 75 SA and Phosal 53 MCT are liquid, and contain phosphatidylcholine in ethanol and safflower oil/or medium-chain triglyceride, thus making the bilayer of LPh + petals liposomes more permeable.

The antioxidant potential of pure methanolic *P. tenuifolia* petal extract has been a subject of our previous work, and it has been shown that the IC_{50} value for the neutralization of DPPH ions was 0.123 ± 0.001 mg/mL, whereas for the $ABTS^+$ ions was 0.099 ± 0.000 mg/mL, thus, when incorporated into liposomes, their antioxidant ability was changed, which may be due to the interactions between the lipids and the extract (Čutović et al., 2023) during the preparation of liposomes, as the bioactive compounds responsible for the neutralization of the $ABTS^+$ ions could possibly be encapsulated more efficiently, than the compounds that have the ability to neutralize DPPH ions in the *in vitro* assay (Nikolic et al., 2018). The chemical composition of the methanolic *P. tenuifolia* petal extract has been previously assessed (Čutović, Batinić, Marković, Radanović, Marinković, Bugarski and Jovanović, 2022), and it has been shown that the extract is rich in phenolic acids and flavonoids, which are compounds widely know for their antioxidant potential (Kim et al., 2012).

The comparison of results in this study to those from the literature is not always possible, due to the difference in their expression (%, mg of antioxidant compound equivalent/ mg or mL of extract, etc.), and methodology.

The obtained results for the ABTS radical scavenging activity for all three extract-loaded liposome types were higher than in the case of rosehip extract-loaded liposomes, where the neutralization of $ABTS^+$ radicals was 45.4 ± 1.8% (Jovanović et al., 2023), which is more than 20% lower than in the case of the least efficient *P. tenuifolia* petal extract-loaded liposome, probably due to differences in composition of the extracts, the content of compounds with antioxidant potential, and used phospholipid mixtures. In other study (Alemán et al., 2019) the ABTS assay was performed on the freeze-dried liposomes containing *Crithmum maritimum* leaves and stems aqueous and ethanolic extracts, and the results were 556.8 ± 4.26 mg vit C eq./g, and 805.1 ± 7.37 mg vit C eq./g, respectively. These results are not comparable to the ones presented in this study, as in this study liposomes were tested right after the preparation whereas theirs were lyophilized, and the expression units also differ. In case of DPPH radical scavenging, the results from this study were higher than in the research with rosehip extract-loaded liposomes (Jovanović et al., 2023), where the neutralization was 57.7 ± 1.7%, probably due to the difference in the chemical composition of the extracts, as well as the encapsulation efficiency of extracts into liposomes. On the other hand, in the work of Dag and Oztop (2017) the antioxidant activity of green tea extract-loaded liposomes determined by the DPPH assay was in the range from 15.051 to 20.451 mg DPPH/L sample, but the results are again not comparable due to differences in expression units.

4. CONCLUSION

In the present research, liposomes containing the dry methanolic extract of *P. tenuifolia* petals, endangered wild growing plants from Serbia, have been developed and characterized from the standpoint of their antioxidant activity, by the radical scavenging methods. The liposomal particles showed a very high level of both the ABTS and DPPH radical scavenging. However, the extract-loaded liposomes have shown to be more efficient in scavenging DPPH ions, due to the presence of antioxidants in the petal extract, more capable of interacting with them, than with $ABTS^+$. The lipid mixture that was used for the preparation of the liposomes with the highest antioxidant capacity is Phospholipon, due to the difference in its chemical composition, in comparison to the other two phospholipid mixtures. This was the first step in assessing the biological activities of *P. tenuifolia* petal extract-loaded liposomes, so further study should be focused on other biological activities for their potential industrial application.

ACKNOWLEDGMENTS

This work was supported by joint funding of the Ministry of Science and Technology of the Republic of Serbia (451-03-1202/2021-09/3; 451-03-47/2023-01/200003; 451-03-47/2023-01/200135; 451-03-47/2023-01/200019).

CONFLICT OF INTEREST

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

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Developed and validated HPLC method for simultaneous analysis of key flavonoids and phenolcarboxylic acids in the hawthorn-based cardiotonic Forticor®

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Received: November 24, 2023
Accepted: December 11, 2023
Published on-line: December 23, 2023
Published: December 25, 2023

Relatively simple, but very reliable, one-step qualitative/quantitative HPLC analysis of four key flavonoids, and a phenolcarboxylic acid, has been introduced. For substrate/sample preparations, either triple percolations, using 70% ethanol-water (V/V), or/and ultrasonic bath methanol extractions, were carried out to obtain the desired isolates yielding quercetin, isoquercitrin, hyperoside, vitexin and chlorogenic acid. The method is linear, over the studied range of 1.05 – 210.00, 6.25 – 250.00, 25.00 – 250.00, 7.50 – 150.00 and 1.04 – 10.40 μg/mL for chlorogenic acid, vitexin, hyperoside, isoquercitrin and quercetin, respectively. The correlation coefficient for each of the analytes was greater than 0.999. The intra-day and inter-day precision of the analysis was below 2.00 and 3.00 %, respectively. The accuracy of the analysis is verified by the standard addition method, using three different concentrations of each component in the tested materials, with recovery values obtained in the range of 98.04 – 102.47% (RSD $\leq$ 1.85%). The detection limits were 0.6, 0.5, 0.5, 0.8 and 0.3 μg/mL chlorogenic acid, vitexin, hyperoside, isoquercitrin and quercetine, respectively. The developed method is convenient in the routine control of hawthorn raw materials and pharmaceutical dosage forms made from hawthorn berries or flower-bearing branches. The new HPLC method may be used for the quality control of the commercially used cardiotonic Forticor® intended for increasing/strengthening cardiac muscles, in order to lower the risks of atherosclerosis, hypertension and congestive heart failure.

Keywords: Hawthorn; extraction; HPLC method; validation; forticor®

https://doi.org/10.61652/leksir2343e171T

1. INTRODUCTION

It is well known that the medicinal plant hawthorn i.e., *Crataegus monogyna* Jacq. (Lindm) *or C. laevigata* (Poir.) D.C., Rosaceae, is an excellent source of naturally occurring and biologically multi-active (cardiotonic, anti-arrhythmic, hypotensive, hypolipidemic, anti-inflammatory, gastro-protective, free-radical-scavenging and somewhat antimicrobial) valuable secondary metabolites, such as various flavonoids and phenolcarboxylic acids (Gheitasi et al., 2022; Li et al., 2022; Lin and Harnly, 2007; Lund et al., 2020; Tadić et al., 2008).

Numerous scientific studies suggest that the consumption of plants abundant in various phenolics may significantly contribute to human health. Over the last few decades, many publications on the analysis of plants phenolics have already appeared. Unfortunately, there is still neither a standardized extraction procedure for sample preparation/handling, nor general analytical methods available, including many variations of High Pressure/Performance Liquid Chromatography (HPLC) currently used to determine all beneficial polyphenols in a single step. Therefore, the need for better systematic approaches persists, and many researchers are impatiently striving to develop more and more robust analytical procedures for simultaneous determination of important subclasses of polyhydroxy aromatics (Lund et al., 2020; Orhan, 2019; Sagaradze et al., 2019).

At present, hawthorn extracts (from *Craetegus* sp.) are used for the treatment of declining cardiac performance, classified by the New York Heart Association (NYHA) as stages I and II. Based upon long-standing traditional usage, *Cratae-*

gus spp., *folium cum flore* has been utilized to relieve symptoms of temporary nervous cardiac complaints (e.g. palpitations, perceived extra heart beat due to mild anxiety) after serious conditions have been excluded (European Medicines Agency - EMA/HMPC, 2016). Dried flowering tops, flowers, leaves and fruits are taken/considered as crude drugs. Most often, medicinally used species are *C. monogyna* Jacq. (Lindm) and *C. laevigata* (Poir.) *D.C.*, *Rosaceae*, while less frequently are *C. pentagyna*, *C. nigra* and *C. azarolus* L. (Cui et al., 2024; Lu et al., 2023; Popovic-Milenkovic et al., 2014; Turnalar Ülger et al., 2023; Wang et al., 2011). Hawthorn species contain flavonoids, procyanidins and (-) epicatechin, and there are qualitative and quantitative differences in the flavonoid composition in the flowers, leaves and fruits of each species. The main flavonoids found in *Crataegus* species are flavonol-O-glycosides, such as hyperoside, flavone-3¬glycosides, like vitexin-2"-O-rhamnoside, as well as acetylvitexin-2"-O-rhamnoside (Tadić et al., 2008). Procyanidins, or condensed tannins, as flavan-3-ol oligomers and/or polymers, create a separate group of flavanoids, and are classified as the type A or B, depending on their interflavonoid linkages. In addition, hawthorn contains phenylpropanoids - phenolic carboxylic acids, such as a chlorogenic and a caffeic one.

Furthermore, the flavonoid glycosides are quantified in both the German and Swiss Pharmacopoeia, using the hydrolysis procedure, and expressing the overall quantity of flavonoids as hyperoside content by the photometric method (*DAB 10*, 1991). The composition data on phenolic acids, among *Crataegus* sp., are also insufficient, and the concentrations of different phenolics in hawthorn extracts are largely unknown. Once again, quantitative separations of phenolic compounds are needed to obtain their exact concentrations, but the polyphenols themselves interfere with the HPLC technology, therefore, in many cases, the additional sample "clean-up" procedures are unavoidable (Brown and Lister, 2014; Naczk and Shahidi, 2004).

In a continuation of our research, we have developed and validated a relatively simple, but very reliable, one-step qualitative/quantitative HPLC analysis of four key flavonoids, such as quercetin, isoquercitrin, hyperoside, vitexin and a phenolcarboxylic acid, chlorogenic acid.

For sample preparations, either triple percolations, using 70% ethanol-water (V/V) (the method used in the cardiotonic product Forticor® capsules, a trade mark in Serbia), or/and ultrasonic bath methanol extractions (as one of the methods commonly applied in investigation of plant extracts), were carried out to obtain the desired isolates. The new all-around procedure, including HPLC analyses, is already being applied to check the quality of the commercially used cardiotonic Forticor®, intended for strengthening cardiac muscles, to lower the risks of atherosclerosis, hypertension, and congestive heart failure.

2. MATERIALS AND METHODS

2.1. Plant extracts

Crataegus raw materials were obtained from the Pomoravlje region, Serbia, where plants are grown according to the principles of Good Agricultural Practice (GAP), while the extracts were produced according to Good Laboratory Practice (GLP). The plant materials were dried, at room temperature, in dark places. All the specimens, namely cm250609 and cl250609 (hawthorn leaf/flower) and cmb110909 and clb110909 (hawthorn berries) were deposited/kept at a Herbarium of Jevremovac Botanical Gardens, Belgrade.

2.2. Re-percolation of hawthorn samples

Dry plant materials were grinded to the size of 180 meshes, and put into percolation devices. Hawthorn leaf/flower (R-HLF) and hawthorn berry extract (R-HB) extracts were obtained by triple percolation with 70% ethanol, at a drug/solvent ratio of 1:1. After all extractions, liquids were evaporated, yielding 15.8 % and 10.5% (w/w) residues, expressed relatively as "dried" starting materials. Such obtained dry extracts (0.05 g of R-HLF and 0.55 g of R-HB, respectively) were dissolved in 10 mL methanol, then filtered through 0.2 μm PTFE syringe filters into glass HPLC vials, and later on analyzed, as described further in the text. These experiments were being repeated for five times, over the periods of three days.

2.3. Ultrasonic extraction of hawthorn plant samples and Forticor capsules content

Dry hawthorn leaf/flower (E-HLF, 0,51 g) and hawthorn berry (E-HB, 2.20 g) plant materials were grinded to the size of 180 meshes and extracted with 10 mL of methanol in ultrasonic baths, during 60 minutes. Forticor capsules homogenized content (grinded to 180 mesh, 450 mg each, on average) were also extracted, but with 50 mL of methanol in ultrasonic baths, during 60 minutes too. After extractions, all plant/Forticor samples were filtered through 0.2 μm PTFE syringe filters into glass HPLC vials, and later on analyzed, as described further in the text. These experiments were being repeated for five times, over the periods of three days.

2.4. Chemicals and reagents

HPLC grade acetonitrile, 85 % orthophosphoric acid and methanol were purchased from Merck (Darmstadt, Germany). All commercially available phenolic reference standards were obtained from Carl Roth (Karlsruhe, Germany). Their purity was declared as follows: chlorogenic acid > 98%, vitexin > 99%, hyperoside > 99%, isoquercitrin > 99% and quercetin > 99%, based on the manufacturer's internal high-precision HPLC method. For the long-term preservation, and to make sure they are to remain reliable, all standards were kept in desiccators and stored in a freezer, always protected from the oxygen and any kind of light(s).

2.5. Preparation of standards and creation of calibration curves

Pure chlorogenic acid, vitexin, hyperoside, isoquercitrin and quercetin, were used for a gradual dissolution in methanol (the approximate amounts were 2.1, 2.5, 2.5, 1.5 and 0.1 mg, respectively) into 10.0 mL volumetric flasks, followed by methanol dilutions in order to prepare final chemical standard solutions for the creation of calibration curves at target concentrations ranged 1.05 - 210.00, 6.25 - 250.00, 25.00 – 250.00, 7.50 – 150.00 and 1.04-10.40 μg/mL, respectively. The equations were ascertained by using a sample linear regression, while the correlation factor for each of the curves was calculated. The actual concentrations were expressed as percentage contents, corrected only for the impurities of the standards, as being declared by the fine chemical supplier. To ensure a complete linearity, calibration curves were always being visually inspected as well. Most relevant data are shown in Figure 2 and Table 1.

2.6. HPLC analyses

"Fingerprinting" of the investigated phenolic compounds was achieved by an Agilent Technologies 1200 HPLC machine, equipped with Lichrospher® 100 RP 18e column (5 μm, 250 x 4 mm), applying gradient elutions of two mobile phases, i.e., "A/B" ("A" – consisting of V/V 500.0 mL of water and 9.8 mL

of 85% phosphoric acid, and "B" - being a pure acetonitrile) at flow-rates of 1 mL/min, with photodiode-array (PDA) detection (UV at 360 nm), always within 70 min. Gradient elution was 89 – 75 % A (0 - 35 min); 75 – 60 % A (35 - 55 min); 60 – 35 % A (55 - 60 min) and 35 – 0 % A (60 - 70 min). The injection volume of standard solutions, as well as of the tested sample extracts, was 4 μL. The identification was based on retention times and overlay curves. Quantification was performed by the external calibration with already mentioned/described standards. Final results were being confirmed by so-called peak purity tests, as shown in Figure 2 and Table 3.

2.7. Method validation

2.7.1. Scopes of the analyses

The limits of the detection (LOD) and limits of quantification (LOQ) for each of the target compounds were being determined over a period of three consecutive days. The "noise" and retention times for the analyzed unidentified compounds were recorded and integrated. The standard deviation and the average values were calculated and used for the estimation of the method's LOD. The LOQ for each of the analytes was defined as the mean value, plus the ten standard deviations, calculated from the measured data, as shown in Table 1 and Table 3.

In accordance to the appropriate guideline, the limit of detection (LOD) for this assay was calculated as three times signal-to-noise ratio (S/N), while LOQ for this assay was calculated as ten times S/N level (*ICH Harmonised tripartite guideline: Validation of Analytical Procedures: Text and Methodology Q2(R1)*, 2014; Tadic et al., 2022).

2.7.2. Precision

For every tested sample, three people e.g., analysts, on three occasions, were engaged to collect the results - in three repetitions! Thus, in total, our analysts randomly ran a matrix of 15 experiments, over three-day periods during 5 business days of each week. Collected data were used to statistically determine the method's precision. All 3 intra-day, inter-day values and the peak areas, were expressed as their relative standard deviations (RSDs), while also ascertained for the target four key flavonoids and a phenolcarboxylic acid, by multiple analyses, as shown in Table 2.

2.7.3. Accuracy

A spike recovery study was used to check the accuracy of the method. Three amounts (0.15, 0.30 and 0.45 mg) were tested in three repetitions for all analytes, except for quercetin which was diluted ten times (0.015, 0.030 and 0.045 mg), to make Samples I, II and III, respectively. For every single analyte, the recovery degree was calculated by dividing the actual with the expected value and multiplying it by 100. The recovery of all tested compounds, at each spike level, was then calculated along with the average and standard deviation.

2.7.4. Stability of standards

At the beginning of the validation, all reference materials were prepared, as described above in the section "Preparation of standards and creation of calibration curves". To further confirm the stability of standards upon storing them, their fresh solutions were repeatedly prepared and checked again three weeks after the study begun. The concentrations were maintained at 100 μg/mL, with an exception, in the case of quercetin (5 μg/mL only), and the results were compared to data obtained after making their original/initial standard solutions, immediately upon receiving fine chemicals from the manufacturer.

3. RESULTS AND DISCUSSION

Identification of flavonoids (quercetin, isoquercitrin, hyperoside, vitexin) and phenolcarboxylic acid (chlorogenic acid), based on retention times and overlay curves with the appropriate standards, and further confirmed by so-called peak purity test is shown in Figure 1 and Figure 2.

Data regarding quantification of the investigated phenolics, obtained using calibration curves (Figure 3), are shown in Table 1.

Table 1. Overall quantification of five investigated phenolics from hawthorn isolates and Forticor capsules: R-HLF – re-percolated hawthorn leaf/flower; E-HLF - extracted hawthorn leaf/flower; R-HB - re-percolated hawthorn berry; E-HB - extracted hawthorn berry.

Samples	Analyzed	Phenolic content (mg/g of dry matter)	RSD (%)
R-HLF	Chlorogenic acid	14.8	1.37
	Vitexin	18.1	1.66
	Hyperoside	10.6	1.08
	Isoquercitin	9.5	0.65
	Quercetin	0.8	2.08
E-HLF	Chlorogenic acid	3.4	1.25
	Vitexin	3.1	1.22
	Hyperoside	2.5	1.82
	Isoquercitin	2.2	0.43
	Quercetin	0.1	0.71
R-HB	Chlorogenic acid	-	-
	Vitexin	0.1	1.8
	Hyperoside	1.2	0.91
	Isoquercitin	0.8	0.88
	Quercetin	0.1	1.81
E-HB	Chlorogenic acid	-	-
	Vitexin	-	-
	Hyperoside	0.3	0.32
	Isoquercitin	0.2	0.17
	Quercetin	-	-
Forticor capsules	Chlorogenic acid	17.1	0.35
	Vitexin	45	0.18
	Hyperoside	14.7	0.41
	Isoquercitin	12.4	0.79
	Quercetin	1.7	0.07

Results regarding method validation are presented in the appropriate tables, namely limits of the detection (LOD) and limits of quantification (LOQ) are shown in Table 2, while method precision and accuracy are shown in Table 3.

This paper referred to a relatively simple, but very reliable, newly developed, and validated one-step qualitative/quantitative HPLC analysis of four key flavonoids, and a phenolcarboxylic acid. During the course, for substrate/sample preparations, either triple percolations, using

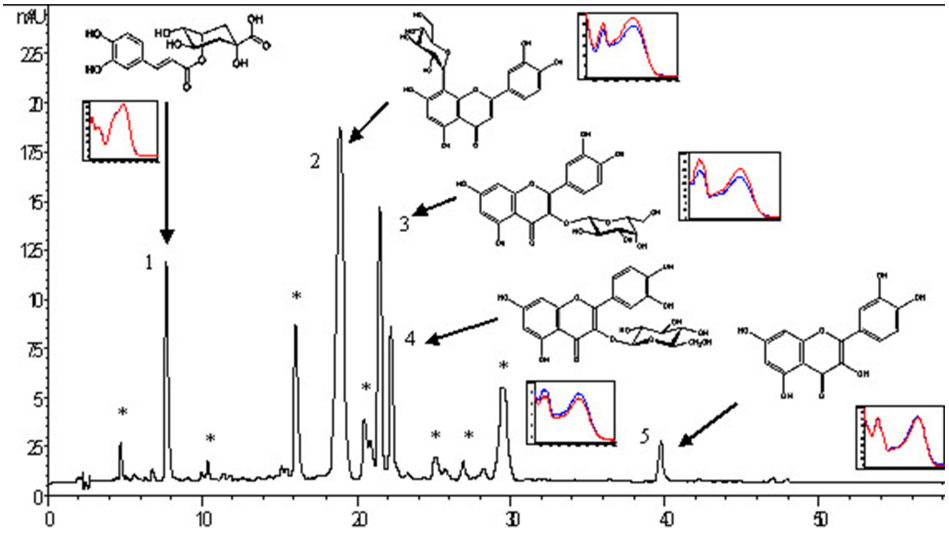

Fig. 1. HPLC analyses "fingerprinting" shown as correlated UV standard/sample detection data overlays: 1. Chlorogenic acid; 2. Vitexin; 3. Hyperoside; 4. Isoquercitin; 5. Quercetin; * Others – unidentified or/and not included.

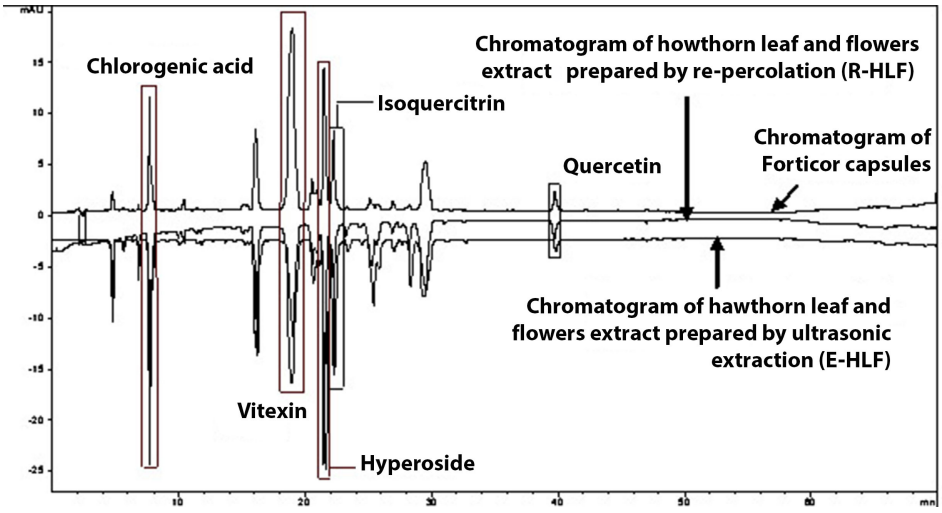

Fig. 2. A spike recovery study - overlay and mirrored.

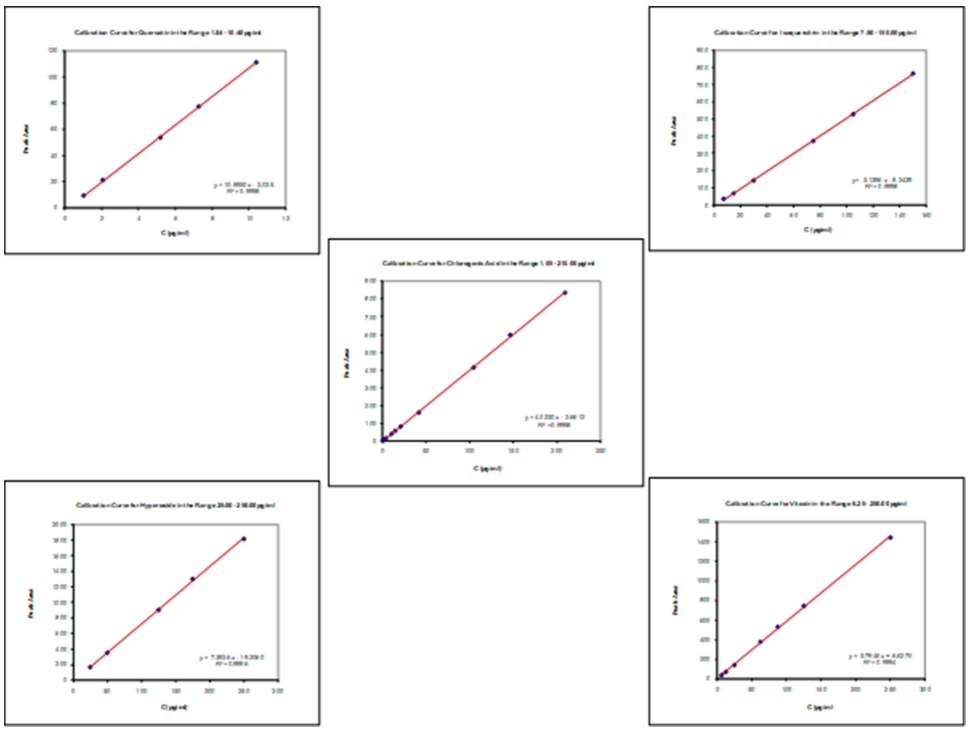

Fig. 3. Calibration curves of five phenolics from investigated hawthorn extracts.

Table 2. Key validation data from calibration curves of five investigated phenolics from various hawthorn isolates: CA – chlorogenic acid; V – vitexin; H – hyperoside; IQ – isoquercitin; Q – quercetin. y – Peak area; x – concentration (μg/ml).

Compound	Regression equation	Correlation coefficient (R)	Linear range (μg/ml)	Limit of detection (LOD) (μg/ml)	Limit of quantification (LOQ) (μg/ml)
CA	y = 4.0230 x – 2.6612	0.9998	1.05 – 210.00	0.6	1.99
V	y = 5.7946 x + 6.627	0.9994	6.25 – 250.00	0.5	1.66
H	y = 7.3936 x – 19.206	0.9996	25.00 – 250.00	0.5	1.66
IQ	y = 5.1396 x – 8.2439	0.9998	7.50 – 150.00	0.8	2.66
Q	y = 10.895 x – 2.0348	0.9998	1.04 – 10.40	0.3	1

Table 3. Validation results for intra-day and inter-day HPLC precision and accuracy: CA – chlorogenic acid; V – vitexin; H – hyperoside; IQ – isoquercitin; Q – quercetin. (R-HLF – re-percolated hawthorn leaf/flower; E-HLF - extracted hawthorn leaf/flower; R-HB - re-percolated hawthorn berry; E-HB - extracted hawthorn berry).

Component matrix	Method precision (% of relative standard deviation)						Method accuracy (% of spike recovery)				
	CA	V	H	IQ	Q	Tested	CA	V	H	IQ	Q
	R-HLF						R-HLF				
Day 1	1.19	1.17	0.88	1.09	0.63	Sample I	101.28	98.99	98.76	99.08	100.9
Day 2	0.87	0.71	0.58	0.53	2.31	Sample II	99.16	99.21	101.4	98.98	100.99
Day 3	0.35	0.52	0.21	0.42	0.18	Sample III	99.14	98.13	100.24	100.46	100.23
Inter-day	1.37	1.66	1.08	0.65	2.08						
	E-HLF						E-HLF				
Day 1	1.7	1.8	1.65	0.55	1.84	Sample I	100.65	100.65	100.09	100.2	101.71
Day 2	1.89	0.57	0.59	0.37	1.04	Sample II	99.08	100.27	100.59	99.25	101.18
Day 3	2.2	1.41	1.1	0.62	0.68	Sample III	100.05	100.76	100.47	100.26	101.5
Inter-day	1.25	1.22	1.82	0.43	0.71						
	R-HB						R-HB				
Day 1	-	1.73	1.33	1.2	1.33	Sample I	-	-	100.47	101.92	101.35
Day 2	-	2.95	0.51	0.54	0.88	Sample II	-	-	100.49	100.3	101.35
Day 3	-	1.71	0.83	0.88	0.39	Sample III	-	-	100.05	101.25	100.17
Inter-day	-	1.8	0.91	0.88	1.81						
	E-HB						E-HB				
Day 1	-	-	1.24	0.67	2.1	Sample I	-	-	98.25	99.03	100.44
Day 2	-	-	2.36	0.01	1.92	Sample II	-	-	100.56	98.67	100.36
Day 3	-	-	1.79	0.34	0.82	Sample III	-	-	100.81	101	101.12
Inter-day	-	-	0.32	0.17	0.85						

Table 4. Validation results for intra-day and inter-day HPLC precision and accuracy: CA – chlorogenic acid; V – vitexin; H – hyperoside; IQ – isoquercitin; Q – quercetin. (Forticor capsules).

Component matrix	Method precision (% of relative standard deviation)						Method accuracy (% of spike recovery)				
	CA	V	H	IQ	Q	Tested	CA	V	H	IQ	Q
	Forticor capsules						Forticor capsules				
Day 1	1.45	1.67	1.84	1.94	1.7	Sample I	99.47	99.7	99.33	99.78	102.48
Day 2	1.12	1.49	1.45	1.07	1.73	Sample II	99.31	99.71	98.04	100.53	100.2
Day 3	1.82	1.92	1.75	1.95	1.42	Sample III	99.5	100.15	100.46	100.8	101.99
Inter-day	0.35	0.18	0.41	0.79	0.07						

70% ethanol-water (V/V), or/and ultrasonic bath methanol extractions, were carried out to obtain the desired isolates yielding quercetin, isoquercitrin, hyperoside, vitexin and chlorogenic acid. Thereby, triple percolation using 70% ethanol-water (V/V) was shown to extract higher yield of target compounds compared to ultrasonic bath methanol extraction in both hawthorn leaves and flowers as well as hawthorn berries (Table 1). The method is linear, over the studied range of 1.05 - 210.00, 6.25 - 250.00, 25.00 – 250.00, 7.50 – 150.00 and 1.04 - 10.40 μg/mL for chlorogenic acid, vitexin, hyperoside, isoquercitrin and quercetin, respectively. The correlation coefficient for each of the analytes was greater than 0.999. The intra-day and inter-day precision of the analysis was below 2.00 and 3.00 %, respectively. The accuracy of the analysis was further verified by adding the fine chemicals, i.e. standards (in three different concentrations), to all tested materials, therefore applying a commonly accepted technique, which resulted in average recovery values obtained in the range between 98.04 - 102.47 % (RSD $\leq$ 1.85 %).

4. CONCLUSIONS

Bringing all of the above to a short conclusion, we firmly claim that one-step, e.g., at once, separations of quercetin, isoquercitrin, hyperoside, vitexin and chlorogenic acid in extracts of hawthorn leaves and flowers as well as hawthorn berries, obtained by two extraction procedures (triple percolation, using 70% ethanol-water (V/V) and ultrasonic bath methanol extraction) representing complex mixtures of a number of individual components. The established linearity, precision and accuracy of the developed and validated method also implied its applicability as a reliable quality test of commercially used cardiotonic Forticor® capsules (a trade mark in Serbia), as the basis for further investigations enabling potential clinical relevance of such multicomponent formulation for increasing/strengthening cardiac muscles, lowering the risks of atherosclerosis, hypertension and congestive heart failure.

ACKNOWLEDGEMENT

The authors thank The Serbian Ministry of Science, Technological Development and Innovations for supporting the project 451-03-68/2023-14/200003, and Eureka E!17236.

FUNDING STATEMENT

The work was funded by the Serbian Ministry of Science, Technological Development and Innovations for supporting the project 451-03-68/2023-14/200003, and Eureka E!17236.

CONFLICT OF INTEREST

None declared.

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Microbiological quality and efficacy of preservatives in marigold-based skin care products

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Received: November 30, 2023
Accepted: December 18, 2023
Published on-line: December 24, 2023
Published: December 25, 2023

The microbiological stability of cosmetic products is crucial, especially in products with high water content, such as creams. Therefore, preservatives are used in order to protect consumers from harmful pathogens that would otherwise invade the cosmetics. Despite their function, the addition of preservatives can be considered controversial in the cosmetics industry. Parabens are frequently used synthetic preservatives in various industries. According to regulatory directives concerning cosmetics (Cosmetic Directive by the European Commission) usage of some parabens (methylparaben, propylparaben, butylparaben, and ethylparaben) is safe and permitted in allowed concentrations. On the other hand, the European Commission prohibited the use of isopropylparaben, isobutylparaben, phenylparaben, benzylparaben and pentylparaben. Therefore, a comprehensive stability study was conducted to evaluate the influence of paraben-based preservative (Gujsol-1®, INCI name: Phenoxyethanol, Methylparaben, Ethylparaben, Propylparaben, Butylparaben) and potassium sorbate in order to preserve plant extracts-based skin-care products, Day and Night creams with marigold extract. Previously, mentioned skin-care products were preserved with the preservative Dekaben C®, which contained paraben mixture with banned isobutylparaben. All tested formulations showed favorable texture and sensory characteristics, desirable mild acidic pH values, satisfactory physical stability and appropriate microbiological quality at the initial point, as well as after storage at elevated temperature or at cyclic stress test conditions and at room temperature during two years. In addition, both preservatives significantly reduced the number of inoculated microorganisms during the challenge test. It can be concluded that potassium sorbate in plant-based creams could be an efficient alternative to paraben-based preservatives.

Keywords: plant-based cosmetics; marigold; parabens; challenge test; storage stability

https://doi.org/10.61652/leksir2343e172S

1. INTRODUCTION

Today, the cosmetics industry represents a growing part of the economy, with a focus on cosmetic products based on environmentally-friendly natural ingredients. This trend is driven by the global transition to a "greener" lifestyle, and so the demand for natural ingredients is increasing. Polyphenolic compounds, essential oils, and plant extracts are natural ingredients that are broadly used in the cosmetics industry (Piccolella et al., 2019). These compounds with proven antioxidant, anti-inflammatory, and soothing properties, could increase the value of cosmetic products, especially skin-care products. Although natural-based cosmetics contain a large amount of bioactive nutrients, they could also disrupt product stability (Rybczyńska-Tkaczyk et al., 2023). Moreover, high water content in cosmetic products, such as creams, favors the development of pathogenic microorganisms, that could affect the quality of products, as well as human health (Barthe et al., 2021). Substances with antimicrobial properties, used as preservatives in cosmetics, could ensure their shelf life and safety (Campana et al., 2006). Despite their function, the addition of preservatives can be considered as one of the most con-

troversial issues in the cosmetics industry. Recently, there has been a growing interest in replacing synthetic chemical preservatives with natural ones, as the increasing use of natural compounds in cosmetic products has raised customer awareness of the negative effects of synthetic preservatives (Rybczyńska-Tkaczyk et al., 2023). Therefore, effective and low-toxicity preservative compounds without side effects should be imperative in modern cosmetology (Rybczyńska-Tkaczyk et al., 2023).

Parabens are synthetic preservatives used in various industries since 1920. They are derived from synthetic esters of *p*-hydroxybenzoic acid (methylparaben, ethylparaben, n-propylparaben, benzylparaben, isobutylparaben, isopropylparaben, n-butylparaben and heptylparaben and their respective sodium salts) (Nowak et al., 2021). Unfortunately, when excessive quantities of cosmetic preparations containing these compounds were used, adverse health outcomes of parabens have been reported and some studies speculated that parabens demonstrated side effects on the endocrine system and even diseases such as cancer, thyroid disorders, skin allergies, or even reproduction and neurological problems (Barthe et al., 2021; Matwiejczuk et al., 2020; Mitra et al., 2021). Despite reports found in literature causing bad marketing reputation of parabens, according to the valid regulatory directives concerning cosmetic (European Commission, n.d.) usage of some parabens such as methylparaben, propylparaben, butylparaben, and ethylparaben is permitted, while some parabens are banned for use in cosmetic products.

Day and Night creams (Neven dnevni krem® and Neven noćni krem®) with marigold flowers (*Calendula officinalis* L., Asteraceae) extract have been produced by the Institute for Medicinal Plants Research "Dr. Josif Pančić" (Belgrade, Serbia), for more than 10 years. Marigold is an annual herbaceous plant abundantly distributed in Central Europe, Middle Eastern countries and the Mediterranean regions, with a long history of use in traditional medicine (Shahane et al., 2023). The 10th European Pharmacopoeia states that ligulate flowers (*Calendulae flos*), which have been detached from the receptacle, represent drug intended for pharmaceutical use. Phytochemical studies of marigold flowers have found that the most important constituents are triterpene pentacyclic alcohols (dominantly faradiol derivatives) and triterpene saponins (calendasaponins A, B, C and D), along with polysaccharides, carotenoids (lutein and zeaxanthine), flavonoids (hyperoside and rutin), coumarins and essential oil (Barnes et al., 2007; Heinrich et al., 2017; Shahane et al., 2023). These pharmacologically active principles could be connected with antioxidant, anti-inflammatory, antimicrobial and wound-healing activities of marigold, which have been demonstrated in numerous *in vitro* and *in vivo* experiments (Barnes et al., 2007; Shahane et al., 2023). On the basis of these attributes, the Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA) approved marigold flowers to be used in different forms of traditional herbal medicinal products for the symptomatic treatment of minor inflammations of the skin, as well as aid in the healing of minor wounds.

However, previously mentioned Day and Night skin care formulations were manufactured and preserved using the mixture under the trade name Dekaben C® (INCI Name: Phenoxyethanol, Methylparaben, Ethylparaben, Butylparaben, Isobutyl paraben, Propylparaben). Prohibition of isobutylparaben usage, one of the ingredients of the used preservative mixture, according to the Cosmetic Directive by European Commission, from the regulatory safety aspect, imposed reformulation of stated products, in terms of preservative replacement.

For this purpose, Day and Night creams were preserved with Gujsol-1® (INCI name: Phenoxyethanol, Methylparaben, Ethylparaben, Propylparaben, Butylparaben) or potassium sorbate instead of preservative Dekaben C®. Gujsol-1® was chosen due to the similarity to the previously used Dekaben C®, but without the presence of the unacceptable isobutylparaben. Potassium sorbate is a potassium salt of sorbic acid, widely used as a preservative in cosmetic products. This cosmetic ingredient provides prolonged shelf life of the product by inhibition of the growth of mold, yeast, and bacteria, and thus prevents spoilage and ensures consumer safety. Considering that it represents salt of unsaturated fatty acid that participate in the normal human fat metabolism. Therefore, it is oxidized into carbon dioxide and water, without accumulation in the human body (Jorge, 2003).

Bearing in mind that preservatives have a crucial influence on the microbiological stability of cosmetic products, comprehensive stability study was carried out in order to evaluate influence of different preservatives (Gujsol-1® and potassium sorbate) on Day and Night skin care formulations with marigold extract.

2. MATERIALS AND METHODS

2.1. Creams preparations

Two types of formulations were prepared, Day Cream with marigold extract-DC and Night Cream with marigold extract-NC, both prepared with two different preservatives, Gujsol-1® (DC-1 and NC-1, respectively) and potassium sorbate (DC-2 and NC-2, respectively).

Marigold propylene glycol extract (0.5 %, w/v) (1:4, solid: solvent ratio) and rose hip propylene glycol extract (0.3 %, w/v) (1:4, solid: solvent ratio), obtained from the production sector of the Institute for Medicinal Plant Research "Dr. Josif Pančić" (Belgrade, Serbia), were used for creams preparations. Extracts were prepared by percolation method with propylene glycol (45%) as a solvent instead of alcohol, as propylene glycol has a beneficial effect on the skin and it is well tolerated in the cream formulations. In addition to both formulation 's composition the following active components were incorporated: avocado oil, shea butter, elastin protein, vitamin E, natural wetting factors. Night cream additionally contained glycine soja (soybean) seed extract. The formulation was made without alcohol addition, paraffin and synthetic dyes in order to grease the skin and impair the natural skin barrier. Day and Night skin-care creams were prepared by continuous mixing of the oil and aqueous phases. Active compounds were added at a temperature below 45 °C in order to preserve the stability of the sensitive active compounds, such as avocado oil, a mixture of extracts, vitamin E, natural moisturizing factor, and glycine soja (soybean) seed extract. Day and Night formulations with marigold extract, assigned as DC-1, NC-1, were prepared with Gujsol-1® (1%) as preservative, while formulations DC-2 and NC-2 were preserved with and potassium-sorbate (0.6%). Formulations DC-1 and DC-2, as well as NC-1 and NC-2 were prepared in laboratory conditions, while control samples (DC-control and NC-control) were prepared by the standard procedure in the production sector of the Institute for Medicinal Plants Research "Dr. Josif Pančić" (Belgrade, Serbia) using Dekaben C® as preservative.

2.2. Stability tests

Stability tests of prepared formulations were performed on both series of reformulated skin care creams with Gujsol-1® (series DC-1 and NC-1) and potassium sorbate (series DC-2 and NC-2), as well as samples with the Dekaben C® (DC-control and NC-control). Samples were filled in the original packaging and divided into three groups, for the purpose of

stability tests according to the following protocol:

2.2.1. 1) Storage at room temperature (during the expected shelf life)

The stability of the samples was evaluated after 24 hours after preparation and then after 7, 19, 90, 180, 270 days, 12, 18 and 24 months of storage of the samples at 25 ± 2 °C.

2.2.2. 2) Accelerated storage with increased temperature

The stability of the samples was monitored after the sample's storage at increased temperature conditions (40 ± 2 °C), without light in the thermostat chamber. The samples were analyzed after 90 and 180 days.

2.2.3. 3) Cyclic stress test

Samples were exposed to a cyclic stress test, where formulations were evaluated initially after preparation and after 18 days. This test was performed 24 hours after samples preparation, and samples were exposed to three different temperature conditions, *i.e.*, at reduced temperature (4 ± 2 °C), room temperature (25 ± 2 °C) and increased temperature (40 ± 2 °C), following six cycles in total.

2.3. Formulation analysis

Organoleptic properties, pH, and physical stability were monitored at defined time points during the stability tests.

2.3.1. Organoleptic characteristics

The color, consistency and homogeneity of the samples were monitored by visual evaluation in the package, by application and smearing on a glass plate. Application characteristics (lubricity, appearance, shine of the smear on the skin, penetration (absorption) of the cream into the skin, subjective feeling of hydration, dryness/oiliness and occlusion) were monitored after applying the product to the skin.

2.3.2. Measurement of pH value

This analysis was performed by the potentiometric method by pH meter (Hanna instruments HI 99161, Portugal), directly in the prepared samples, by immersing the electrode of the pH meter in the tested samples. Before measurements, the apparatus was calibrated with standard buffers pH 7.0 and pH 4.0.

2.3.3. Centrifugal tests

An adequate mass of formulations was put into plastic cuvettes, and then subjected to centrifugation (3000 rpm, for 30 min), in order to examine potential separation of the water and oil phases (Centrifuge, Hermle Z206 A).

2.4. Microbiological stability of skin-care formulations

Prepared creams should be applied on face skin and thus should meet certain requirements regarding microbiological integrity (purity), according to the Regulation on Cosmetic Products (*Official Gazette of RS, 60/2019, 47/2022, 21/2023*, 2023). According to this Regulative, microbiological quality acceptance criteria required in cosmetic products for adults should be:

- the total number of aerobic microorganisms (bacteria plus yeast and molds) should not be greater than 10^3 CFU/g or CFU/mL

Candida albicans Absence in 1 g or 1 mL

Staphylococcus aureus Absence in 1 g or 1 mL

Pseudomonas aeruginosa Absence in 1 g or 1 mL

Escherichia coli Absence in 1 g or 1 mL

2.5. Challenge tests

A test of the preservative's effectiveness (challenge tests) of the tested formulations was also performed. The antimicrobial properties of the preservative are considered adequate if under the test conditions, there is a significant decrease or no increase in the number of microorganisms in the inoculated preparation after the defined period.

The test microorganisms for analysis were selected according to the recommendation of the ISO 11930 standard:

Bacteria:

Escherichia coli ATCC 8739

Pseudomonas aeruginosa ATCC 27853

Staphylococcus aureus ATCC 25923

Yeast:

Candida albicans ATCC 10231

Mold:

Aspergillus brasiliensis (*A. niger*) ATCC 16404

Creams inoculated with calibrated inoculum, which was prepared from relevant ATCC strains were evaluated. Suspensions of selected microorganisms in the inoculum for contamination were made in adequate liquid media (Mueller Hilton Broth and Tryptone Soya Broth). After overnight incubation, the number of cells in the inoculum was measured by spectrophotometric method *via* optical density (o.d.). The number of cells in the suspension was adjusted for both bacteria (10^7 CFU/mL), yeast and mold (from 10^4 to 10^6 CFU/mL). Successive decimal dilutions of the calibrated suspension were made in the diluent. The number of test microorganisms in the suspension was determined by seeding 1 mL of the appropriate decimal dilutions in duplicate on the appropriate substrates. The plates were incubated at 32 ± 2.5 °C for 48 h (bacteria and *C. albicans*) and at 25 ± 2.5 °C for 5 days for *A. brasiliensis*.

According to the ISO Standard 11930:2019 procedure, calibrated inoculum (0.2 mL) was added to cream formulations (20 g) to obtain a certain number of microorganisms in the final concentration. In addition, inoculation with mixed cultures of all test microorganisms was also performed, in duplicate. All creams were mixed after inoculation to ensure a homogeneous distribution of the inoculum. The inoculated creams were stored in the premises of the microbiological laboratory of the Institute for Medicinal Plants Research "Dr. Josif Pančić" (Belgrade, Serbia), where analyses were performed at each specific interval in accordance with the ISO Standard method. Samples were stored at room temperature (25 °C) which support the growth of microorganism and their possible reaction with preservative in the samples.

Before starting the test, it was necessary to check zero, *i.e.* initial determination of the microbiological purity (integrity) of the prepared samples.

The number of viable microorganism's existent in creams was performed at specific intervals in accordance with each strain, 7, 14, 28 days after inoculation, by plate count method. The microbiological stability of the creams and the antimicrobial potential of the investigated preservatives were checked even after 50 days of storage. According to the regulations, microbiological stability of the creams and the antimicrobial potential of the investigated preservatives, Gujsol-1® and potassium sorbate should be considered appropriate if the abundance of

all microorganisms decrease in all tested intervals.

3. RESULTS AND DISCUSSION

3.1. Organoleptic characteristics

Fig. 1. Organoleptic characteristics of Day and Night cream formulations after the period of cyclic stress tests of 18 days (from the left to the right: DC-1, DC-2, NC-1, NC-2, NC-control and DC-control, respectively).

According to the results of organoleptic analysis, all tested formulations were white to light orange homogeneous, glossy creams, with a pleasant, mild smell. Moreover, all creams were easily spread on the skin, leaving a gentle feel. It was evident that during real storage and cyclic temperature stress tests, there were no changes in the organoleptic characteristics of creams (Figures 1 and 2). On the other hand, during storage under increased temperature a slight change of creams color (*i.e.*, slightly darker color) was observed. It is known that color changes could be caused by chemical reactions (oxidation, reduction and hydrolysis), as well as chemical interactions between formulation ingredients, which are particularly induced at high temperature conditions (Kamaruzaman and Yusop, 2021).

3.2. Evaluation of pH value

With regard to the effectiveness and stability of preparations intended for topical applications, the pH value of creams represents an important parameter that should be determined. In the current Book of cosmetic products (*Official Gazette of RS, 60/2019, 47/2022, 21/2023*, 2023), the pH value of the cosmetic product should be in the range of 3.0 to 10.0. It has been established that the skin surface has a mild acidic pH, within the range of 4.0 to 6.0, which is associated with a number of physiological roles, such as defense mechanism against harmful microorganisms and activation of enzymes responsible for the skin barrier function (Buraczewska and Lodén, 2004). The results of the pH values of the examined creams are shown in Table 1 and Figure 3. Generally, the pH values of the creams differed in dependence on the formulation, while storage conditions had a low impact on this parameter. The lowest pH value was estimated for NC-1 (3.22) whereas the highest pH value was determined for DC-2 (6.08), which can be ascribed to intrinsic characteristics of preservatives. Namely, in case of

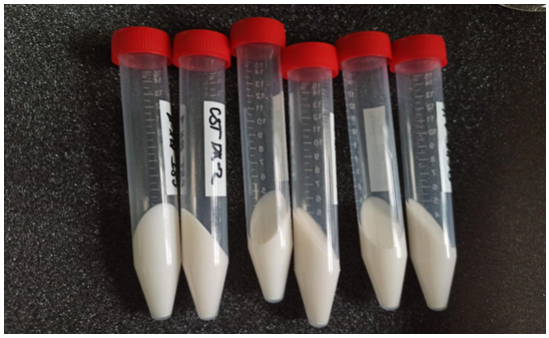

Fig. 2. Day and Night cream formulations after the centrifugation test after the period of 18 months storage (from the left to the right: DC-1, DC-2, NC-1, NC-2, NC-control and DC-control, respectively).

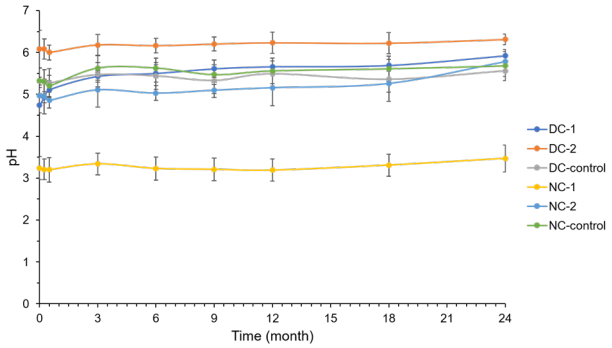

Fig. 3. pH of investigated Day Cream (DC) and Night Cream (NC) formulations during 24 months under real storage conditions.

Gujsol-1® contained creams, DC-1 and NC-1, the measured pH values were lower in comparison to creams with potassium sorbate, DC-2 and NC-2. During the storage of samples at room (25 ± 2 °C) and elevated (40 ± 2 °C) temperature, a mild increase of pH values was observed. The noticed changes in pH values may be due to the chemical reaction which has been occurred between the ingredients of creams, followed by formation of different classes of chemical compounds (aldehydes, acids, alcohols). Moreover, literature data indicate that fluctuations in the pH could be affected by nature of plant extract, temperature at the time of the measurement of pH and product contamination (Kamaruzaman and Yusop, 2021; Sathya et al., 2017). Also, after the cyclic temperature stress test, almost the same level of pH was measured in creams compared to the initial point.

3.3. Centrifuge assay

In order to evaluate the physical stability of creams, a centrifuge assay was performed. The centrifugation test is simulating the influence of gravity on the investigated samples and no signs of phase separation or other physical instabilities after centrifugation were found in any of the tested samples obtained during real storage, accelerated storage, and cyclic temperature stress testing. The results obtained in this assay indicate that creams have satisfactory physical stability during 24 months at room temperature, as well as during 6 months at elevated temperature.

3.4. Microbiological stability

A key aspect of cosmetic products' safety is related to their microbiological quality and stability. Cosmetic products should not represent a potential risk to the customer's health throughout their shelf time period. Although cosmetic products are not sterile, they should not be contaminated with pathogenic microorganisms, and the content of non-pathogenic (saprophytic) microorganisms must be at an appropriate (low) level (Kim et al., 2020).

The microbial contaminants of cosmetics can originate from a) contaminated raw materials, b) the specific production process of cosmetics, c) the sanitary conditions of the environment and equipment (*i.e.*, improper air conditioning, reuse of utensils, etc.) as well as d) poor personal hygiene. Water and raw materials of animal, plant, and mineral origin *per se* are among the main sources of bacterial contamination. Moreover, consumers store most cosmetic products in the bathroom, where the temperature and humidity are elevated, which affects the development of microorganisms (Kim et al., 2020; Krajišnik and Đekić, 2018; Neza and Centini, 2016).

Several studies have shown that the most frequently found microorganisms in cosmetics are *P. aeruginosa, S. aureus, E. coli*, and *Bacillus* species, as well as other bacteria, mold, and yeasts

Table 1. pH of investigated Day Cream (DC) and Night Cream (NC) formulations under accelerated storage conditions and cyclic stress test conditions.

	Cyclic stress test		Accelerated storage		
	0 days	18 days	0 days	90 days	180 days
DC-1	4.74 ± 0.18	5.31 ± 0.15	4.74 ± 0.18	5.88 ± 0.21	6.01 ± 0.21
DC-2	6.08 ± 0.21	5.99 ± 0.13	6.08 ± 0.21	6.17 ± 0.41	6.11 ± 0.28
DC-control	5.32 ± 0.13	5.41 ± 0.29	5.32 ± 0.13	5.87 ± 0.26	5.91 ± 0.16
NC-1	3.22 ± 0.18	3.13 ± 0.11	3.24 ± 0.18	3.30 ± 0.17	3.48 ± 0.14
NC-2	4.97 ± 0.29	5.72 ± 0.17	4.97 ± 0.29	5.71 ± 0.19	5.68 ± 0.31
NC-control	5.33 ± 0.12	5.41 ± 0.25	5.33 ± 0.12	5.69 ± 0.14	5.80 ± 0.08

(Budecka and Kunicka-Styczyńska, 2014). Fungal multiplication in cosmetics is more rapid and evident than bacteria, and fungal infections can be caused by *Candida, Aspergillus*, and *Penicillium* species. Microorganisms such as *P. aeruginosa*, *S. aureus*, *C. albicans* and *A. brasiliensis* were restricted by EU Pharmacopoeia as most founded contaminants pretentiousness microbial spoilage in cosmetics and risk to the consumer health.

The consequence of the growth and reproduction of microorganisms in cosmetic products can cause degradation of ingredients and disruption of the organoleptic characteristics of the products that manifest as a visible presence of microorganisms and/or the appearance of an unpleasant odor, change in pH, texture, viscosity or color, discoloration, destruction of emulsion systems (Orus and Leranoz, 2005). Microorganisms can survive in harsh conditions and are resistant to various antimicrobial agents. Since microorganisms are ubiquitous in nature, they can be easily transported to the cosmetic environment through human hands.

The current European regulatory framework in the field of cosmetic products requires manufacturers to provide results of preservative efficacy testing (challenge test), as well as to ensure microbiological stability data that are documented in the safety report. It is also important to identify the microbiological stability and durability of cosmetics through the storage period and to determine the potential risk of contamination during consumer use.

In our study, creams with preservatives potassium sorbate and Gujsol-1® were experimentally evaluated in order to investigate the microbiological stability of the DC-1, DC-2, DC-control, NC-1, NC-2, NC-control during storage and application. Day and night skin-care formulations with both tested preservatives were microbiologically stable immediately after production as well as in each tested point after storage for a certain period at elevated temperature, and during cyclic stress test. At the zero point of the test, immediately after the formulation production, a certain but allowed total number of aerobic mesophilic microorganisms (bacteria plus yeasts and molds) was detected in DC-2 (Table 2). In the following testing points their number was completely reduced by the activity of preservatives. Furthermore, all investigated formulations stored at room temperature were microbiologically stable for two years.

This result has indicated that Day and Night skin-care formulations, with both preservatives, potassium sorbate and Guisol-1®, had good microbiological quality during the entire period of storage (2 years). Regardless of whether the formulations were exposed to room temperature, elevated temperature or cyclic stress test conditions, as previously explained, the applied preservatives provided them excellent microbiological quality throughout the test period.

3.5. Challenge tests

Given that EU Regulation 1223/2009 does not provide recommendations for a specific test that can be used to test the effectiveness of preserving cosmetic products (preserving microbiological purity), several tests are in use: Pharmacopoeia (Ph. Eur. 11.0), ISO 11930, Koko test, etc. Generally, ISO Standard 11930 ("Evaluation of the antimicrobial protection of a cosmetic product" or "Challenge test") is an overall standard, which is used to evaluate the anti-microbial stabilization of a cosmetic product.

Before the Challenge test performance, the microbiological quality of the samples was checked according to the Regulation on cosmetic products (*Official Gazette of RS, 60/2019, 47/2022, 21/2023*, 2023), which was zero point of initial examination. According to this Regulation, the number of certain microorganisms was determined before treatment (initial - zero test), presented in Table 2. The obtained results indicated that all creams were microbiologically approved. The conservation efficiency test is based on the "provoking" formulation in the final packaging by suitable microorganisms' inoculum. Namely, after the storage of inoculated preparation at a prescribed temperature, samples were analyzed at certain time intervals by counting microorganisms detected in the samples. The antimicrobial properties of the preparation are adequate if, in the test conditions, there is a significant decrease or no increase in the number of microorganisms in the inoculated preparation after the examination period at the defined temperatures.

The results of the tests at seven-day intervals are shown in Table 3. After seven days of inoculation, the presence of all microorganisms in a certain number in both cream formulations with both preservatives was confirmed. After 14 days of storage, the complete absence of *E. coli* and *P. aeruginosa* was established in the Day and Night creams due to the action of both tested preservatives, Gujsol-1® and potassium sorbate. The number of other tested microorganisms was significantly reduced. Results of the challenge test of the investigated formulations after 28 days, demonstrated a reduction of the number of all inoculated test microorganisms to a minimum by the action of both tested preservatives. After 50 days of storage, only the presence of *S. aureus* in formulations with potassium sorbate and *A. brasiliensis* in formulations with both preservatives was determined in very small numbers. Similar results were obtained in creams inoculated with a mixture of all tested microorganisms, where the total number of inoculated microorganisms was completely eliminated or reduced to a minimum with both tested preservatives.

Table 2. Microbiological quality of skin-care formulations with marigold extract before treatment (initial - zero test) according to the Regulation of cosmetic products (*Official Gazette of RS, 60/2019, 47/2022, 21/2023*, 2023)

Type of testing	Reference values	DC-1	DC-2	NC-1	NC-2
Total aerobic mesophilic microorganisms (bacteria plus yeasts and molds)	$<10^3$ CFU/g ili mL	$<10^2$	$2x10^2$	$<10^2$	$<10^2$
Candida albicans	absence in 1 g or 1 mL	not found	not found	not found	not found
Pseudomonas aeruginosa	absence in 1 g or 1 mL	not found	not found	not found	not found
Escherichia coli	absence in 1 g or 1 mL	not found	not found	not found	not found
Staphylococcus aureus	absence in 1 g or 1 mL	not found	not found	not found	not found

Table 3. Challenge test.

Test microorganisms	Initial inoculum	Obtained values			
		DC– 1	DC – 2	NC– 1	NC – 2
***Escherichia coli* ATCC 8739**	10^7				
After 7 days		$2x10^3$	$2x10^4$	$3x10^3$	10^4
After 14 days		/	/	/	2x102
After 28 days		/	/	/	/
***Pseudomonas aeruginosa* ATCC 27853**	$2x10^7$				
After 7 days		$2x10^2$	10^2	$2x10^2$	$3x10^2$
After 14 days		/	/	/	/
After 28 days		/	/	/	/
***Staphylococcus aureus* ATCC 25923**	$3x10^7$				
After 7 days		$3x10^4$	$5x10^5$	$2x10^4$	10^5
After 14 days		$2x10^2$	$4x10^3$	$2x10^2$	$2x10^4$
After 28 days		20	20	30	$5x10^3$
After 50 days		/	/	10	$2x10^2$
***Candida albicans* ATCC 10231**	$2x10^6$				
After 7 days		$3x10^3$	$2x10^3$	$5x10^3$	$2x10^4$
After 14 days		$5x10^2$	$2x10^2$	$3x10^2$	$3x10^3$
After 28 days		10^2	$<10^2$	$<10^2$	10^2
After 50 days		/	/	/	/
***Aspergillus brasiliensis* ATCC 16404**	10^4				
After 7 days		10^3	$2x10^3$	10^2	$3x10^2$
After 14 days		$5x10^2$	$4x10^2$	$2x10^2$	10^2
After 28 days		$2x10^2$	10^2	10^2	$<10^2$
After 50 days		10	$<10^2$	10	/
Mixed inoculation					
After 7 days		5x 10^3 *S. aureus* 10^2 *P. aeruginosa* $7x10^3$ *C. albicans* $6x10^2$ *E. coli* $7x10^2$ *A. niger* 5x 10^3 *S. aureus*	$3x10^2$ *A. niger* $5x10^3$ *E. coli* 10^2 *P. aeruginosa* $7x10^3$ *C. albicans* 5x 10^3 *S. aureus* $5x10^2$ *S. aureus*	$>10^4$ *S. aureus* $2x10^2$ *A. niger* 10^3 *C. albicans* $2x10^2$ *A. niger* $<10^2$ *P. aeruginosa*	$2x10^2$ *A. niger* $2x10^3$ *E. coli* $6x10^3$ *C. albicans* $2x10^2$ *P. aeruginosa* $3x10^4$ *S. aureus* $<10^2$ *E. coli*
After 14 days		$3x10^2$ *C. albicans* $2x10^2$ *A. niger*	$<10^2$ *A. niger*	$4x10^3$ *S. aureus;* 10^2 *A. niger;*	10^4 *S. aureus*
After 28 days		10^2 *C. albicans* 30 *S. aureus*	20 *S. aureus* 10^2 *A. niger*	10^3 *S. aureus*	10^3 *S. aureus*
After 50 days		/	/	10 *S. aureus*	10 *S. aureus*

Based on the obtained results, it can be concluded that both preservatives significantly reduced the number of inoculated microorganisms, indicating that they can be used as effective preservatives in cosmetic products with herbal extracts, such as Day and Night cream with marigold extract.

4. CONCLUSION

Preservatives are substances with a crucial influence on the microbiological stability of cosmetic products. This study highlighted the influence of paraben-based preservative blend (Gujsol-1®) and potassium sorbate, on the stability of creams containing marigold flowers propylene glycol extract. All tested formulations showed favorable texture and sensory characteristics, desirable pH values, satisfactory physical stability and appropriate microbiological quality at the initial point, as well as during the entire period of storage of two years. On the basis of these results, it can be concluded that potassium sorbate could be an efficient alternative to paraben-based preservatives, such as Gujsol-1®, in formulations containing plant extracts.

ACKNOWLEDGMENT

This research work was supported by the Ministry of Science and Technological Development and Innovation of the Republic of Serbia, contract numbers 451-03-47/2023-01/200003.

CONFLICT OF INTEREST

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

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Antimicrobial activity of *Hyssopus officinalis* L. subsp. *aristatus* (Godr.) Nyman (Lamiaceae) essential oils from Montenegro and Serbia

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Received: November 30, 2023
Accepted: December 12, 2023
Published on-line: December 24, 2023
Published: December 25, 2023

In this study, antimicrobial activity of essential oils extracted from the aerial flowering parts (herbs) of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman (Lamiaceae) collected from five different locations in Montenegro, or purchased in Serbia, were investigated. In addition, their antibacterial activity in combination with antibiotics was studied. The antimicrobial activity against selected standard bacterial and yeast strains was investigated using the broth microdilution method. Two standard antibiotics were used for comparison: the aminoglycoside antibiotic amikacin and the cephalosporin antibiotic ceftriaxone. The antimicrobial activity of the essential oil-amikacin combination was investigated using the checkerboard assay. The main components of the essential oils were 1,8-cineole, *cis*-pinocamphone, β-pinene and limonene in varying quantities. Most of the tested essential oils showed no significant antimicrobial activity. However, an essential oil rich in *cis*-pinocamphone showed moderate activity against both *Staphylococcus aureus* and *Escherichia coli* (MIC = 400 μg/mL). The overall effect of the essential oils and antibiotic combinations against *E. coli* or *S. aureus* ranged from additive (FICI = 0.625) to indifferent (FICI = 1.5), depending on the source of the essential oil.

Keywords: *Hyssopus officinalis*; essential oil; antimicrobial activity; additive effect

https://doi.org/10.61652/leksir2343e173M

1. INTRODUCTION

The genus *Hyssopus* L. (Lamiaceae) comprises 13 recognized plant species, which are mainly distributed in the temperate climate zone of Eurasia, from the Mediterranean region via Central Asia to Mongolia. In Montenegro and Serbia, the genus is monotypic; there is only one species, *Hyssopus officinalis* L. subsp. *aristatus* (Godr.) Nyman. The species is mainly distributed in the Mediterranean region, and the places in Serbia (Pirot and Nišava regions) are one of the northernmost points of its distribution range (Diklić and Janković, 1974; Gbif, 2023).

In Montenegro, hyssop is an unprotected plant species, according to the Rulebook on the Detailed Manner and Conditions of Collection, Use and Trade of Unprotected Wild Animal, Plant and Mushroom Species Used for Commercial Purposes (*Official Gazette of Montenegro*, 2010), in contrast to Serbia, where it is a protected wild plant species, according to the Rulebook on the Designation and Protection of Strictly Protected and Protected Wild Species of Plants, Animals and Fungi *Official Gazette of the Republic of Serbia, 5/2010, 47/2011, 32/2016 and 98/2016; Anex II*. Hyssop is frequently used in folk medicine. It is also used in the food and cosmetics industry, as well as an ornamental plant. The use of the hyssop herb and its preparations (infusions, syrups, tinctures, extracts) for various purposes is well documented in folk medicine - as a carminative, stomachic, tonic, diaphoretic, emmenagogue, expectorant, antiseptic, muscle relaxant; for digestive and intestinal problems, loss of appetite, stomach pain and cramps; for urinary tract infections; for the treatment of respiratory diseases such as tuberculosis, asthma, chronic catarrh and bronchitis, coughs,

sore throats, respiratory infections, fever and respiratory irritations associated with the common cold, etc. It is also valued for the treatment of rheumatic pain, bruises, wounds, burns, frostbite, skin irritations, anxiety and hysteria, toothache, earache, for regulating blood pressure and night sweats (Charles, 2012; Judžentienė, 2016; Milovanović, 1975; Özer et al., 2006; Tucakov, 2010; Venditti et al., 2015).

Despite the numerous data on traditional use, scientifically sound information on hyssop herb use is rather limited. The competent institutions and associations, such as the European Medicines Agency (EMA), European Scientific Cooperative on Phytotherapy (ESCOP), Commission E of the German Federal Ministry of Health, as well as the World Health Organization (WHO) have not yet published official monographs regulating the use of herbal medicinal products based on *H. officinalis*. Moreover, there is no official information in modern pharmacopeias about the specific pharmacopoeial quality of herbal medicinal products from the aerial parts of *H. officinalis*.

Within the concept of rational phytotherapy, pharmacognostic studies of the wild hyssop herb are a necessary step towards the rational use of this herbal medicinal product. In general, antimicrobial activity is one of the most studied effects of various essential oils. The alarming increase in bacterial resistance to antibiotics has become an urgent challenge for the medicine and veterinary medicine, as well as for the pharmaceutical and food industries (Krist et al., 2015; Llor and Bjerrum, 2014). The search for antimicrobial agents from medicinal plants represents a promising alternative to synthetic chemicals. In this context, plant constituents, especially essential oils and their main components, have attracted a lot of attention.

According to the available literature data, the studies on the combined use of hyssop essential oil with antibiotics are scarce. Therefore, a better understanding of the antimicrobial activity of hyssop essential oils from Montenegro and Serbia has aroused our scientific interest.

2. MATERIAL AND METHODS

2.1. Plant material, essential oil isolation and chemical analysis

The essential oils tested in this study were obtained earlier by Mićović et al. (2021) using hydrodistillation of the aboveground flowering parts (herb) of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman, collected at five different locations in the territory of Montenegro or purchased from a local provider in Serbia (Table 1).

The chemical compositions of the essential oils were analyzed by GC-FID and GC-MS, and their chromatographic profiles were described in detail in the publication by Mićović et al. (2021).

2.2. Antimicrobial activity of isolated essential oils

The antimicrobial activity of the essential oils was investigated using the broth microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). Six standard strains of microorganisms were used for the study: Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633), Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* NCIMB 9111, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027) and one yeast strain (*Candida albicans* ATCC 10231).

The experiment was performed on 96-well microtiter plates with serial dilutions of the tested essential oil samples dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) in concentration 1000 μg/mL and further diluted in Mueller-Hinton broth (Torlak, Serbia) to the tested concentrations (25-500 μg/mL). Fresh, overnight cultures of microorganisms were suspended in sterile physiological saline solution, and suspensions with a turbidity of 0.5 McFarland (density of approximately 2 x 10^8 CFU/mL) were obtained. The suspensions were further diluted (in Mueller-Hinton broth for bacterial strains and in Sabouraud dextrose broth; Torlak, Serbia for *C. albicans*) to a final density of 2 x 10^6 CFU/mL. In each well, 100 μL of the corresponding essential oil solution and 100 μL of bacterial or fungal suspension were mixed.

The results (minimum inhibitory concentration - MIC) were interpreted after incubation of the plates at 35 °C for 24 hours. Two standard antibiotics were used for comparison: the aminoglycoside antibiotic amikacin (Sigma-Aldrich, USA) and the cephalosporin antibiotic ceftriaxone (Sigma-Aldrich, USA). The concentration range of the antibiotics was between 0.01 and 8.0 μg/mL. Minimum inhibitory concentrations were determined by the absence of turbidity in the well of the microtiter plate, indicating inhibition of the growth of the inoculated microorganism. The minimum concentration of essential oil at which no turbidity occurred actually represented the MIC value. Each test was repeated three times, and the average values of the results obtained were calculated.

2.3. Interaction of essential oils with amikacin

Checkerboard method was used to evaluate combined effects of the essential oils and amikacin. In brief, the method was performed in 96-well polystyrene microtiter plates, by pouring decreasing concentrations of tested essential oils and twofold dilutions of examined antibiotic, lower than the recorded MICs, into horizontal and vertical wells, respectively (Langeveld et al., 2013). The bacterial suspension was prepared as described earlier (10^6 CFU/mL). Each well was filled with the same amounts of tested agents (50 μL) and a bacterial suspension to a final volume of 200 μL. The last two vertical rows of the microtiter plate represented the positive control and contained only the bacterial suspension without antimicrobial agent. After the incubation of plates for 24 h at 35 °C, MICs were determined as the lowest concentrations of combinations,where visible growth was absent.

To assess the interaction of the essential oil with the antibiotic, the fractional inhibitory concentration index (FICI) was calculated. The fractional inhibitory concentration (FIC) of each substance in the mixture is obtained by dividing the MIC of that substance in the mixture by the MIC of the pure substance (e.g. FIC of essential oil = MIC of essential oil in the mixture with antibiotic / MIC of essential oil).

The FICI value represents the sum of the FIC of the essential oil and the FIC of the antibiotic, and is interpreted as follows (Hu et al., 2002; Orhan et al., 2005):

1. FICI $\leq$ 0.5 indicates synergy;
2. 0.5 < FICI $\leq$ 1 indicates additivity;
3. 1 < FICI $\leq$ 2 indicates indifference (no effect) and
4. FICI $\geq$ 2 indicates antagonism.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial activity of isolated essential oils

The MIC of investigated hyssop essential oils on the tested bacterial strains was mostly > 500 μg/mL and significantly weaker than the same feature of antibiotics ceftriaxone and amikacin used for comparison (Table 2).

Slightly better activity, *i.e.* a lower MIC value compared to those mentioned, was found in cases of *Escherichia coli* (samples 1, 3 and 5) and *Staphylococcus aureus* (sample 3). The

Table 1. Data on investigated plant material and essential oils.

Sample	Origin	Site of collection	Geographic coordinates	Altitude (m)	Voucher Specimen	Main essential oil - constituents
1	Commercial (Serbia)	Southeastern Serbia	N/A	N/A	N/A	1,8-cineole (67%), limonene (8%), β-pinene (7%), methyl eugenol (5%)
2	Wild-growing (Montenegro)	Kuči	N42°31′55″ E19°24′07″	870	1420263	1,8-cineole (42%), β-pinene (9%), limonene (8%), cis-pinocamphone (6%)
3	Wild-growing (Montenegro)	Šavnik	N42°57′16″ E19°05′59″	880	1420261	cis-pinocamphone (23%), methyl eugenol (19%), β-pinene (16%), limonene (16%), 1,8-cineole (10%)
4	Wild-growing (Montenegro)	Piva	N43°9′25″ E18°50′46″	750	1420162	methyl eugenol (28%), limonene (24%), β-pinene (16%), cis-pinocamphone (15%), trans-pinocamphone (8%)
5	Wild-growing (Montenegro)	Piperi	N42°34′23″ E19°16′0.8″	800	1420259	1,8-cineole (38%), limonene (22%), cis-pinocamphone (15%), β-pinene (10%)
6	Wild-growing (Montenegro)	Cuce	N42°35′19″ E18°47′40″	820	1420260	1,8-cineole (56%), limonene (15%), methyl eugenol (14%), β-pinene (5%)

Table 2. Minimum inhibitory concentrations of investigated essential oils and selected antibiotics (DMSO < 1%).

Microorganism (strain)	**Minimum inhibitory concentration (MIC; μg/mL)**							
	1	**2**	**3**	**4**	**5**	**6**	**Ceftriaxone**	**Amikacin**
Staphylococcus aureus ATCC 6538	>500	>500	**400**	>500	>500	>500	4	4
Bacillus subtilis ATCC 6633	>500	>500	>500	>500	>500	>500	2	0.5
Escherichia coli ATCC 8739	**400**	>500	**400**	>500	**400**	**500**	2	1
Klebsiella pneumoniae NCIMB 9111	>500	>500	>500	>500	>500	>500	4	0.25
Salmonella Typhimurium ATCC 14028	>500	>500	>500	>500	>500	>500	2	2
Pseudomonas aeruginosa ATCC 9027	>500	>500	>500	>500	>500	>500	8	2
Candida albicans ATCC 10231	500	500	500	500	500	500	n.t.	n.t.

n.t.– not tested.

growth of *Candida albicans* was inhibited at a minimum concentration of 500 μg/mL for all samples.

The essential oils 1 (commercial sample, Serbia) and 6 (Cuce, Montenegro) had similar compositions: they were high both in 1,8-cineole and β-pinene, but low in *cis*-pinocamphone. In contrast, the essential oils 3 (Šavnik, Montenegro) and 5 (Piperi, Montenegro) did not have similar chemical profiles: the dominant component in sample 5 was 1,8-cineole, while *cis*-pinocamphone was the predominant component in sample 3. In all cases, the essential oils samples 1, 3, 5 and 6 showed moderate antimicrobial activity against the *E. coli* strain, while 3 was effective against both *E. coli* and *S. aureus*.

The observed antimicrobial activities of the essential oils are probably due to the dominant compounds 1,8-cineole and *cis*-pinocamphone. These results are consistent with some previously published data in the literature, not only for the essential oil of *Hyssopus officinalis*, but also for some other commercially used essential oils such as cajuput oil, eucalyptus oil, sage oil and similar, as well as pure substances (Aguilar-Rodríguez et al., 2022; Jiang et al., 2021; Kizil et al., 2010; Mazzanti et al., 1998; Wińska et al., 2019). Although the biological properties of essential oils and the extent of their effect are closely related to their main constituents and their high concentrations, whole essential oils often exhibit greater antibacterial activity than their main constituents alone; therefore, the influence of the other constituents should not be ignored (Yap et al., 2014).

There are also reports in the literature on the antimicrobial effect of hyssop essential oils against the fungal strain *C. albicans* (Hristova et al., 2015; Kizil et al., 2010; Mazzanti et al., 1998; Venditti et al., 2015). For example, the study by Hristova et al. (2015) showed that a commercially available hyssop essential oil from Bulgaria with *cis*-pinocamphone, β-pinene and *trans*-pinocamphone as dominant components exhibited antifungal activity against *C. albicans* with a MIC of 210 μg/mL. The mechanism of antifungal activity of hyssop essential oil is thought to be related to increased permeability of fungal cells and disruption of normal membrane transport acting on the membrane ATPase (Hristova et al., 2015). We can only speculate why the essential oils of *H. officinalis* did not show similar activity against *C. albicans* in our study, but it seems reasonable to attribute the activity to *cis*-pinocamphone, as its concentration did not exceed 23% in the essential oils we tested, in contrast to the results of Hristova et al. (2015), where the content of this component reached even up to 50% in some samples.

3.2. Interaction of essential oils with amikacin

In combination therapy, the combined effect of two or more drugs is greater than the sum of their individual effects, which leads to a synergistic result. This is in contrast to an additive effect, where the combined effect is equal to the sum of the individual effects, and an indifferent effect, where there is no interaction between the drugs. Antagonism occurs when the combined effect is less than when the two drugs are used individually (Iseppi et al., 2021; Yap et al., 2014).

One strategy to combat antibiotic resistance is to find compounds that can counteract the antibiotic-destroying enzymes produced by bacteria (for example, clavulanic acid can inhibit β-lactamase enzymes that break down penicillin-type antibiotics). This led to the development of amoxicillin/clavulanate, a combination preparation that is effective against many bacteria that are resistant to amoxicillin alone. However, the effectiveness of combination drugs can be limited by the development of resistance to the new drug, as the widespread use of clavulanic acid has led to the emergence of resistant variants of bacteria. This underlines the need for continuous research and development of new antibiotics and resistance-breaking compounds (Monserrat-Martinez et al., 2019; Yap et al., 2014).

Numerous essential oils have antimicrobial properties that are of great importance in various scientific and industrial fields, including medicine, agriculture and cosmetology. Of the roughly 250 commercially available essential oils, about a dozen have remarkable antimicrobial potential. Essential oils appear to be a promising alternative to synthetic compounds, especially in view of the increasing resistance of pathogenic microorganisms (Iseppi et al., 2021; Wińska et al., 2019).

The combination of conventional antibiotics and essential oils is a relatively new concept. In some cases, such as in this study, essential oils have been found to enhance the antimicrobial effect of conventional antibiotics, even when they do not have a significant inhibitory effect on their own.

When essential oils and antibiotics were combined and tested against antibiotic-resistant bacteria, a significant reduction in antibiotic concentrations was observed in many cases, thus minimizing the adverse effects of these drugs (Iseppi et al., 2021; Yap et al., 2014). A key advantage of combining antibiotics with essential oils is that they can target different bacterial molecules. This can lead to new treatment options to overcome the growing problem of microbial resistance.

Essential oils are intrinsically multi-component plant products. In most cases, they are rather intricate blends of a number of different compounds, such as hydrocarbons, aromatic or aliphatic alcohols, acids and their derivatives, aldehydes and ketones, phenolics and phenylpropanoids, that account for their wide range of pharmacological and therapeutic effects (Bunse et al., 2022; Caneschi et al., 2023). Therefore, the antimicrobial activity of essential oils is probably based on a combination of mechanisms, unlike many conventional antimicrobials that have a single point of action.

In many cases, essential oils and their components easily penetrate the cell membranes of bacteria due to their lipophilic nature and cause irreversible damage to the cell architecture, leading to disruption of various cellular processes and ultimately to cytolysis and cell death. The interaction of essential oils with the bacterial cell wall could be used to enhance antibiotic activity, facilitate their penetration and allow a reduction in therapeutic doses (Bunse et al., 2022; Caneschi et al., 2023; Iseppi et al., 2021; Yap et al., 2014). This also makes it less likely that they will lead to the development of resistant bacteria, and this is the reason for all the effort put into studying the combined activity of essential oils and antibiotics (Yap et al., 2014).

The results of our study of the combined effect of essential oils with the antibiotic (amikacin) are shown in Table 3.

For all combinations, the MIC of the essential oil and the antibiotic in combination was, as expected, lower than that of the individual active ingredients. However, the true indicator of the activity of the combination is the FICI value (Table 4).

Our study showed that commercial sample of essential oil (sample 1) with 1,8-cineole as the main component had an additive effect with the antibiotic (amikacin) against the *E. coli* strain, while the sample 3 of essential oil rich in *cis*-pinocamphone had an additive effect with amikacin against both *S. aureus* and *E. coli* strains. According to the available information, this is the first study to investigate the combined use of hyssop essential oil with an antibiotic.

4. CONCLUSIONS

The available literature data on the antimicrobial activity of hyssop preparations are diverse and depend on numerous factors that ultimately influence the composition of the essential oil, such as plant subspecies/variety, habitat, extraction method, etc.

Table 3. Antimicrobial activity of hyssop essential oils in combination with the antibiotic (amikacin; DMSO < 1%).

Microorganism (strain)	Minimum inhibitory concentration (MIC; μg/mL)					
	1/amikacin	2/amikacin	3/amikacin	4/amikacin	5/amikacin	6/amikacin
S. aureus ATCC 6538	200/0.5	200/0.5	200/0.5	200/1	200/1	200/1
E. coli ATCC 8739	200/0.5	200/0.5	200/0.5	200/1	200/1	200/1
K. pneumoniae NCIMB 9111	400/0.0625	400/0.0625	400/0.0625	200/0.5	200/0.5	200/0.5
S. Typhimurium ATCC 14028	200/4	200/4	300/2	300/2	300/1	300/1

Table 4. Fractional inhibitory concentration indices (FICI) for active essential oils.

Combination	Bacteria	FICI	
		Value	Interpretation
1/amikacin	*E. coli*	1	Additivity
3/amikacin	*S. aureus*	0.625	Additivity
3/amikacin	*E. coli*	1	Additivity
5/amikacin	*E. coli*	1.5	Indifference
6/amikacin	*E. coli*	1.4	Indifference

This study has shown that hyssop essential oil has some potential to act as an antimicrobial agent. Moderate activity of certain samples of the tested essential oils against *S. aureus* and *E. coli* strains was shown, which could also be related to the use of hyssop in traditional medicine for mild respiratory and urinary tract infections; also, their additive effect with the antibiotic (amikacin) was demonstrated. In addition, it was also shown that essential oil of hyssop has antimicrobial potential against the fungus *C. albicans*.

Further research in this direction is certainly needed to clarify the mechanism of action and to find out which main components or combinations of components are responsible for the antimicrobial activity.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Science, Technological Development and Innovation, Republic of Serbia through a Grant Agreement with the University of Belgrade – Faculty of Pharmacy No: 451-03-47/2023-01/ 200161.

CONFLICT OF INTEREST

None declared.

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Alternaria leaf spot on *Paeonia peregrina* and *Paeonia tenuifolia* in Serbia

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Received: November 24, 2023
Accepted: December 19, 2023
Published on-line: December 25, 2023
Published: December 25, 2023

Paeonia peregrina* Mill. and *Paeonia tenuifolia* L. are endangered species of herbaceous plants with high ornamental and medicinal values. There are a few natural habitats in Serbia where these plants spontaneously grow: Krivi Vir, Skrobnica, Golina, Pirot, Deliblato Sands, and Sokobanja, in the municipality of Knjaževac. In July 2021, the symptom of leaf spots was observed on *P. peregrina*, while spots on leaves, stems, and lower branches on *P. tenuifolia* in the localities of Pirot and Deliblato Sands. Disease incidence was estimated to 32% in Pirot and 25-35% in Deliblato Sands. Therefore, the aim of the study was to identify the causal agent of leaf spot on *P. peregrina* and *P. tenuifolia* in Serbia by molecular identification and characterization, and morphological characterization. For molecular identification and characterization, nuclear ribosomal internal transcribed spacer (ITS) region, partial translation elongation factor 1-alpha (*EF-1α*), beta-tubulin (*TUB2*) and histone 3 (*H3*) genes were amplified using primer pairs ITS1/ITS4, EF1-728F/EF1-986R, T1/Bt2b, and H3-1a/H3-1b, respectively. Morphological characterization of the representative isolates was done on potato dextrose agar (PDA) and potato carrot agar (PCA) media at 22 °C under an 8 h light:16 h dark regime. Based on the molecular and morphological characteristics of the obtained isolates, *Alternaria alternata* was identified as the causal agent of leaf spot on *P. peregrina and P. tenuifolia*. In Serbia, to the best of our knowledge, this is the first report of *A. alternata* causing a leaf spot of *P. tenuifolia*.

Keywords: *Alternaria alternata*; herbaceous peonies; molecular identification; molecular characterization; morphological characterization

https://doi.org/10.61652/leksir2343e176M

ABBREVIATIONS

PDA – potato dextrose agar
PCA – potato carrot agar
ITS – nuclear ribosomal internal transcribed spacer
EF-1α – translation elongation factor 1-alpha gene
TUB2 – beta-tubulin gene
H3 – histone 3 gene
NaClO – 1% sodium hypochlorite solution
ML – Maximum likelihood tree
UV – ultraviolet
CTAB – cetyltrimethylammonium bromide
NCBI – the National Center for Biotechnology Information

1. INTRODUCTION

There are 34 species in the genus *Paeonia*, which are grouped into two subgenera (Moutan and herbaceous peonies) and seven sections (Hong, 2021). Herbaceous peonies make up one of five sections that comprise the subgenus *Paeonia* (Prijić et al., 2023). Temperate Asia, southern Europe, and western North America are habitats for *Paeonia peregrina*, known as the Balkan or Kosovo peony, and *Paeonia tenuifolia*, known as the fern leaf or steppy peony (*The Plant List*, 2023). In Serbia, their collection from nature is protected by law and requires permission from the Ministry of Environmental Protection of the Republic of Serbia.

Alternaria spp. can be found as saprophytic, endophytic, and pathogenic species in agricultural crops and products, soil, and organic matter (Neergaard, 1945; Pavón Moreno et al.,

2012; Polizzotto et al., 2012). The species are located in semi-arid and humid areas, and can affect leaves, stems, flowers, and fruits of several plant species (Deshpande, 2002). Also, *Alternaria* spp. are well-known as post-harvest pathogens that cause disease in over 100 host plants (Thomma, 2003). The species can survive on infected crop debris, seeds, and weeds in the form of mycelia or conidia, and can be transmitted by insects, wind, rain, irrigation, etc. Conidia are carried by air currents to the aerial parts of plants throughout the growing season (Battilani et al., 2009; Laemmlen, 2002). *Alternaria* spores are one of the most important airborne allergens (Thomma, 2003). The production of melanin, particularly in the spores, and the production of host-specific toxins in the case of pathogenic species are two distinguishing characteristics of *Alternaria* species (Thomma, 2003). The species of the genus, host plant, and combination of right temperature and humidity are important factors in the external environment for the development of infection. The spores penetrate the host's tissue through stomata, cuticle, or wounds. The first symptoms of infection appear after 2–3 days on the leaves and stems of the host plant. Black necrotic lesions surrounded by chlorotic halos are characteristic of leaf spots. Leaf spot can lead to reduced leafy crops. Additionally, it may cause the host to abscise leaves, thus lowering photosynthetic potential and crop yield indirectly (Armitage, 2013; Battilani et al., 2009).

Despite the increase in peony production and consumption, available information on peony diseases and their treatment is limited. *Alternaria* spp. has been found on herbaceous and tree peonies in Canada (Coulson, 1923), the United States (Anonymous, 1960), China (Zhang, 2003), Korea (Cho and Shin, 2004), Bulgaria (Bobev, 2009), and the Netherlands (Andersen et al., 2009). The Texas Plant Disease Handbook (Anonymous, n.d.) describes the leaf spots as irregularly shaped, purplish-brown to reddish-brown. Later, the leaves may turn yellow and fall off (Texas Plant Disease Handbook). Coulson (1923) described *Alternaria* spp. as causing yellow leaf spots, irregularly shaped with a clearly defined, dark margin. Leaf spots caused by *Alternaria suffruticosae*, *A. suffruticosicola*, and *A. tenuissima* were circular or irregularly shaped, brown to black (Zhang et al., 2008). Cheng et al. (2018) recorded diversity of fungi of *Aspergillus*, *Alternaria*, and *Penicillium* on the leaf, stem, and root of *Paeonia lactiflora* Pallas. *Alternaria* sp., especially *A. alternata*, showed the maximum frequency of colonization and isolation (10.95% and 31.68%), similar to the results of previous reports (Jennings et al., 2002; Lin et al., 2007; Xiao et al., 2013). In addition, *A. tenuissima* was identified as the causal agent of red leaf spot disease on *P. lactiflora* in China (Sun and Huang, 2017). The first symptoms on the leaves appeared as small, circular, reddish to dark brown, necrotic spots that gradually enlarged and became irregular. The spots grew together, and the leaves withered, dried up, and defoliated. This disease destroys the aesthetic value of the Chinese peony and potentially reduces the yield and quality of its medicinal properties, affecting the economic importance of this plant.

The identification of *Alternaria* species is difficult and at present, molecular and phylogenetic analyses are essential for recognizing species (Lawrence et al., 2015; Woudenberg et al., 2015). Different loci have been used for molecular identification and characterization, among which are the nuclear ribosomal internal transcribed spacer (ITS) region (Lawrence et al., 2015; Matić et al., 2019; Woudenberg et al., 2015), partial translation elongation factor 1-alpha (*EF-1α*) (Lawrence et al., 2015; Siciliano et al., 2018; Woudenberg et al., 2015), beta-tubulin (*TUB2*) (Lawrence et al., 2015; Matić et al., 2019; Peever et al., 2004; Siciliano et al., 2018), and histone 3 gene (*H3*) (Garibaldi et al., 2018a; Matić et al., 2019; Wang et al., 2019; Zhang et al., 2020; Zhao et al., 2016).

In Serbia, *A. alternata* has been reported on *P. peregrina* originating from the location of Bogovo Guvno (Mikić et al., 2023), while there isn't information about this pathogen on *P. tenuifolia* (Farr and Rossman, 2023).

2. MATERIALS AND METHODS

2.1. Plant collection and fungal isolation

In July 2021, leaf spot were observed on *P. peregrina* and *P. tenuifolia* in the natural habitats in Pirot (43° 07′ N; 22° 26′ E 666) and Deliblato Sands (44° 57′ N; 21° 03′ E 167) areas, Serbia. The spots appeared on leaves and sporadically on stems of *P. peregrina*, while identical spots, white mycelia and rot appeared on stems and lower branches of *P. tenuifolia*. The percentage of infected plants at a given locality were calculated by counting the numbers of healthy and symptomatic plants separately. In total, 197 and 100 individual plants of the single populations of *P. peregrina* and *P. tenuifolia* were found on about 0.5 ha. Sixty-three plants of *P. peregrina* and 50 plants of *P. tenuifolia* were symptomatic.

Symptomatic plants were transplanted into pots and transferred to the laboratory. Isolation of the pathogen was done from symptomatic leaves and stems. Leaf fragments (5x5 mm) of five symptomatic plants and stem fragments (5x5 mm) of ten symptomatic plants were cut from the margins of the lesions and the healthy tissue. Leaf and stems fragments were washed, surface disinfected with 75% ethanol for 1 min, 1% sodium hypochlorite solution (NaClO) for 2 min, rinsed three times in distilled water, dried on sterilized filter paper, and placed on potato dextrose agar (PDA) in Petri dishes (90 mm) (Sun et al., 2022). After seven days of incubation at 25 °C, developed colony fragments were transferred to PDA to obtain pure cultures.

2.2. Pathogenicity test

The pathogenicity was tested by artificial inoculation leaves of plant origin. Two wounded and two non-wounded detached leaves of healthy *P. peregrina* obtained from the natural habitat were used. The leaves were placed onto the filter paper moistened in Petri dishes, wounded with a sterile needle, and mycelial fragments (5 mm) of 7-day-old culture on PDA were placed on the adaxial surface of the leaves. Control leaves were treated the same way, but sterile PDA fragments were used (Fang et al., 2023). Petri dishes with inoculated leaves and control dishes were incubated at 21±2 °C under natural daylight conditions. The test was repeated once. Re-isolation of the pathogen was performed as described above.

2.3. DNA extraction, PCR amplification and amplicon sequencing

DNA extraction was performed by the cetyltrimethylammonium bromide (CTAB) protocol proposed by Day and Shattock (1997) from 7-day-old cultures grown on PDA. For molecular identification and characterization, the nuclear ribosomal internal transcribed spacer (ITS) region, partial translation elongation factor 1-alpha (*EF-1α*), beta-tubulin (*TUB2*), and histone 3 gene (*H3*) were amplified using primer pairs ITS1/ITS4 (White et al., 1990), EF1-728F/EF1-986R (Carbone and Kohn, 1999), T1/Bt2b (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997), and H3-1a/H3-1b (Glass and Donaldson, 1995), respectively. Each 25 μL PCR mix contained 1 μL of template DNA, 1xPCR Master Mix (Thermo Scientific, Vilnius, Lithuania) and 0.4 μM of each primer. Conditions for amplification of ITS were: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min. Conditions

for *EF-1α* and *TUB2* amplifications were: initial denaturation at 95 °C for 8 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s and elongation at 72 °C for 1 min. Conditions for *H3* amplification were: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 60 s, annealing at 58 °C for 1 min and elongation 72 °C for 1 min; repeat protocol for 32 cycles. Final elongation occurred for 10 min at 72 °C. PCR products (5 μL) were observed in 1.2% agarose gel, stained in ethidium bromide, and visualized with UV transilluminator. Amplified products were purified and commercially sequenced (Macrogen Inc., Seoul, South Korea) using the same reverse primers as for amplification. The obtained sequences were assembled using Pregap4 from the Staden programme package (Staden et al., 1998). For species identification, the obtained sequences were compared with those publicly available in NCBI's GenBank database using MegaBLAST (http://www.ncbi.nlm.nih.gov/). Sequences for each locus were aligned using Clustal X (Thompson, 1997), under MEGA version 7 (Kumar et al., 2016). The sequences of isolate were deposited in the National Center for Biotechnology Information (NCBI) GenBank.

2.4. Phylogenetic analyses

Evolutionary history was inferred based on combined analyses of the two (ITS and *EF-1α*) loci of two representative isolates obtained in this study, reference isolates of *Alternaria* spp. and *Alternaria alternantherae* CBS 124392 as an outgroup,using Maximum likelihood (ML) method (MEGA X) (Kumar et al., 2018). Sequences used for phylogeny are presented in Table 1. For the ML, the best nucleotide substitution model was determined using the "find best model" option in MEGA X. For tree inference, the nearest-neighbor-interchange heuristic method was employed, with the initial trees being automatically generated by Neighbor-Join and BioN Jalgorithms. To estimate the statistical significance of the inferred clades, each tree was bootstrapped 1,000 times.

2.5. Morphological characterization

Macromorphological characteristics of two representative isolates (colony color, texture, and growth rate) were observed on PDA and PCA 40 cm below cool white fluorescent illumination at 23 °C for seven days (Blagojević, 2020; Simmons, 2007). The colony diameter was measured every day in two diagonal directions. Mean values were obtained for every day from three replicates with three plates each. The growth rate was calculated and expressed as the diameter of the colony after seven days. Micromorphological characteristics of selected isolates (length, width, shape, size, color of conidia, conidiophores, dimensions of the conical beak, and sporulation pattern) were observed on seven-day-old cultures grown on PCA at 22 °C under cool white fluorescent illumination and an 8/16 h light/dark regime (Simmons, 2007). Microscopic preparations were prepared in lactic acid and viewed under a microscope at 10x and 20x magnifications (Olympus CX43, Olympus, Hamburg, Germany). Photographs of conidiophore and conidia and measurements were obtained using the camera Axiocam ERc 5s, Zeiss, and software ZEN 2 (blue edition), Jena, Germany.

3. RESULTS AND DISCUSSION

3.1. Symptoms, isolates and pathogenicity

Circular or irregular, light to dark brown spots with defined dark margins appeared on the leaves and sporadically stems of *P. peregrina* during the stage of follicle formation, whereas on *P. tenuifolia* identical spots first appeared from the base at the bud stage and finally white mycelia and rot appeared at the base of the stems and lower branches at the seed maturity stage (Figure 1). The spread of spots on leaves causing the plants to wilt and die. Disease incidences in the areas of Pirot and Deliblato Sands were 32% and 25-35%, respectively.

Following a seven-day incubation, five isolates of *P. peregrina* and five isolates of *P. tenuifolia* were obtained from symptomatic plants. In the pathogenicity test, on wound-inoculated leaves, leaf necrosis and mycelia appeared on the adaxial surface of the leaves, as well as necrosis along the leaf nerve, while non-wounded leaves and control leaves remained symptomless (Figure 2). The pathogen was reisolated from inoculated leaves and showed identical morphological characteristics as the original isolate, thus completing Koch's postulates. From each host, a representative isolate with proven pathogenicity was selected for further molecular and morphological characterization (isolate PpPili from *P. peregrina* and isolate SBDPst from *P. tenuifolia*).

3.2. Molecular identification

Following the amplification of ITS, *EF-1α*, *TUB2* and *H3*, PCR products of expected size (600, 300, 600 and 400 bp, respectively) were obtained for the two selected isolates. Comparison of the obtained sequences showed that isolates PpPili and SBDPst were 100% similar (identical) in ITS and *EF-1α*, but differed in 1 nt in *TUB2* and 1 nt in *H3* gene regions. BLAST analysis showed that both isolates were 100% similar with *A. alternata* ex-type CBS 916.96, established by Woudenberg et al. (2013) and Woudenberg et al. (2015), in ITS (AF347031) and *EF-1α* (KC584634). Also, sequences of both regions of obtained isolates were identical with several corresponding sequences of *A. alternata* isolates (e.g. CBS 115188, CBS 113013, CBS 117143, CBS 119115, CBS 918.96, IT44, IT61) (Matić et al., 2020; Woudenberg et al., 2015). In *TUB2*, isolate PpPili was 100% similar with *A. alternata* ex-type CBS 916.96 (=EGS 34.016; MF070244) (Siciliano et al., 2018) and other published *A. alternata* sequences of *TUB2* region (Garibaldi et al., 2019; 2020), while isolate SBDPst was 99% similar with *A. alternata* ex-type CBS 916.96 and 100% similar with CBS 918.96 and CBS 115152 (Siciliano et al., 2018). In *H3*, isolate PpPili was 100% similar with *A. alternata* isolate IT44 (MG182429) (Garibaldi et al., 2018a), while isolate SBDPst was 100% similar with *A. alternata* isolate IT61 (MF997593) (Garibaldi et al., 2018b). Based on ITS, *EF-1α*, *TUB2* and *H3*, obtained isolates were identified as *A. alternata* which is in accordance with Woudenberg et al. (2015), Garibaldi et al. (2019; 2020; 2018a), Siciliano et al. (2018), and Matić et al. (2020).

3.3. Phylogeny

The phylogenetic analysis, based on Maximum likelihood method and Kimura 2-parameter model, of concatenated ITS and *EF-1α* sequences of *Alternaria* spp. grouped two representative isolates, PpPili and SBDPst, with reference isolates of *A. alternata* (Figure 3), confirming the identification.

3.4. Macromorphology and micromorphology

After seven days, isolate PpPili formed circular, cottony, light brown to brown colonies, with a white border on PDA (diameter 60.3 mm), and circular, grayish-brown colonies on PCA (diameter 83.7 mm). Conidia were brown, ovoid, or ellipsoid with three to four transverse and one to three longitudinal septa (16.01-27.74 x 5.50-14.41 μm; average 20.83 x 9.41 μm, n = 50). Conidia formed in chains of 2-7, mostly 5-6. Dimensions of the conical beak were 0.70-5.27 x 1.53-4.57 μm; average 2.55 x 2.68 μm, n = 50. Conidiophores were simple, dark brown, and unbranched (27.86-187.31 x 1.91-7.73 μm; average 59.88 x 4.39 μm, n = 50) (Figure 4).

Table 1. Fungal isolates used for phylogeny.

Isolate	Host/Location	GenBank accession numbers		Reference
		ITS	EF-1α	
Alternaria alternata				
PpPili	*Paeonia peregrina*/Pirot	OP562152	OP643688	This study
SBDPst	*Paeonia tenuifolia*/Deliblatska peščara	nd	nd	This study
CBS 916.96^{T}	*Arachis hypogaea*, India	AF347031	KC584634	(Woudenberg et al., 2015)
CBS 115188	*Citrus clementina*, South Africa	KP124349	KP125125	(Woudenberg et al., 2015)
CBS 113013	*Malus domestica*, South Africa	KP124341	KP125117	(Woudenberg et al., 2015)
CBS 117143	*Capsicum annuum*, Italy	KP124355	KP125131	(Woudenberg et al., 2015)
CBS 119115	*Prunus sp.*, Greece	KP124360	KP125136	(Woudenberg et al., 2015)
CBS 918.96 (ex *A. tenuissima*^{R})	*Dianthus chinensis*, UK	AF347032	KC584693	(Woudenberg et al., 2015)
CBS 115152	*Psychotria serpens*, China	KP124348	KP125124	(Woudenberg et al., 2015)
REIS 68	*Ceratostigma willmottianum*, Italy	MN565914	MN627329	(Matić et al., 2020)
IT44	*Echinacea purpurea*, Italy	MG182428	MN627319	(Matić et al., 2020)
IT61	*Mentha* × *piperita*, Italy	MF997592	MN627337	(Matić et al., 2020)
Alternaria alstromeriae				
CBS 118809^{T}	*Alstroemeria sp.*, Australia	KP124297	KP125072	(Woudenberg et al., 2015)
CBS 118808^{R}	*Alstroemeria sp.*, USA	KP124296	KP125071	(Woudenberg et al., 2015)
Alternaria alternantherae				
CBS 124392	*Solanum melongena*, China	KC584179	KC584633	(Woudenberg et al., 2015)
Alternaria arborescens				
CBS 102605^{T}	*Solanum lycopersicum*, USA	AF347033	KC584636	(Woudenberg et al., 2015)
CBS 109730	*Solanum lycopersicum*, USA	KP124399	KP125177	(Woudenberg et al., 2015)
Alternaria gaisen				
CBS 632.93^{R}	*Pyrus pyrifolia*, Japan	KC584197	KC584658	(Woudenberg et al., 2015)
CBS 118488^{R}	*Pyrus pyrifolia*, Japan	KP124427	KP125206	(Woudenberg et al., 2015)
Alternaria longipes				
CBS 540.94^{R}	*Nicotiana tabacum*, USA	AY278835	KC584667	(Woudenberg et al., 2015)
CBS 121332^{R}	*Nicotiana tabacum*, USA	KP124443	KP125222	(Woudenberg et al., 2015)

Legend: T - ex-type isolate; R - representative isolate; nd - not deposited.

Fig. 1. Symptoms of leaf spot on natural infected plants of *Paeonia peregrina* (left) and *Paeonia tenuifolia* (right).

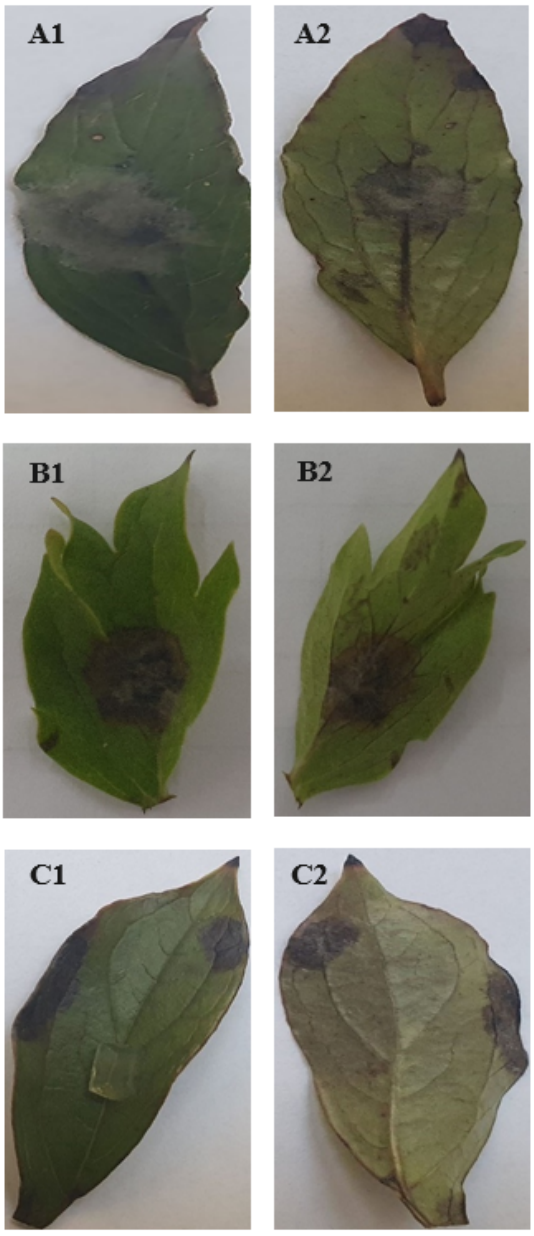

Fig. 2. Pathogenicity of obtained isolates on detached leaves of *Paeonia peregrina* (A and B) - the inoculated leaves with mycelial fragments (surface A1, B1; reverse A2, B2) and the control leaves of *Paeonia peregrina* with sterile PDA (surface C1 and reverse C2).

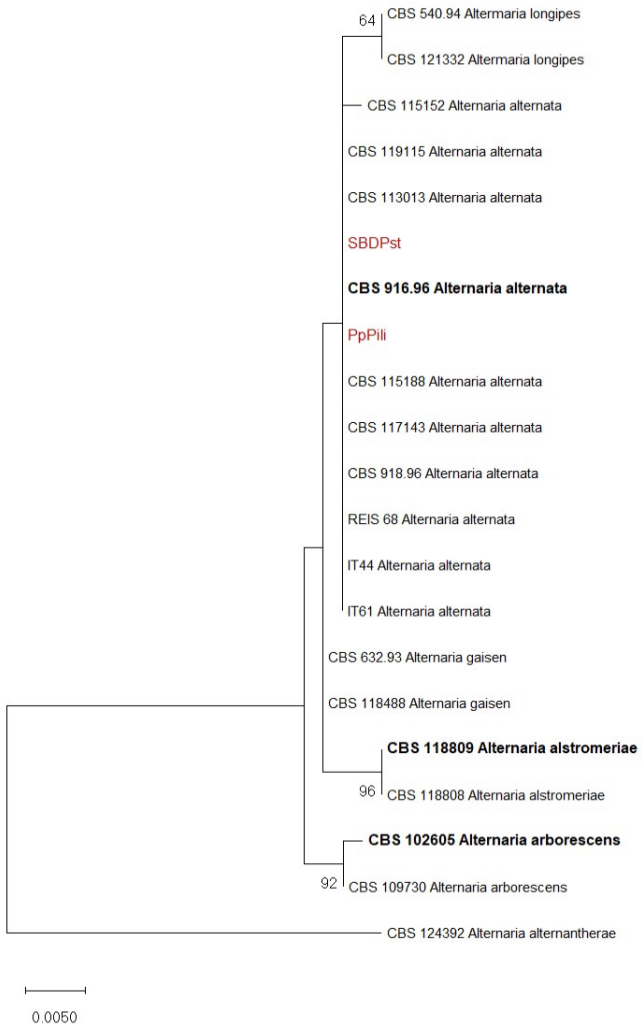

Fig. 3. Phylogenetic tree obtained from the maximum likelihood analysis of concatenated ITS and *EF-1α* sequences of *Alternaria* spp. Numbers on the branches represent bootstrap values obtained for 1,000 replicates (only values above 60% are shown). Isolates obtained in this study are shown in red and ex-type isolates are shown in bold. The tree was rooted to *Alternaria alternantherae*. The scale bar represents the number of nucleotide substitutions per site.

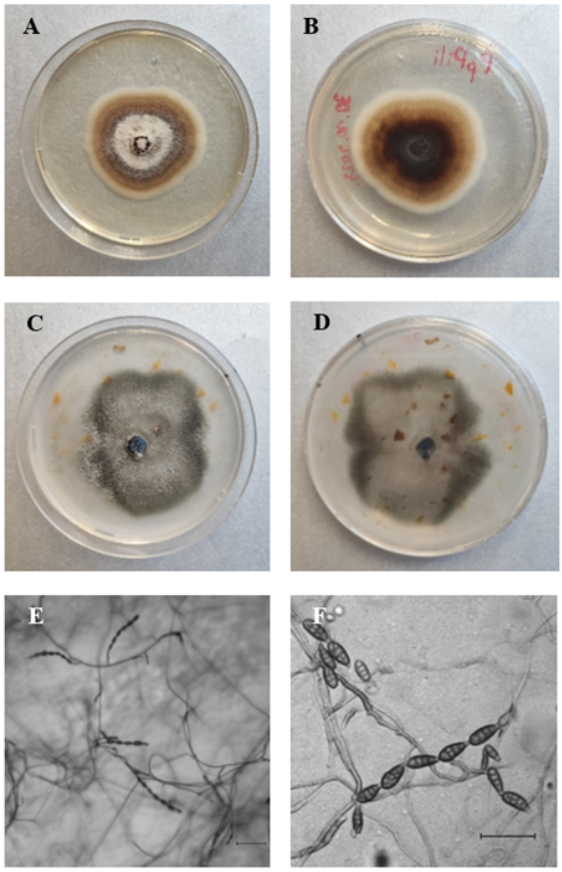

Fig. 4. Morphological characteristics of isolate PpPili from *Paeonia peregrina*: 7-day-old-culture on PDA (surface A, reverse B); and 7-day-old-culture on PCA (surface C, reverse D), sporulation pattern (E), conidia (F). In (E) scale bar 100 μm, while in (F) scale bar 20 μm.

After seven days, isolate SBDPst formed circular, cottony, white, or light brown colonies on PDA (diameter 45.3 mm). On PCA, colonies were circular, grayish-brown (diameter 85.5 mm), and brown, ovoid or ellipsoid conidia formed in chains of 3-10, mostly 3-8. Conidia had zero to four transverse and zero to three longitudinal septa, and were 13.56-24.27 x 5.7-14.3 μm in size (average 18.93 x 9.67 μm, n = 50), with a conical beak 1.00-5.52 in long (average 2.82) x 0.65-3.34 in wide (average 1.73) μm (n = 50). Conidiophores were simple, dark brown, and unbranched, 12.64-53.15 x 1.29-5.97 μm in size (average 25.98-2.36, n = 50) (Figure 5).

Observed morphological characteristics corresponded to the genus *Alternaria* (Simmons, 2007). According to the sporulation pattern the isolates could be identified as an *A. tenuissima* morphospecies, but with regards to a comprehensive revision by Woudenberg et al. (2015) and recently, Dettman and Eggertson (2021) which show lack of phylogenetic divergence in support of morphological differences and the fact that morphospecies are synonymized with *A. alternata*, the isolates were identified as *A. alternata*.

In this study, Alternaria leaf spot was described on two herbaceous peony species *P. peregrina* and *P. tenuifolia* in Serbia. Isolated fungi whose pathogenicity was proven, were identified as *A. alternata.* In Serbia, there has been a report of Alternaria leaf spot affecting wild herbaceous peony (Mikić et al., 2023), but other diseases have not been identified in these plant species. In the United States, Garfinkel and Chastagner (2019) described Alternaria leaf spot and other diseases on the herbaceous peony species *P. lactiflora* Pallas.. Also, Park et al. (1997) examined the effect of different pathogenic fungi (*Alternaria* sp., *Erysiphe aquilegiae,* and *Cronartium flaccidum*) on the growth and root yield of *P. lactiflora*; brown, dark-brown to black spots were observed on leaves, and finally the with-

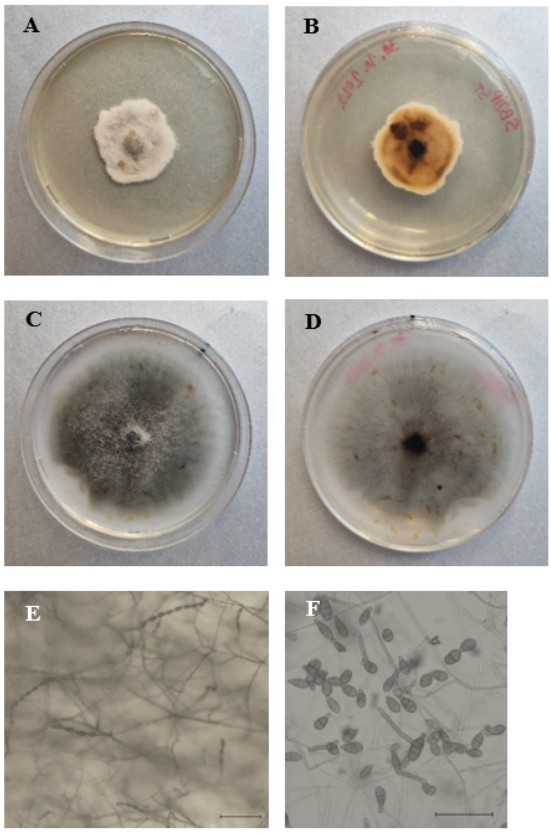

Fig. 5. Morphological characteristics of isolate SBDPst from *Paeonia tenuifolia*: 7-day-old-culture on PDA (surface A, reverse B); and 7-day-old-culture on PCA (surface C, reverse D), sporulation pattern (E), conidia (F). In (E) scale bar 100 μm, while in (F) scale bar 50 μm.

ering of the above-ground part of the plant. The symptoms of *A. alternata* on tree peonies described by Shi et al. (2015) were similar with our findings of *A. alternata* on herbaceous *P. peregrina*. Zhang et al. (2008) reported *A. suffruticosae*, *A. suffruticosicola*, and *A. tenuissima* on woody peony – *Paeonia suffruticosa* cultivar Andrews.

4. CONCLUSION

Alternaria spp. are present in humid and semi-arid regions, and can infect a wide range of plant crops through leaves, stems, flowers, and fruits. The fungus was isolated from infected plants collected at the localities Pirot and Deliblato Sands, and identified as *A. alternata* based on molecular characteristics. The ML phylogenetic tree of concatenated ITS and *EF-1α* sequences, and ITS, *EF-1α*, and *TUB2* sequences of *Alternaria* spp., showed that two isolates obtained in this study clustered among *A. alternata.* After the pathogenicity test, the pathogen was reisolated from inoculated leaves and showed identical morphological characteristics as the original isolate, thus completing Koch's postulates. Based on the observed morphological characteristics and sporulation pattern the isolates can be identified as *A. tenuissima* morphospecies, but this morphospecies in recent taxonomy has been synonymized with *A. alternata*. In Serbia, to the best of our knowledge, this is the first report of *A. alternata* infecting herbaceous wild peony species. Further investigations of *A. alternata* should elucidate the importance of the disease it causes on peonies and propose possible control measures to preserve this important, rare, and protected plant species in its natural habitats in Serbia.

ACKNOWLEDGMENTS

This manuscript was supported by the Ministry of Science and Technology of the Republic of Serbia (451-03-1202/2021-09/02 and 451-03-47/2023-01/200003) and the National Key R&D Program of China (2021YFE0110700).

CONFLICT OF INTEREST

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

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Antibacterial and antibiofilm potential of carvacrol against oral *Streptococcus* spp. isolates

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Received: November 20, 2023
Accepted: December 19, 2023
Published on-line: December 25, 2023
Published: December 25, 2023

Dental caries is one of the most prevalent public health problems. It is caused by a well-organized bacterial structure *i.e.*, biofilm, where the cariogenic bacteria *Streptococcus mutans* is recognized as the primary pathogenic factor. Furthermore, the first phase of dental biofilm onset is predominantly associated to bacteria of the *Streptococcus* genus. This research aimed to assess the antibacterial and antibiofilm potential of monoterpene carvacrol, against relevant tooth caries-associated isolates, including *Streptococcus mutans*, *Streptococcus sanguinis*, *Streptococcus mitis*, and *Streptococcus gordonii*. Microdilution test was used for the assessment of the antimicrobial potential of carvacrol, and the minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined. The capacity of carvacrol to disrupt pre-formed biofilm and prevent its formation was evaluated in crystal violet assay. Tested monoterpene achieved notable antimicrobial activity, with MICs ranging from 1.22 mg mL^{-1} to 2.44 mg mL^{-1}, and MBCs from 1.22 mg mL^{-1} to 19.52 mg mL^{-1}. Screening of the prevention of biofilm biomass formation revealed that carvacrol has the potential to prevent biofilm formation of the *S. sanguinis*, *S. gordonii* (41% and 47% of inhibition, at concentration 4.88 mg mL^{-1} respectively) and *S. mutans* (at higher tested concentrations 59% of inhibition, at concentration of 19.52 mg mL^{-1}). Biofilm disruption also occurred in case of *S. mitis* and *S. sanguinis* biofilms (40% and 46% of biomass decrease, respectively, at concentration of 2.44 mg mL^{-1}). According to the study results, carvacrol could be valuable as an active antibacterial agent and incorporated in adjunct to oral hygiene maintenance.

Keywords: carvacrol; antibacterial potential; antibiofilm potential; *Streptococcus* spp.

https://doi.org/10.61652/leksir2343e177N

ABBREVIATIONS

MIC – Minimal inhibitory concentration
MBC – Minimal bactericidal concentration
WHO – World Health Organization
EO – Essential oil
FEMA – Flavor and Extract Manufacturers Association
FDA – Food and Drug Administration

1. INTRODUCTION

Dental caries, as a bacterially derived disease, is one of the most common public health problems (WHO). It is caused by a well-organized bacterial structure known as biofilm, in which the cariogenic bacteria *Streptococcus mutans* have been identified as the primary pathogenic agent (Kolenbrander et al., 2010). In addition, the initiation of dental biofilm formation, provided by the colonization of oral pathogens, is primarily attributed to *Streptococcus* genus bacteria (Sbordone and Bortolaia, 2003). It is common knowledge that complete elimination of biofilm through standard dental care techniques (*e.g.*, brushing and flossing) is not often possible. Therefore, additional oral hygiene products are required, being used in the form of mouthwashes or oral gels. These products contain active antibacterial components, and among them, compounds based on chlorhexidine are the most widely used due to their proven effectiveness (Karamani et al., 2022; Pérez-Nicolás et al., 2023). Even though chlorhexidine-based formulations are undisputedly effective, some side effects may occur including changes in taste, numbness or discomfort in the tongue and lips, as

well as dry mouth and teeth discolouration (Poppolo Deus and Ouanounou, 2022). Within dentistry, the efficacy of herbal extracts and essential oils (EOs) in preventing plaque and maintaining dental hygiene has been well-documented. These natural compounds possess promising activity against various oral infections and oral inflammation, used as both preventive measures and treatment options (Muresan et al., 2023).

EOs are secondary metabolites produced by different organs of aromatic plants. They are complex mixtures of terpenes and aromatic and aliphatic compounds of low molecular weight (Bakkali et al., 2008; Marinković et al., 2022). Numerous authors have reported their antioxidant, anti-inflammatory, antiviral, cytotoxic but also antimicrobial activity (Bakkali et al., 2008; Dorman and Deans, 2000). In the last few decades inhibitory potential of EOs against bacteria was in focus and their activity against oral ones was confirmed many times (Freires et al., 2015). Among them EOs rich in monoterpene carvacrol, such as *Zataria multiflora* and *Origanum vulgare* oils proved their efficiency against *Streptococcus* spp. (Aires et al., 2020; Yazdanian et al., 2022).

Previous studies demonstrated carvacrol's antibacterial activity against *S. mutans* (Fernández-Babiano et al., 2022; Hoş et al., 2023; Hwang et al., 2004; Mathela et al., 2010; Miladi et al., 2017; Park et al., 2003; 2012; Wang et al., 2016; Zhang et al., 2023). However, results about its activity against *S. sanguinis* and *S. mitis* are still scarce (Fernández-Babiano et al., 2022; Hoş et al., 2023). Moreover, up to our knowledge, there is no information about carvacrol antibacterial potential against *S. gordonii*. Concerning antibiofilm activity, carvacrol was suggested as the one with antibiofilm potential against oral isolates including *S. mitis, S. sanguinis, S. mutans,* and *S. gordonii* (Maquera Huacho et al., 2019). For that reason in this study we monitored both, the antibacterial activity and antibiofilm activity of carvacrol against all four isolates belonging to *Streptoococcus* species.

2. MATERIAL AND METHODS

2.1. Bacterial strains and cultivation

Clinical isolates tested in the study, originated from patients at the Department of Pediatric and Preventive Dentistry, School of Dental Medicine, University of Belgrade, Serbia. Bacterial collecting and identification were undertaken according to the Declaration of Helsinki and approved by the Ethical Committee No 36/7. The bacteria cultivation was carried out by streaking frozen stock cultures onto Mueller Hinton Agar plates (MHA, Torlak, Belgrade, Serbia) followed by 48 h incubation at 37 °C. Individual colonies were grown overnight in Tryptic Soy Broth (TSB, Oxoid, Basingstoke, Hampshire, United Kingdom), at 37 °C. The optical density of bacterial suspensions was adjusted spectrophotometrically at 600 nm to achieve a value of 0.2 corresponding to bacterial inoculum of 1×10^8 CFU mL^{-1}. All microbial procedures were carried out in a sterile environment.

2.2. The assessment of the antibacterial potential of carvacrol

Antibacterial potential of the monoterpene carvacrol was examined by the use of the broth-microdilution assay. Minimal inhibitory concentrations (MICs) as well as minimal bactericidal concentrations (MBCs) were determined according to the method previously outlined by (Vasilijević et al., 2019). Carvacrol was prepared by dissolving it in Tween 80 (2:3 *v/v* ratio) and subsequently serially diluted two-fold in TSB medium across the columns of 96-well microtiter plates. Its concentration range was 0.61 – 78.08 mg mL^{-1}. Thereafter, an inoculum containing 2*10^4 CFU/well (10^5 CFU mL^{-1}) of bacteria was added to each well. Resazurin was used as a growth indicator, and it was introduced at a final concentration per well of 0.0675 mg mL^{-1}. In the assay, the negative control was the solvent Tween 80, while a positive control was the most commonly used antibiotic in dentistry (amoxicillin). Minimal bactericidal concentrations were determined by plating from the wells with no noticeable growth onto Mueller Hinton Agar plates. The experiment was performed in triplicate and repeated two times.

2.3. The assessment of the antibiofilm potential of carvacrol

The assessment was provided using crystal violet assay on 96-well flat-bottom plates (Stepanović et al., 2000) in order to investigate the carvacrol activity in the prevention of the biofilm formation as well as against already formed biofilm.

2.3.1. Prevention of biofilm formation

The impact of antibacterial agents on biofilm formation was determined according to previously described in Marinkovic et al. (2021). (2021). The bacterial cell suspensions (10^5 CFU/well) of four oral *Streptococcus* spp. isolates have been incubated simultaneously with the carvacrol (tested concentrations 2.44 – 19.52 mg mL^{-1}, selected in accordance with the results of microdilution assay), at 37 °C, for 48 hours, in 96-well microtiter plates. Tryptic soy broth enriched with 0.5 % glucose has been used as a growth medium. After the 48 h required for the biofilm formation, the medium was aspirated and planktonic cells have been removed by twice washing with sterile saline. The cells have been air-dried and then the biofilm has been stained with 0.1 % crystal violet (Bio-Merieux, France). After 15 minutes, the wells have been washed, while the remaining stain, bounded to biofilm, was suspended in 96 % ethanol. The absorbance was read at 570 nm. The percentage of the inhibition of biofilm formation was calculated according to the Equation (1):

$$I = 100\% \times (AC - AT) \div AC \quad \textbf{(1)}$$

AC and AT are absorbances at 570 nm of control and treatment, respectively.

2.3.2. Disruption of the pre-formed biofilm

In order to provide biofilm formation, inoculums of 2×10^5 CFU/well of each strain were added into polystyrene microtiter plates and incubated 48 h at 37 °C in order to allow biofilm formation. After that biofilms were exposed to the carvacrol in the same concentration range as previously mentioned. The disruption of biofilms was spectrophotometrically quantified at 570 nm, as described in previous section.

In both assays Tween 80 was used as negative control. Concerning its possible impact on biofilm forming prevention and biofilm disruption, its activity was taken into account in expressing carvacrol activity.

2.4. Statistical analysis

Results are presented as means ± standard deviation (SD). Group comparisons were performed using the One-Way Analysis of Variance test. All data were analysed using SPSS 20.0 (IBM Corporation) statistical software. All p values less than 0.05 were considered significant.

3. RESULTS

The inhibitory potential of carvacrol (**Table 1**) was the same against *S. mitis, S. gordonii,* and *S. mutans* (MIC=1.22 mg mL^{-1}), and slightly lower against *S. sanguinis* (MIC=2.44 mg mL^{-1}). On the other hand, bactericidal effect differed among four isolates where *S. gordonii* (MBC=1.22 mg mL^{-1}) was the most sensitive to its activity, while it was just an opposite in case of *S. mutans* (MBC=19.52 mg mL^{-1}).

Table 1. Antibacterial potential of carvacrol against four *Streptococcus* spp. isolates.

	Carvacrol		Positive control (amoxicillin)	
	MIC[a]	MBC[b]	MIC	MBC
	mg mL $^{-1}$		μg mL $^{-1}$	
S. mitis	1.22	2.44	8	16
S. sanguinis	2.44	9.76	2	4
S. mutans	1.22	19.52	4	4
S. gordonii	1.22	1.22	16	32

[a] MIC – Minimal inhibitory concentration; [b] MBC – Minimal bactericidal concentration.

Concerning carvacrol antibiofilm potential, concentration of 4.88 mg mL^{-1} successfully inhibited its formation (41% and 47% for the *S. sanguinis* and *S. gordonii*, respectively). However, carvacrol effect on *S. sanguinis* biofilm formation was antagonistic: although inhibitory potential was detected for the conc. 4.88, higher concentrations significantly stimulate biofilm establishment. Inhibition of the *S. mutans* biofilm formation was successful when higher concentrations were tested ($\geq$9.76 mg mL^{-1}), while none of the tested concentrations had an impact on *S. mitis* biofilm Figure 1.

In case of the biofilm disruption, carvacrol was effective against *S. mitis* and *S. sanguinis*, inducing decrease of biofilm biomass for 40% and 46%, respectively, both at concentration 2.44 mg mL^{-1}. In addition, the concentration 4.88 mg mL^{-1} reduced biofilm of *S. sanguinis* (48%). However, carvacrol could not affect already composed biofilm of *S. mutans* and *S. gordonii* regardless tested concentration Figure 2.

4. DISCUSSION

In this study, antibacterial and antibiofilm properties of the EO monoterpene carvacrol have been investigated. The selection of this monoterpene was provided in accordance to following: (1) some preparations commonly used in oral hygiene maintenance, such as Listerine, contain similar EO constituents (Marinković et al., 2023), and (2) carvacrol is generally recognized-as-safe (GRAS category) by the Flavor and Extract Manufacturers Association (FEMA) and the Food and Drug Administration (Magi et al., 2015). Furthermore, up-to date investigations of the antibacterial potential of EOs, containing carvacrol along with the studies exploring carvacrol solely, suggested its promising antibacterial and antibiofilm activity (Fernández-Babiano et al., 2022; Hoş et al., 2023; Hwang et al., 2004; Mathela et al., 2010; Miladi et al., 2017; Park et al., 2003; 2012; Wang et al., 2016; Zhang et al., 2023).

In the present study, antibacterial and antibiofilm potential of carvacrol was screened, with the initial hypothesis that, if the *Streptococcus* spp. biofilm formation could be prevented or already formed biofilm removed, then the use of carvacrol may reduce the incidence of tooth decays. To evaluate the antibacterial potential of carvacrol against the bacteria relevant to caries, the study focused on *S. mitis, S. sanguinis, S. gordonii* and *S.mutans*. *S. mutans* is broadly recognized as pathogenic and cariogenic bacteria. Other selected bacteria, *i.e. S. mitis*, *S. sanguinis* and *S. gordonii* are considered as commensals or bacteria with dual, beneficial, or detrimental roles in the host homeostasis (do Rosário Palma et al., 2018). However, they additionally showed some cariogenic potential that has been related to their capability to decrease oral pH, and consequently contribute to caries development (Banas et al., 2016). Moreover, these species are also recognized as pioneers in biofilm onset (Kolenbrander et al., 2010). Taking into account aforementioned these bacteria could be considered as an adequate model for testing antibacterial/antibiofilm activity of test substances intended to be used in oral hygiene maintenance.

A pre-screening of antibacterial properties of carvacrol was performed using a microdilution assay and it revealed that carvacrol possess some antibacterial potential (MIC 1.22–2.44 mg mL^{-1}). Slight differences in sensitivity among strains were observed, however more in the bactericidal effect in comparison to inhibitory one. Literature review showed that activity of carvacrol against *S. mutans* was explored and MIC ranged between 16 μg mL^{-1} and 2mg mL^{-1} Fernández-Babiano et al. (2022); Hoş et al. (2023); Hwang et al. (2004); Mathela et al. (2010); Miladi et al. (2017); Park et al. (2003; 2012); Wang et al. (2016); Zhang et al. (2023) which was in line with the present results. The only available information about the activity of carvacrol against *S. sanguinis* and *S. mitis* responded to MIC=93.4 μg mL^{-1} (Fernández-Babiano et al., 2022) and 1.90 mg mL^{-1} (Hoş et al., 2023), respectively, and the latter one is in line with our results. The activity of carvacrol against *S. gordonii* was for the first time monitored in this study. Possible explanation of the distinction in strain response compared to present study could be attributed to the genotypic and phenolic variances among the tested strain used in different studies (Marinkovic et al., 2021).

The screening of the prevention of biofilm biomass formation, revealed that carvacrol can prevent biofilm formulation of the *S. sanguinis*, *S. gordonii* and *S. mutans,* while the disruption was also occurred in case of biofilms of *S. mitis* and *S. sanguinis.* This is in agreement with the studiy presented by Maquera Huacho et al. (2019) that demonstrated the potential of carvacrol to prevent as well as to disrupt mature biofilms of aforementioned isolates (Maquera Huacho et al., 2019).

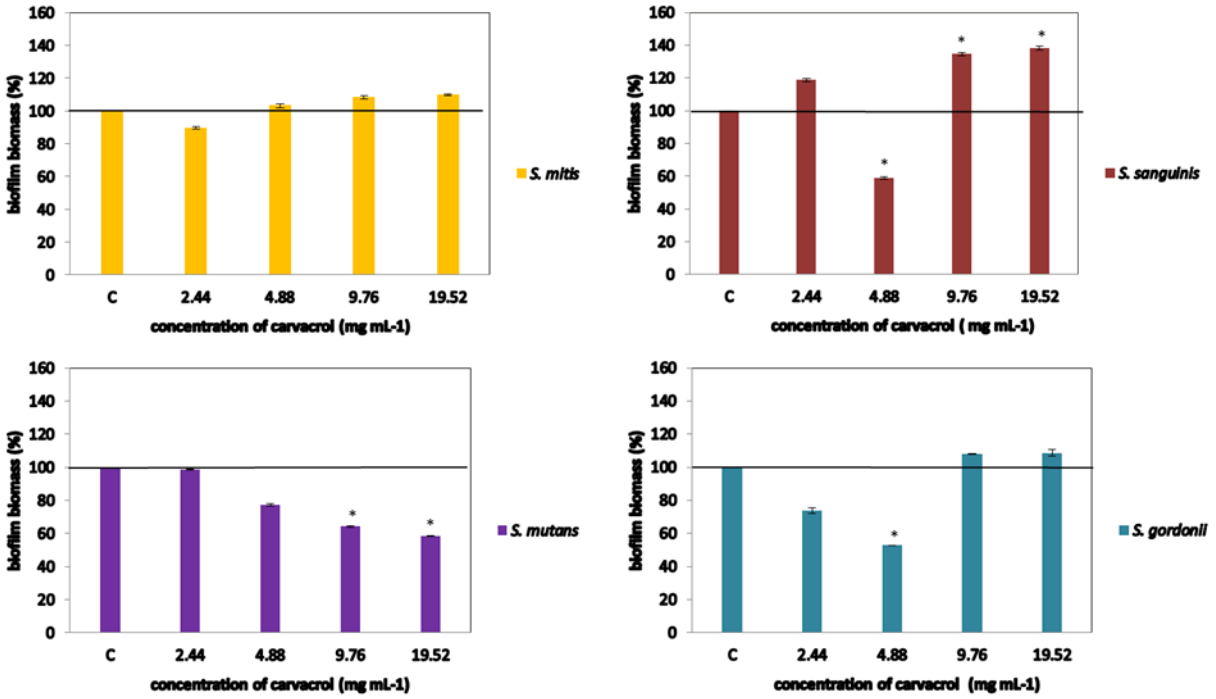

Fig. 1. Carvacrol-induced prevention of four *Streptococcus* isolates biofilm formation.
The results are expressed as the mean values ± standard deviation of two individual experiments, each performed in five replicas. Statistical significance was tested using the one-way ANOVA: *Statistical difference in the biofilm biomass compared to the untreated control (100 % biofilm biomass), p <0.05.

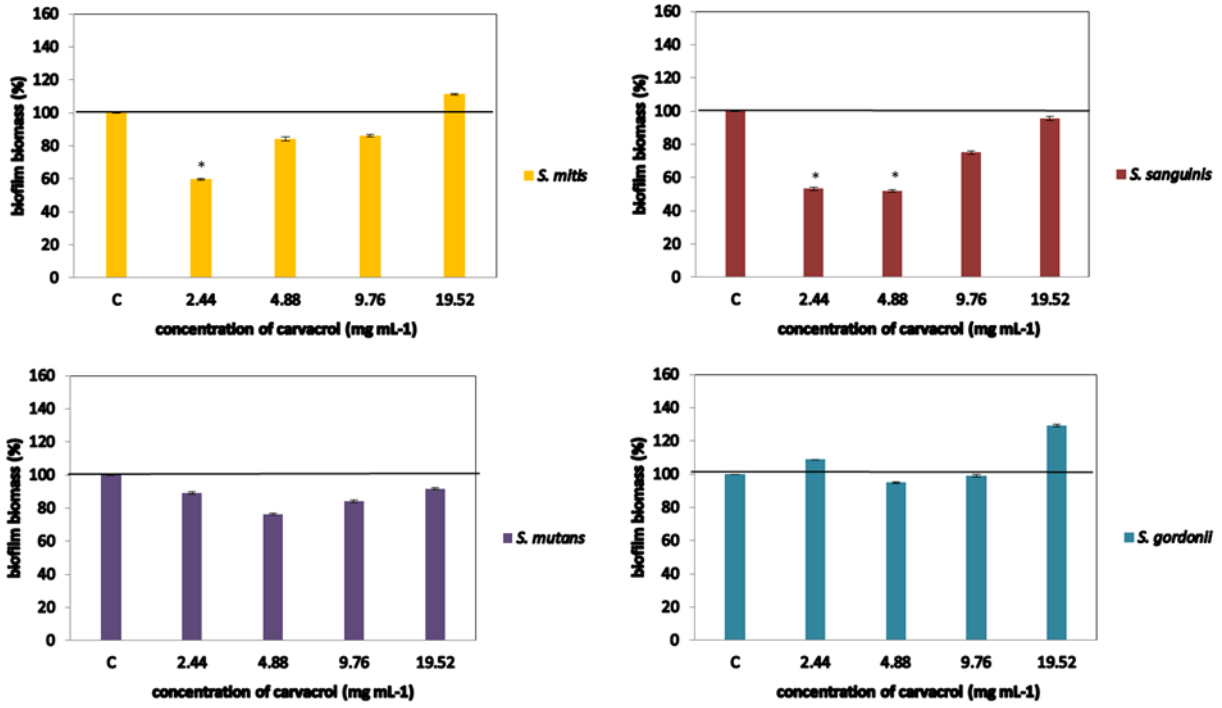

Fig. 2. Carvacrol-induced disruption of four *Streptococcus* isolates' pre-formed biofilms.
The results are expressed as the mean values ± standard deviation of two individual experiments, each performed in five replicas. Statistical significance was tested using the one-way ANOVA: *Statistical difference in the biofilm biomass compared to the untreated control (100 % biofilm biomass), p <0.05.

5. CONCLUSION

The evaluation of the antibacterial properties confirms that carvacrol has substantial antibacterial potential towards *Streptococcus mitis, Streptococcus mutans, Streptococcus gordonii,* and *Streptococcus sanguinis*. Carvacrol proved to be capable of preventing formation of the *S. sanguinis*, *S. gordonii,* and *S. mutans* biofilms. Furthermore, it also successfully disrupted biofilms of *S. mitis* and *S. sanguinis*. In light of the premise that prevention of *Streptococcus* species biofilm formation or removal of their pre-formed biofilms could affect dental caries prevalence, carvacrol may be a suitable candidate for incorporation into oral hygiene products.

ACKNOWLEDGMENT

Authors thank the Ministry of Science, Technological Development, and Innovation of the Republic of Serbia for the financial support to the research through institutional funding (Contract numbers 451-03-47/2023-01/200017);

CONFLICT OF INTEREST

None declared.

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Usage areas, biological activities and volatile oil compounds of *Matricaria aurea* and *Matricaria chamomilla*

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Received: November 5, 2023
Accepted: December 10, 2023
Published on-line: December 19, 2023
Published: December 25, 2023

Plants are natural products responsible for many biological activities. It is quite common to use plants that attract attention with their nutritive properties within the scope of complementary medicine. *Matricaria* species, which have naturally distributed, are among the plants used in traditional medicine. Revealing the biological activities of plants is very important in terms of their potential use. In this study, the usage areas, chemical contents and biological activities of *Matricaria* species reported in the literature were compiled. According to the findings, it was seen that it was used for health and cosmetic purposes. In addition, it has been observed that *Matricaria* species have biological activities such as antioxidant, antimicrobial and anticancer. It was observed that the dominant compounds from different *Matricaria* species were 1,5-Bis (dicyclohexylphosphino)-pentane (3.95-44.7%) and α-bisabolol (2.2-56.86%). According to the literature data, *Matricaria* species are an important natural resource. As a result, it is predicted that *Matricaria* species may be an important source in pharmacological studies.

Keywords: antioxidant; antimicrobial; chamomile; *Matricaria*; traditional medicine

https://doi.org/10.61652/leksir2343e156Y

1. INTRODUCTION

Plants are highly significant materials that are utilized by humans for various purposes, either by obtaining them from their natural habitats or through cultivation (Mohammed et al., 2022). Plants are considered a fundamental component of human dietary lists due to their ability to contain vitamins, minerals, and essential nutrients within their structures (Dastagir et al., 2022; Sevindik et al., 2017). Throughout history, humans have utilized plants for a multitude of purposes, including but not limited to shelter, warmth, equipment, sustenance, and medicinal applications. Plants constitute the basis for combating diseases in both developing and underdeveloped countries worldwide (Dayangaç et al., 2021; Mohammed, Günal, Şabik, Akgül and Sevindik, 2020). In this context, it is highly important to evaluate not only the nutritional properties of plants but also their potential from a medicinal perspective (Khan et al., 2023; Mohammed, Günal, Pehlivan, Doğan, Sevindik and Akgül, 2020). Numerous studies have reported that plants possess a variety of activities such as antioxidant, anti-inflammatory, anticancer, antimicrobial, antiproliferative, hepatoprotective, and DNA-protective (Abbas et al., 2022; Benkhnigue et al., 2023; Mazher et al., 2023; Mohammed et al., 2018; 2019; Unal et al., 2022; Uysal et al., 2023). In this study, the reported usage areas, chemical contents, and biological activities of *Matricaria* species in the literature have been compiled.

1.1. *Matricaria* species and their usage areas

Matricaria, a member of the Asteraceae family, is one of the more important medicinal plant species native to Southern and Eastern Europe. In addition to these regions, Germany, Hungary, France, Egypt, Russia and Brazil are also areas where it is cultivated. It is known that the first emergence of it in history was during the Babur period in India. Plants belonging to the *Matricaria* species are annual plants with thin needle-shaped roots that penetrate the soil in a straight manner. The

stem of the plant is characterized by its branching structure, with a high degree of branching observed. It is capable of attaining heights ranging from 10 to 80 centimeters, and its leaves are long and narrow. It is also known that the number of petals in a flower is two or three. The flower heads have been arranged separately, with a diameter range from 10 to 30 millimeters. The flower heads have a stem, and are heterogeneous in nature. The flowers of this species consist of five-toothed, golden-yellow tubules measuring 1.5-2.5 mm in length, which are always terminated by a glandular tube. The petals on 11-27 white flowers are arranged concentrically and measure 6-11 mm in length and 3.5 mm in width. The fruit has a yellowish-brown rind (Franz et al., 2005; Handa et al., 1957; Ivens, 1979; Svab, 1979). The literature documents the various methods and techniques employed for different purposes in the utilization of *Matricaria* species. Upon examination of the traditional application of the *Matricaria* genus, it is observed that various parts of the plant, including the flower, leaf, stem, and whole plant, are utilized. The utilization of various techniques such as infusion, boiling, steam inhalation, bathing, and compress have been preferred among plants application methods. It has been observed that there are various health applications for conditions such as diabetes, nerve disorders, diarrhea, angina, thrush, abscess, painful menstrual periods, gastralgia, digestive disorders, female genital infections, kidney stones, sedation, antiseptic, nausea, gastrointestinal disorders, eye irritation, sciatic pain, oral, throat, ear, and skin infections, spasm conditions, colds, sprains, fractures, liver disorders, skin and mucous membrane inflammation, burn treatment, and cough. In addition, it has applications in facial cleansing, tea consumption, oral hygiene, and shampoo fragrance (Di Novella et al., 2013; Eddouks et al., 2017; Idm'hand et al., 2020; Kozuharova, 2013; Marković et al., 2021; Mikou et al., 2015; Mrabti et al., 2019; Naceiri Mrabti et al., 2021; Parada et al., 2009; Pieroni, 2017; Tuttolomondo et al., 2014).

2. BIOLOGICAL ACTIVITIES

Plants are natural resources responsible for many biological activities (Mohammed et al., 2021). In this study, the biological activity studies of *Matricaria* species reported in the literature were compiled and shown in Table 1.

2.1. Antioxidant activity

Free radicals are oxidant compounds that are produced as a result of metabolic activities (Krupodorova and Sevindik, 2020). Elevated levels of oxidizing compounds can be highly detrimental to living organisms. The antioxidant defense system plays a role in preventing or suppressing these harmful effects (Bal et al., 2017). In cases where the antioxidant defense system is insufficient, oxidative stress occurs (Eraslan et al., 2021). Serious illnesses such as cancer, cardiovascular diseases, multiple sclerosis, neurodegenerative diseases, Alzheimer's, and Parkinson's may manifest in individuals as a result of oxidative stress (Bal et al., 2023; Saridogan et al., 2021; Selamoglu et al., 2020). Supplemental antioxidants play a crucial role in reducing the potential effects of oxidative stress (Akgül et al., 2022). Within this scope, a compilation of studies on the antioxidant activity of *Matricaria* species reported in the literature, has been presented (Table 1). The volatile oils of the leaves, stems, flowers, and roots of *Matricaria aurea* collected from Tunisia were reported to exhibit antioxidant activities as determined by ABTS and DPPH tests (Kheder et al., 2014). They compared the obtained results with the synthetic antioxidant Trolox value. The results of the study indicate that the DPPH assay exhibited a range of inhibition values between 1.06% and 24.53%. Furthermore, it has been reported that the ABTS assay exhibits ion neutralization values ranging from 0.1% to 21.2% (Kheder et al., 2014). According to a study conducted in Iran, it has been reported that the ethyl acetate and methanol solvents of *M. aurea* exhibit high antioxidant activity as determined by the DPPH radical scavenging assay and phosphomolybdenum reduction assay (Yousefbeyk et al., 2022). It has been reported that the water and other solvents of the flowers of *M. aurea* collected from Jordan exhibit inhibitory effects on linoleic acid peroxidation, with inhibition percentages of 91.2% and 77%, respectively. Furthermore, it has been reported that the DPPH values of water and other solvents were 92.8% and 90.3%, respectively. Additionally, the obtained results were evaluated in liposome model systems (Mohammad Al-Ismail and Aburjai, 2004). It has been reported that the IC_{50} values of the methanol and ethanol solvents of *M. aurea* collected from Jordan were 51.5 and 19.8 μg/mL, respectively, while the FRAP test results were 954.33 and 814.83 μM/mg, respectively (Tarawneh et al., 2008). It has been reported that the EC_{50} value of the volatile oil of *M. chamomilla* collected from Bosnia and Herzegovina on the DPPH test was 2.07 mg/mL after a 90-minute incubation period (Stanojevic et al., 2016). The LC_{50} values, known as the potency, of the essential oil and methanol extract of *M. chamomilla* collected from Djibouti were reported to be 4.18 and 1.83 μg/mL, respectively. It was also reported that the inhibition potential of β-carotene-linoleic acid system was 12.69% and 11.37%, respectively. To compare the results, butylated hydroxytoluene (BHT) was used as a positive control (Abdoul-Latif et al., 2011). In a study conducted in South Korea, it was reported that ethanol extract was more effective than aqueous extract in the antioxidant protection factor of *M. chamomilla* (Cho et al., 2005). DPPH test result of essential oil obtained from *M. chamomilla* sample was 533.89 μg/mL, the iron ion chelating ability was 943.61 μg/mL, and the β-carotene bleaching test result was 31.01%. It was also reported that the DPPH test result of the honey obtained from the *M. chamomilla* sample was 1945.38 μg/mL, the iron ion chelating ability was 1773.78 μg/mL, and the β-carotene bleaching test result was 745.54%. Butylated hydroxytoluene (BHT) was used as a positive control to compare the results. The BHT value was reported to be 14.24 $\pm$ 1.32 μg/mL (Qasem et al., 2022). It has been reported that the volatile oil of *M. chamomilla* collected from Egypt exhibits a DPPH activity of 91.7% at a concentration of 400 μg/mL. Butylated hydroxytoluene (BHT) was used as a positive control to compare the results. The BHT value was reported to be 75.6% at a concentration of 400 μg/mL (Ali, 2013). According to the literature, it has been observed that *Matricaria* species have antioxidant potential.

2.2. Antimicrobial activity

In recent years, the increase in the number of diseases caused by microorganisms draws attention (Baba et al., 2020). The general reason for this is thought to be the increase in the number of resistant microorganisms due to unconscious drug use. In recent years, researchers have turned to the discovery of new antimicrobial drugs (Mohammed et al., 2023). Due to the possible side effects of synthetic drugs, people have started to prefer the use of natural antimicrobial drugs. In this context, plants are a very important resource for the discovery of new antimicrobial drugs (Bal et al., 2017; Islek et al., 2021). It has been reported that the MIC values of the essential oil of different parts of *M. aurea* collected from Tunisia against *Vibrio harveyi, Vibrio vulnificus, Pseudomonas aeruginosa, Vibrio alginolyticus, Vibrio fluvialis, Micrococcus luteus, Escherichia coli, Staphylococcus aureus, Salmonella wien, Bacilus cocus, Vibrio parahaemolyticus, Candida albicans, Candida tropicalis, Candida parapsilolis* and *Candida glabrata* vary about 50->1000 μg/mL of the leaves, 20->1000 μg/mL of flowers, 10->1000 μg/mL of

Table 1. Biological activity of *Matricaria aurea* and *Matricaria chamomilla*.

Plant species	Biological activities	Extracts	Localities	References
Matricaria aurea (Loefl.) Sch.Bip.	Antioxidant, antimicrobial, analgesic, anti-inflammatory	Ethanol, ethyl acetate, methanol, chloroform, acetone, water	Saudi Arabia, Tunisia, Jordan, Iranian	(Kheder et al., 2014; Khodadadi et al., 2011; Mohammad Al-Ismail and Aburjai, 2004; Qnais, 2011; Rizwana et al., 2016; Tarawneh et al., 2008; Yousefbeyk et al., 2022)
Matricaria chamomilla L.	Antioxidant, antimicrobial, angiotensin, antiaflatoxigenic, anticancer, anti-inflammatory, antidiabetic,	methanol, water, ethanol	Bosnia and Herzegovina, Pakistan, Djibouti, South Korea, Morocco, Egypt	(Abdoul-Latif et al., 2011; Ali, 2013; Cho et al., 2005; Mehmood et al., 2015; Qasem et al., 2022; Stanojevic et al., 2016)

roots parts and 35->1000 μg/mL of the stems (Kheder et al., 2014). It has been reported that chloroform, ethyl acetate, acetone, ethanol and methanol extract of the flowers parts of *M. aurea* collected from Saudi Arabia are effective between 0.2-100 mg/mL against *Staphylococus aureus, Bacillus subtilis, Streptococcus pyogenes, Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia, Alternaria alternata, Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Fusarium solani* and *Colletotrichum gleosporoides* (Rizwana et al., 2016). In a study conducted in Iran, it was reported that ethyl acetate and methanol extract of *M. aurea* showed high activity against *Bacillus subtilis* (MIC 1.56 and 12.5 mg/mL) and *Staphylococcus aureus* (MIC 0.78 and 12.5 mg/mL) (Yousefbeyk et al., 2022). It has been reported that the essential oil of *M. chamomilla* collected from Bosnia and Herzegovina has a 13.3-40 mm zone of inhibition against *Listeria monocytogenes, Escherichia coli, Salmonella enterica* and *Staphylococcus aureus* (Stanojevic et al., 2016). It has been reported that the MIC values of the essential oil and methanol extract of *M. chamomilla* collected from Djibouti against *Bacillus cereus, Enterococcus faecalis, Escherichia coli, Listeria innocua, Salmonella enterica, Shigella dysenteria, Staphylococcus aureus, Staphylococcus camorum, Proteus mirabilis, Pseudomonas aeruginosa, Streptococcus pyogenes, Candida albicans, Aspergillus niger* and *Aspergillus* sp. are between 1-16 μg/mL of the essential oil and 25-200 μg/mL of the methanol extract (Abdoul-Latif et al., 2011). In a study conducted in South Korea, it was reported that water extract of *M. chamomilla* leaves did not have antimicrobial activity against *Helicobacter pylori*, but ethanol extract revealed mild antimicrobial activity as a 9.42 mm clean zone (Cho et al., 2005). It has been reported that the essential oil of *M. chamomilla* collected from Morocco has an inhibition zone of 11.20-22.97 mm, and honey obtained from the *M. chamomilla* sample of 8.10-13.90 mm against *Escherichia coli, Proteus mirabilis, Salmonella enterica Typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Listeria monocytogenes, Candida albicans, Trichophyton rubrum* and *Aspergillus niger* (Qasem et al., 2022). It has been reported that ethanol and methanol extract of *M. aurea* collected from Jordan do not show any effect against *Candida albicans, Aspergillus niger, Penicillium chrysogenum, Aspergillus nidulans* (Tarawneh et al., 2008). In this context, according to literature data, it has been observed that *Matricaria* species have important antimicrobial potentials.

2.3. Other activities

In a study conducted in South Korea, it was reported that the angiotensin converting enzyme inhibitory effect of *M. chamomilla* was 57.98% in water extract and 91.36% in ethanol extract (Cho et al., 2005). It has been reported that the essential oil of *M. chamomilla* collected from Egypt has a cell growth inhibition of 7-89% against the MCF-7 cell line. In addition, in the same study, it was reported that the essential oil of *M. chamomilla* completely inhibited aflatoxin B1 (AFB1) production at 800 ppm (Ali, 2013). The anti-diabetic effect of the essential oil of *M. chamomilla* collected from Morocco has been reported as α-glucosidase IC_{50} value of 265.57 μg/mL and α-amylase IC_{50} value as 121.44 μg/mL (Qasem et al., 2022). In addition, α-glucosidase IC_{50} value of honey obtained from the *M. chamomilla* sample has been reported as 1351.02 μg/mL and α-amylase IC_{50} value as 981.44 μg/mL. In the same study, it was reported that the anti-inflammatory effect was 64% in essential oil and 76% in honey at 100 mg/kg after 6 hours (Qasem et al., 2022). It has been reported that the ethanol extract of *M. aurea* collected from Jordan has analgesic effects in rats, using the cold water tail flick test and using tail dip, tail clip, acetic acid-induced writhing and formalin pain tests (Qnais, 2011). The anti-inflammatory result of the LC_{50} values, expressed as the effect potential, of the hydro-alcoholic extract of *M. aurea* collected from Iran was reported to be 1305 μg/mL (Khodadadi et al., 2011). It has been reported that *M. chamomilla* collected from Pakistan has antidiarrheal, antisecretory and antispasmodic activities mainly mediated by weak Ca^{++} and K^{+} -channels activation (Mehmood et al., 2015).

3. CHEMICAL CONTENTS

Plants synthesize many biologically active compounds in their bodies. These compounds are responsible for many different pharmacological activities (Akgül et al., 2022; Selamoglu et al., 2017). In this study, the chemical compounds of *Matricaria* species reported in the literature were compiled. The obtained results are shown in Table 2.

It has been reported that the main components in the essential oil content of *M. aurea* species are pinane (0.03-1.25%), octahydrocoumarin 5,75,7-dimethyl (0.05-19.16%), caryophyllene oxide (0.06-1.54%), β-Farnesol (0.17-2.04%), α-Bisabolol oxide (1.16-4.64%), 2,5-Bis1 ,1-8 dimethylethyl-thiophene (0.02-10.95), 1,5-Bis (dicyclohexylphosphino)-pentane (3.95-44.7%), 2-Ethoxy-6-ethyl-4,4,5-trimetethyl 1,3-dioxa -4-sila-2-boracyclohex-5-ene (6.49-37.99%) and n-eicosane (0.42-6.59%) (Kheder et al., 2014). The main components in the essential oil content of *M. chamomilla* species are limonene (0.99-1.22%), (E)-β-farnesene (1.62-53.45%), bisabolol oxide B (1.2-35.63%), azulene-7ethyl-1,4 dimethyl (4.1-19.27%), bisabolol oxide A (2.19-47.7%), 8-Isobutyryloxy isobornyl isobutyrate (11.15-14.03%), α-bisabolol (2.2-56.86%), trans-trans-farnesol (15.64%), cis-β-farnesine (7.12) %), α-cubebene (2.69%), germacene D (2.5-6.2%), bicyclogermacrene (1.7%), cis-z- α-bisabolene expoxide (9.8%), cis -ene-yne-dicycloether (4%), trans-ene-yne-dicycloether (3.3%), α-farnesene (8.3-9.3%), spiroether (5.6 %), cis-bicycloether (5.0%) and spathulenol (0.81%) (Ali, 2013; Ayoughi et al., 2011; Göger et al., 2018; Pirzad et al., 2006; Rahmati et al., 2011; Rathore and Kumar, 2021; Satyal et al., 2015; Stanojevic et al., 2016; Tolouee et al., 2010; Tsivelika et al., 2018). 1.5-Bis (dicyclohexylphosphino)-

Table 2. Volatile oil compounds (%) of *Matricaria aurea* and *Matricaria chamomilla*.

Plant species	Geographic regions	Used Parts	Chemical contents	References
Matricaria aurea(Loefl.) Sch.Bip.	Tunisia	Aerial, stem	Pinene (0.03%-1.25%), octahydrocoumarin 5,75,7-dimethyl (0.05%-19.16%), caryophyllene oxide (0.06%-1.54%), β-Farnesol (0.17%-2.04%), α-Bisabolol oxide (1.16%-4.64%), 2,5-Bis1,1-8 dimethyleth) thiophene (0.02%-10.95), 1,5-Bis (dicyclohexylphosphino)-pentane (3.95%-44.7%), 2-Ethoxy-6-ethyl-4,4,5- trimetethyl 1,3-dioxa-4-sila-2-boracyclohex-5-ene (6.49%-37.99%), n-eicosane (0.42%-6.59%)	(Kheder et al., 2014)
Matricaria chamomilla L.	Iranian, Egypt, Turkey, Bosnia and Herzegovina, Serbia, India, Greece, Nepal,	Aerial parts	limonene (0.99%-1.22%), (E)-β-farnesene (1.62%-53.45%), bisabolol oxide B (1.2%-35.63%), azulene-7ethyl-1,4 dimethyl (4.1%-19.27%), bisabolol oxide A (2.19%-47.7%), 8-Isobutyryloxy isobornyl isobutyrate (11.15%-14.03%), trans-trans-farnesol (15.64%), cis-β-farnesene (7.12%), α-cubebene (2.69%), germacene D (2.5%-6.2%), bicyclogermacrene (1.7%), α-bisabolol (2.2%-56.86%), cis-z-α-bisabolene epoxide (9.8%), cis-ene-yne-dicycloether (4%), trans-ene-yne-dicycloether (3.3%), α-farnesene (8.3%-9.3 %), spiroether (5.6 %), cis-bicycloether (5.0%), spathulenol (0.81%)	(Ali, 2013; Ayoughi et al., 2011; Göger et al., 2018; Pirzad et al., 2006; Rahmati et al., 2011; Rathore and Kumar, 2021; Satyal et al., 2015; Stanojevic et al., 2016; Tolouee et al., 2010; Tsivelika et al., 2018)

pentane (3.95-44.7%) was found to be the highest compound detected in *M. aurea*, while α-bisabolol (2.2-56.86%), was the highest detected compound in *M. chamomilla*. In this context, it is thought that the value range of the compounds determined according to literature data will be a reference source for future studies. In addition, it is thought that the presence of all the identified compounds together will help speed up the studies and provide information about the major compounds.

4. CONCLUSION

In this study, the usage areas, chemical contents and biological activities of *Matricaria* species were compiled according to the literature data. According to the findings, these plant species are widely used for health and cosmetic purposes. It has also been reported as a very important source of antioxidant and antimicrobial activity. In addition, it was observed that the dominant compound among *Matricaria* species was 1,5-bis(dicyclohexylphosphino)–pentane (3.95-44.7%) and α-bisabolol (2.2–56.86%), It has been determined that it can be an important natural source for the mentioned compounds.

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CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд.

615:633

LEKOVITE sirovine = Natural Medicinal Materials
: the journal that covers all areas of medicinal plants research
/ editor-in-chief Milan Lukić. - 1951, br. 1- . - Belgrade
: Institute for Medicinal plants research "Dr. Josif
Pančić", 1951- (Belgrade : Službeni glasnik). - 30 cm

Godišnje. - Drugo izdanje na drugom medijumu:
Lekovite sirovine (Online) = ISSN 2560-3965
ISSN 0455-6224 = Lekovite sirovine
COBISS.SR-ID 5443330