Original Research

Antioxidant activity and metal content: a study on *Dictamnus albus*

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Abstract

Dictamnus albus is a perennial plant of warm, dry, and sunny habitats that have been traditionally used as a medicinal plant since ancient times. Our study focused on examining the phenolic compound content, antioxidant capacity, and concentrations of Fe, Cu, Zn, Mn, and Ni in the leaves of *D. albus*. The concentration of phenolic compounds in the leaves of *D. albus* was 5.40±0.09 mg GAE/g_{DW}. The leaf ethanol extract of *D. albus* exhibited significant antioxidant activity, as demonstrated by its ability to scavenge DPPH radicals, with an IC_{50} value of 38.20±0.46 µg/mL. The ethanol extract showed a slightly lower capacity to scavenge H_2O_2, with an IC_{50} value of 912±40 µg/mL. For the first time, we demonstrated that *D. albus* extract has a significant capacity to scavenge hydroxyl radicals (·OH). After adding *D. albus* extract, the residual ·OH radicals percentage was 39%, compared to 13% when using a 2 mM Trolox standard. In addition, the ethanol extract of *D. albus* showed the ability to reduce Fe^{3+} and Cu^{2+}, indicating the extract's ability to inhibit oxidative processes. Furthermore, *D. albus* extract can chelate Fe and thus prevent the Fenton reaction. The metal content in the leaves of *D. albus* was as follows: Fe 44.16±0.685 mg/kg, Cu 6.06±0.253 mg/kg, Zn 21.64±0.571 mg/kg, Mn 22.01±0.413 mg/kg, and Ni 1.21±0.112 mg/kg. Our results showed that the ethanol extract of *D. albus* has significant antioxidant capacity, that the concentrations of Fe, Cu, Zn, Mn, and Ni were below permissible doses for medicinal plants, and that the extract can contribute to the daily intake of these essential elements. These findings suggest that *D. albus* extract could be used in the therapy of diseases associated with oxidative stress.

Keywords

phenolic compounds; micronutrients; hydroxyl radical; oxidative stress

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Antioksidativna aktivnost in vsebnost kovin: študija na *Dictamnus albus*

Izvleček

Dictamnus albus je trajnica toplih, suhih in sončnih rastišč, ki se že od nekdaj tradicionalno uporablja kot zdravilna rastlina. V naši študiji smo se osredotočili na preučevanje vsebnosti fenolnih spojin, antioksidativne zmogljivosti in koncentracij Fe, Cu, Zn, Mn in Ni v listih *D. albus*. Koncentracija fenolnih spojin v listih *D. albus* je bila 5,40±0,09 mg GAE/gDW. Etanolni izvleček listov *D. albus* je pokazal pomembno antioksidativno aktivnost, demonstrirano z zmožnostjo odstranjevanja radikalov DPPH z vrednostjo IC_{50} 38,20±0,46 µg/mL. Etanolni izvleček je imel nekoliko manjšo sposobnost za odstranjevanje H_2O_2 z vrednostjo IC_{50} 912±40 µg/mL. Prvič smo dokazali, da ima izvleček *D. albus* pomembno sposobnost odstranjevanja hidroksilnih radikalov (·OH). Po dodatku izvlečka *D. albus* je bil odstotek preostalih ·OH radikalov 39 % v primerjavi s 13 % pri uporabi 2 mM standarda Trolox. Poleg tega je etanolni izvleček *D. albus* pokazal sposobnost redukcije Fe^{3+} in Cu^{2+}, kar kaže na sposobnost izvlečka, da zavira oksidativne procese. Izvleček *D. albus* kelatira tudi Fe in tako prepreči Fentonovo reakcijo. Vsebnost kovin v listih *D. albus* je bila naslednja: Fe 44,16±0,685 mg/kg, Cu 6,06±0,253 mg/kg, Zn 21,64±0,571 mg/kg, Mn 22,01±0,413 mg/kg in Ni 1,21±0,112 mg/kg. Naši rezultati so pokazali, da ima etanolni izvleček *D. albus* pomembno antioksidativno sposobnost, da so koncentracije Fe, Cu, Zn, Mn in Ni pod dovoljenimi odmerki za zdravilne rastline in da lahko izvleček prispeva k dnevnemu vnosu teh bistvenih elementov. Te ugotovitve kažejo, da bi se lahko izvleček *D. albus* uporabljal pri zdravljenju bolezni, povezanih z oksidativnim stresom.

Ključne besede

fenolne spojine; mikrohranila; hidroksilni radikali; oksidativni stres

Introduction

Medicinal plants have been used worldwide from ancient times to the present day for the treatment of various diseases, including cardiovascular disorders, inflammatory conditions, and cancer risk reduction. In addition to their role in traditional medicine, these plants are widely utilized in the pharmaceutical, cosmetic, and food industries (Škrovánková et al., 2012; Astutik et al., 2019; Salmerón-Manzano et al., 2020). One of the plants traditionally used for the treatment of various diseases is *Dictamnus albus* L. (Rutaceae). This is a bushy perennial plant species with a natural range that extends from Europe to southwestern Siberia and western Himalaya (Townsend, 1968; Diklić, 1973; Euro+Med, 2006-onwards; POWO, 2024). It grows in dry and sunny habitats, including rocky slopes, dry meadows, light thickets, openings in deciduous forests, and along roads from lowlands to lower mountainous areas (Diklić, 1973). The plant is characterized by a strong scent reminiscent of lemon. Since ancient times, plants referred to as "*Dictamnus*," which include several species from the Rutaceae and Lamiaceae families, have been used for medicinal purposes. However, since the 8th century, the most used species among these plants has been *Dictamnus albus*. Traditionally, all parts of the plant have been employed in medicinal treatments, with the root and root bark being the most commonly utilized components (Martínez-Francés et al., 2015). *D. albus* exhibits a wide range of biological activities, acting as an antipyretic, antiseptic, antispasmodic, cardiosedative, contraceptive, diuretic, emmenagogue, mutagenic, nervine, phototoxic, propeptic, sedative, stimulant, tonic, uterotonic, and vermifuge (Duke et al., 2002). For example, the leaves are commonly used in the form of tea to treat digestive issues and expel worms, while a tincture made from the leaves is applied externally to treat rheumatism (Gelenčir, 1989; Sarić, 1989; Popović et al., 2014). *D. albus* is also recognized for its use in veterinary care; shepherds, for example, have used fresh leaves to induce heat in goats (González et al., 2020). The bioactive compounds detected in *D. albus* have been classified into several groups, including quinoline alkaloids, limonoids, sesquiterpenes and their glycosides, flavones, and coumarins. Among these, quinoline alkaloids and limonoids are considered the most characteristic compounds (Lv et al., 2015).

Activated forms of oxygen, commonly known as reactive oxygen species (ROS), include free radicals such as hydroxyl ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$), alkoxyl ($RO^{\bullet}$), and peroxyl ($RO_2^{\bullet}$) radicals. These free radicals are highly unstable and reactive due to their unpaired electron in the outer orbit. While hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are not free radicals, they can trigger free radical reactions (Jomova et al., 2023). Under normal physiological conditions, ROS are synthesized and play vital roles in energy production, detoxification, redox regulation of protein phosphorylation, ion channel activity, transcription factor regulation, and immune responses (Brieger et al., 2012; Škrovánková et al., 2012).

ROS-related diseases can result from either a deficiency (e.g., chronic granulomatous disease, certain autoimmune disorders) or an excess of ROS (Brieger et al., 2012). Overproduction of ROS accelerates oxidative processes, leading to cellular damage and, eventually, cell death. ROS have been linked to the development of numerous chronic and degenerative diseases, such as cancer, cardiovascular, respiratory, neurodegenerative, and digestive disorders (Brieger et al., 2012; Liu et al., 2018).

The human body has endogenous antioxidants that combat ROS, such as enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase) and non-enzymatic antioxidants (glutathione, urea, vitamin E, and melatonin). Exogenous antioxidants, derived from the secondary metabolites of medicinal plants, also play a crucial role in defending the body against oxidative stress (Brieger et al., 2012; Akanni et al., 2014; Liu et al., 2018).

Antioxidants from medicinal plants belong to different chemical classes, among which phenolic compounds are one of the most important. Phenolic compounds exert their antioxidant capacity through various mechanisms: they act as reducing agents, hydrogen-donating agents, singlet oxygen quenchers, and metal chelators (Vuolo et al., 2019; Tatipamula and Kukavica, 2021). Under certain conditions (high pH, high transition metal ions concentrations, and presence of O_2), phenolic compounds can exhibit pro-oxidative properties, which should be considered when using them for therapeutic purposes or treatment. Low molecular weight phenolic compounds (gallic acid) exhibit pro-oxidative properties more easily than phenolic compounds with higher molecular weight (condensed tannins), which have almost no pro-oxidative properties (Škrovánková et al., 2012). Antioxidant components may act independently or in plant extracts where it is possible to manifest synergism.

Micronutrients like iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), and nickel (Ni) are essential for plant metabolism in optimal concentrations that differ depending on the plant species. Both lower concentrations (deficit) and increased concentrations (toxicity) negatively affect the plant's growth and development. Essential micronutrients for the human body are Fe, Mn, Cu, Zn, and selenium (Se). Iron is a key component of haemoglobin and an important cofactor for several human enzymes, such as catalase. It is found in high concentrations in erythrocytes and muscle cells. However, excessive iron intake can lead to hepatotoxicity and negatively affect the cardiovascular system, while iron deficiency results in anaemia. Copper is another essential element in the human body, playing a crucial role in transcription factors and enzymes. It regulates oxidation-reduction reactions, energy production, neurotransmitter synthesis, and iron metabolism. Excessive copper levels can lead to symptoms such as vomiting, dermatitis, abdominal pain, and diarrhoea. Chronic copper overload may result in Wilson's disease, characterized by the accumulation of free copper in the liver, brain, and kidneys. On the other hand, copper deficiency can lead to aneurysms, vascular damage, and impaired nutrient transport. Manganese is a component of metalloenzymes responsible for oxidizing cholesterol and fatty acids. A manganese deficiency can cause bleeding disorders, while excessive levels may lead to speech disorders, leg cramps, and encephalitis. Zinc is an essential element in human physiology, involved in transcription factors and enzymes like superoxide dismutase and carboxypeptidase. However, excessive zinc intake can result in toxic effects on blood lipoprotein levels and the immune system, induce copper deficiency, and lead to cell apoptosis. Zinc deficiency is also associated with suppressed immune function. Both zinc and copper play critical roles in glucose metabolism and cholesterol regulation as enzyme cofactors. Nickel is essential for iron metabolism but becomes toxic at elevated concentrations. Excessive exposure to nickel has been linked to an increased risk of hypertension, neurological deficits, cardiovascular disease, developmental delays in childhood, and lung cancer (Brima, 2018; Sulaiman et al., 2024).

The sources of metals available to plants can come from various origins, including natural sources (such as rocks, aerosols, and dust particles), domestic sources (biofuel combustion, electrical waste, and both natural and

chemical waste), agricultural waste (such as pesticides and chemical fertilizers), industrial sources (mine products, chemical waste, and thermal power waste), as well as other sources (fossil fuel combustion, open dump yards, and pharmaceutical waste) (Vinogradova et al., 2023).

The availability of these metals to plants and their accumulation in plant tissues can significantly impact the quality of medicinal plants, particularly by altering the concentration of active substances, such as phenolic compounds. Although the concentration of biologically active compounds in plants is species-specific, it is also influenced by environmental factors (Vinogradova et al., 2023). The uptake of metals from the soil is significantly influenced by soil properties such as water content, pH, organic matter content, and soil structure (Morgan and Connolly, 2013). The qualitative and quantitative composition of these compounds and micronutrients depends on the plant species' metabolic characteristics, habitat, and exposure to various abiotic, biotic, and anthropogenic factors.

Our work focuses on examining the antioxidant capacity of the ethanolic extract of *Dictamnus albus* leaves, with the aim of exploring its potential use in the prevention of diseases associated with oxidative stress. To the best of our knowledge, there are no previous studies reporting the concentrations of metals such as Fe, Cu, Zn, Mn, and Ni in *D. albus* leaves. Therefore, the present study aims to determine these metal concentrations, given their potential health benefits at optimal levels and the risk of toxicity at elevated concentrations.

Materials and Methods

Chemicals

DEPMPO (5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide, Focus Biomolecules, USA); H_2O_2 (Lach-ner, Czechia); $FeSO_4x7H_2O$ (Merck, Germany); dH_2O (Lonza, Belgium); $HClO_4$ (Lach-ner, Czechia); HNO_3 (Lach-ner, Czechia); Ethanol (MB IMPEKS d.o.o.); DPPH (2,2-diphenyl-a-picryl-hydrazyl,); TPTZ (2,4,6-tripyridyl-s-triazine, Sigma-Aldrich, Germany); Na_2CO_3 (Lach-ner, Czechia); Folin Ciocalteu reagens (Sigma-Aldrich, Germany); Na_2HPO_4 (Semikem, B&H); NaH_2PO_4 (Lach-ner, Czechia); Ferrozine (Sigma-Aldrich, Germany); $CuCl_2$ (ZORKA Pharma Šabac, Serbia); Neocuproine (Sigma-Aldrich, Germany); CH_3COOH (Lach-ner, Czechia); CH_3COONa (ZORKA Pharma Šabac, Serbia), Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich, Germany); Gallic acid (Sigma-Aldrich, Germany).

Plant material

Leaves of the *Dictamnus albus* were collected during the flowering phase in May 2020 in the eastern Herzegovina region (Mosko, Bileća, Republic of Srpska, Bosnia and Herzegovina). The plants were growing in a sunny and dry site, on the edge of a rocky meadow, far from larger settlements and traffic roads. One fully developed middle stem leaf was collected from each of a total of 15 individuals. The specimens were identified using dichotomous keys (Townsend, 1968; Diklić, 1973; Nikolić, 2020). The nomenclature is in accordance with Euro+Med Plant Base (2006-onwards). A voucher specimen is stored in the herbarium collection of the Institute for Nature Conservation of Vojvodina province in Novi Sad (Herbarium Code: PZZP) (Thiers, 2024).

Preparation of plant extracts

The leaves were dried at room temperature in the shade in an airy place. For all analyses, we used dry plant material. After drying, the plant material was ground to powder with an electric mill and used for extraction. 80% ethanol was used as a solvent, and the extraction ratio was 10 g of plant tissue and 200 mL of 80% ethanol. The homogenate was then sonicated for 5 minutes and mixed for 30 minutes on a magnetic stirrer (750 rpm, VELP SCIENTIFICA). Then, the homogenate was filtered (filter paper, ALBET, 73 g/m^2, pore size 30-40 microns), and the filtrate was labelled as extract 1 (E1). The remaining residue was re-extracted with 100 mL of 80% ethanol, and the sonication and mixing were repeated. After filtering, extract 2 (E2) was separated. Extracts E1 and E2 were combined and evaporated (IKA Rotary Evaporator RV 8 V–C) at 45 °C to a total volume of 30 mL. The extracts were stored until analysis at -18 °C.

Determination of the content of total phenolic compounds

The content of total phenolic compounds (TPC) was determined according to Singleton and Rossi (1965) by measuring the absorbance at 724 nm (Shimadzu UV-1800). The reaction mixture contained 100 µL of plant extract and 450 µL of Folin-Ciocâlteu reagent (diluted 1:3 with distilled

water). After incubation for 5 minutes at room temperature (23°C), 450 µL of 1M Na2CO3 was added. The mixture was well vortexed (Velp Scientific Zx3) and then incubated for 60 minutes in the dark at room temperature. The quantification of TPC was calculated based on the calibration curve for gallic acid and expressed as gallic acid equivalent (GAE) per g_{DW} (dry weight).

Determination of the ability to remove DPPH radicals

The ability to remove DPPH (2,2-diphenyl-a-picrylhydrazyl) radicals was determined by the method given by Liyana-Pathiranana and Shahidi (2005). The sample contained 0.5 mL of 0.136 mM ethanol solution of DPPH radical and 0.5 mL plant extracts of different concentrations (2.5, 10, 25, 50, 75 µg/mL, in relation to the concentration of phenolic compounds). Different concentrations of plant extract were prepared in 80% ethanol. The sample was mixed and incubated for one hour in the dark at room temperature (23°C). The control contained 0.5 mL of 0.136 mM DPPH solution and 0.5 mL of 80% ethanol. After incubation, the absorbance was measured at 517 nm (Shimadzu UV-1800). The percentage of inhibition was calculated according to the equation:

$$\% \text{ inhibition} = \frac{A_C - A_S}{A_C} \times 100$$

Where AC is the absorbance value of the control and As is the absorbance value of the sample.

The IC_{50} was determined from a standard curve constructed as a dependence of the percentage of inhibition on the concentration of phenolic compounds in the extract and expressed in units of µg/mL.

Ability to scavenge H_2O_2

The ability of the extract to scavenge hydrogen peroxide was determined according to Ebrahimzadeh et al. (2010). The sample contained 920 µL of Na-phosphate buffer 0.067 M, 30 µL of 0.5 M H_2O_2 (prepared in 0.067 M Na-phosphate buffer pH 6.4), and 50 µL of plant extract of different concentrations (25, 500, 1000, 1250, 1500 µg/mL, in relation to the concentration of phenolic compounds). Different concentrations of plant extract were prepared in 80% ethanol. The samples were mixed and incubated at room temperature (23°C) for 30 minutes in the dark. After incubation, the absorbance was measured at 230 nm (Shimadzu UV-1800). The control contained 970 µL of Na-phosphate buffer 0.067 M pH 6.4 and 30 µL of 0.5 M H_2O_2. The sample blank (A_{Sb}) contained 50 µL of extract of different concentrations and 950 µL of Na-phosphate buffer.

The percentage of inhibition was calculated according to the equation:

$$\% \text{ inhibition} = \frac{A_C - (A_S - A_{Sb})}{A_C} \times 100$$

Where Ac is the absorbance value of control, As is the absorbance value of the sample, and A_{Sb} is the absorbance value of the sample blank.

The IC_{50} was determined from a standard curve constructed as a dependence of the percentage of inhibition on the concentration of phenolic compounds in the extract and expressed in units of µg/mL.

Ability to scavenge ·OH radicals

The scavenging ability of the extracts against hydroxyl radicals was assessed using a solution containing the extract, combined with the Fenton reaction and the spin trap 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) (Milutinović et al., 2023).

The reaction mixture (total volume 29 µL) consisted of the extract (1 µL, previously 1000 times diluted in water), deionized water (25 µL), H_2O_2 (2 µL, final concentration 0.35 mM), and DEPMPO (1 µL, final concentration 3.5 mM). This mixture was placed into a gas-permeable Teflon tube, followed by the immediate addition of $FeSO_4$ (1 µL, final concentration 0.15 mM). EPR spectra were recorded after 2 minutes at room temperature using a Bruker Biospin Elexsys II 540 spectrometer. The experimental setup employed the following operating parameters: centre field 3500 G, microwave power 10 mW, microwave frequency 9.85 GHz, modulation frequency 100 kHz, modulation amplitude 1 G. Control experiments were conducted by replacing the extract with an equivalent amount of solvent. The ·OH radical scavenging activity (AA) of the extracts was calculated using the following equation:

$$AA = \frac{(I_C - I_S)}{I_C} \times 100$$

Where Ic and Is are the double integral values for the control and sample, respectively, derived from the EPR spectra (using Xepr software).

Determination of Fe chelating ability

Determination of Fe chelating ability was performed using the method Carter (1971) gave, with certain modifications. The samples contained 937.5 µL of 80% ethanol, 12.5 µL of 1 mM $FeSO_4$, and 50 µL of plant extracts (concentrations of TPC 300 µg/mL). The samples were mixed and incubated for 10 minutes at room temperature (23°C), after which 50 µL of 2 mM ferrozine was added. The samples were then mixed and incubated for 10 minutes in the dark. The absorbance was measured at 562 nm (Shimadzu UV-1800). The control, instead of the plant extract, contained 80% ethanol. The percentage of Fe chelation was calculated according to the equation:

$$\% \text{ chelating} = \frac{A_C - A_S}{A_C} \times 100$$

Where Ac is the absorbance value of the control and As is the absorbance value of the sample.

Determination of Fe^{3+} reduction ability

FRAP (ferric reducing antioxidant power) was performed according to the method developed by Benzie and Strain (1996). The sample (concentration 500 µg/mL) with a volume of 0.2 mL was mixed with 1.8 mL of FRAP working reagent, incubated for 10 minutes at 37 °C, and the absorbance was measured at 593 nm (Shimadzu UV-1800). As a sample blank, 1.8 mL of FRAP working solution and 0.2 mL of distilled water were used. A calibration curve was constructed for $FeSO_4x7H_2O$ in a concentration range of 0.1-2 mM. The capacity of the samples to reduce Fe^{3+} results was presented as mmol Fe^{2+}/L of the sample.

Determination of Cu^{2+} reduction ability

The ability of ethanol plant extracts to reduce Cu^{2+} was determined using a method proposed by Apak et al. (2004). Samples were prepared by mixing 0.3 mL of 0.01 M $CuCl_2$, 0.3 mL of 10 mM acetate buffer (pH 7), 0.3 mL of 7.5 mM ethanolic neocuproine solution, and 0.05 mL of the ethanolic plant extract. The mixtures were vortexed and incubated at room temperature (23°C) for 30 minutes. After incubation, the absorbance was measured at 450 nm (Shimadzu UV-1800). A blank sample was prepared similarly, with 0.05 mL of ethanol replacing the plant extract. Trolox was used as a standard at concentrations ranging from 20 to 300 µg/mL, and the results are expressed as µg of Trolox equivalents per mL of extract.

Determination of metal contents in plant samples

Quantification of elements (Fe, Cu, Zn, Mn, and Ni) contents in the examined plant samples was done in the extracts obtained after the destruction of the plant tissues with concentrated nitric acid, supplemented with 30% H_2O_2 and 70% $HClO_4$ (Perquel et al., 1993) by atomic absorption spectrophotometry (AAnalyst 400, Perkin Elmer, USA). The selected wavelengths of elements to be determined were as follows: Fe: 248.33, Cu: 324.75, Zn: 213.70, Mn: 279.48, and Ni: 232.00. Standard solutions were prepared using a single element stock solution (1000 mg/L, Perkin Elmer, USA) and appropriate diluent extractant. All analytical procedures were done in three biological replicates and made with glassware prewashed with 10% HNO_3. Quality control of acid digestion and element quantification was performed by analyses of certified referent material (ERM®-CD218, ryegrass) and recovery values in the acceptable range (85-103%) for each element. Results are expressed as mg of metal content per kg of dry weight (mg/kg DW)

Data analyses

All data were analyzed using GraphPad Prism 8, employing One-way ANOVA and paired t-tests for statistical comparisons. Correlations between phenolic compound content, antioxidant activity (measured by various methods), and metal content were determined using Pearson's correlation coefficient (r), calculated in Microsoft Excel. The significance levels were defined as follows: *p<0.05, ***p<0.001 and ****p<0.0001.

Results and Discussion

Phenolic content and antioxidant properties of the plant extract

One of the major health problems in developing countries is malnutrition (deficiencies, excesses, or imbalances in an intake of energy and/or nutrients), which is the aetiology of various diseases (Liu et al., 2018; Liu et al., 2021). In their review paper, Liu et al. (2018) emphasized the connection

between nutrition and the occurrence of oxidative stress associated with cardiovascular diseases, neurodegenerative disorders, cancer, digestive diseases, and ageing. Antioxidants can mitigate or prevent oxidative processes through various mechanisms. They are defined as compounds that neutralize free radicals and other reactive molecules, halt chain reactions, and repair oxidative damage (Apak et al., 2007). Phenolic compounds, multifunctional antioxidants, as part of the diet or as supplements can be crucial for neutralizing ROS, reducing oxidative stress, and preventing the development of various diseases (Rahman et al., 2021). Numerous beneficial effects for human health of *Dictamnus albus* and its related species *D. dasycarpus* Turcz. (with a native range from southeastern Siberia to China and Korea) extracts are known such as neuroprotective, anti-allergy activity, protection of cardiovascular activity, improvement of gastrointestinal activity, and anti-inflammatory activity (Qin et al., 2021; İlgün and Karatoprak, 2022). The positive effects of the extract are based on the action of plant secondary metabolites, among which phenolic compounds play an important role. A total phenolic content (TPC) was measured in the ethanol extract of *D. albus*, and the results are shown in Table 1. The concentration of phenolic compounds in leaves of *D. albus* was 5.4±0.09 mgGAE/gDW. İlgün and Karatoprak (2022) measured TPC in the methanolic extracts of *D. albus* herb and root. Their results indicated a higher phenolic content in the herbal extract (77.13±5.73 mg GAE/g extract) compared to the root extract (43.81±9.49 mg GAE/g extract). The differences in phenolic compound concentrations may be due to variations in extraction methods, solvents used, and the units of measurement (e.g., fresh or dry plant mass, extract mass). Additionally, these results can be influenced by the geographical origin of the plants as well as the biotic and abiotic stresses to which the plants were exposed in their natural habitats (Shi et al., 2022; Bilgin and Şahin, 2023; El Kamari et al., 2024).

The two main mechanisms by which antioxidants inactivate ROS are via electron transfer (ET) and hydrogen atom transfer (HAT), although in some cases, the two mechanisms cannot be clearly distinguished. Through which mechanism the antioxidant will act depends on its structure, solubility, distribution coefficient, and the type of solvent (Çelik et al., 2010). Using different methods to evaluate antioxidant capacity gives us a clearer insight into the possibilities of plant extracts for the removal of ROS. Therefore, we evaluated the antioxidant capacity of *D. albus* using several different methods.

The electron donation ability of plant metabolites (e.g. phenolic compounds) can be measured by DPPH radical scavenging abilities (Akanni et al., 2014). The ethanolic extract of Dictamnus albus with an IC_{50} value of 38.20±0.46 µg/mL showed a great ability to inhibit DPPH radicals (Table 1). *D. albus* extracts from Serbia showed a lower ability to remove DPPH radicals compared to our sample with IC_{50} values of 59.80±1.53 µg/mL for the methanol extract and IC_{50} values of 76.48±2.30 µg/mL for the ethanol extract (Pavlović et al., 2018). The *D. albus* herbal extract from Turkey at a concentration of 4 mg/mL showed the ability to remove DPPH radical with a value of 56.86±2.4%, higher than root *D. albus* extract (34.56±2.77%). Another study showed a significantly higher antioxidant activity of *D. albus* leaf extract than the root. Cao et al. (2022) showed an IC_{50} for a leaf extract of 0.13±0.23 µg/mL, while for root extract, the measured IC_{50} value was 3.68±0.56 µg/mL.

Significant DPPH radical scavenging activity was demonstrated for the methanol extract of *Dictamnus dasycarpus* root bark from China for different years (Liu et al., 2021). The authors showed that DPPH radical scavenging activity was concentration-dependent from 1.6 to 14.4 µg/mL with IC_{50} values in the range of 1.287-9.758 µg/mL, whereby the scavenging activity was gradually enhanced with the prolonging of growth years. Differences in the anti-

Table 1. The phenolic content (TPC) and antioxidant activity of the ethanol extract of *Dictamnus albus* leaves were measured using different methods. Results are expressed as mean value ± SD (n = 3).

Tabela 1. Vsebnost fenolov (TPC) in antioksidativna aktivnost etanolnega izvlečka listov *Dictamnus albus*, izmerjena z različnimi metodami. Rezultati so izraženi kot povprečna vrednost ± SD (n = 3)

TPC	DPPH scavenging ability	H_2O_2 scavenging ability	Ability to reduce Fe^{3+}	Ability to reduce Cu^{2+}	Ability to chelate Fe
(mg GEA/g_{DW})	IC_{50} (µg/mL)	IC_{50} (µg/mL)	mmol/L Fe^{2+}	µg Trolox/mL	300 µg/mL (%)
5.40±0.09	38.20±0.46	912±40	975±52	164±8	53.62±2.84

oxidant abilities of the extract may arise from differences in the qualitative and quantitative composition of the extract (Ren et al., 2020).

Hydrogen peroxide in a Fenton-type reaction with Fe produces a hydroxyl radical. The superoxide anion radical can also react with Fe^{3+}, whereby Fe^{2+} is regenerated, which can participate in the Fenton reaction. Hydroxyl radical is one of the most powerful ROS in biological systems. It reacts with cell membranes' polyunsaturated fatty acids, causing damage and "leakage" of membranes. The hydroxyl radical is capable of oxidative damage to every molecule of the biological system and contributes to carcinogenesis, mutagenesis, and cytotoxicity (Akanni et al., 2014). The mutagenic capacity of ·OH is a consequence of their interactions with DNA and, therefore, plays an important role in the development of cancer (Akanni et al., 2014). It is known that the reducing properties of an extract could serve as a measure of its antioxidant action by donating hydrogen atoms to break the free radical chain. In the ethanol extract of *Dictamnus albus*, the ability to remove H_2O_2 was with IC_{50} values of 912±40 µg/mL (Table 1).

In addition, the ethanol extract of *Dictamnus albus* showed the ability to remove ·OH radicals (measured as % residual hydroxyl radical after the addition of the extract) (Figure 1). After the addition of *D. albus* extract, the percentage of residual ·OH was 39%, indicating a lower ability to remove ·OH radicals compared to the 2 mM Trolox standard (13%). However, this removal capacity was significantly higher compared to the control (dH_2O), which represents 100% residual ·OH and is not shown in the figure. Our results indicate that *D. albus* extract has a significant ability to remove OH radicals, which may indicate its potential role in preventing oxidative DNA damage and lipid peroxidation (Laughton et al., 1989; Khennouf et al., 2010; Sevgi et al., 2015).

The determination of the antioxidant potential of the plant extract as the ability to reduce Fe^{3+} and Cu^{2+} is based on the theoretical assumption that any good reductant is a good antioxidant. Fe^{3+} reduction is used as an indicator of plant extract for its electron-donating capacity (Petkov et al., 2022). At a TPC concentration of 500 µg/mL, *Dictamnus albus* shows the ability to reduce Fe^{3+} with a value of 975±52 mmol/L Fe^{2+} and reduce Cu^{2+} with a value of 164±8 µgTrolox/mL at a TPC concentration of 300 µg/mL (Table 1). Literature data point out that the ability of plants to reduce Fe^{3+} ions varies greatly from species to species (Nwozo et al., 2023). The authors showed that the range of values of the FRAP method for different medicinal plants is very

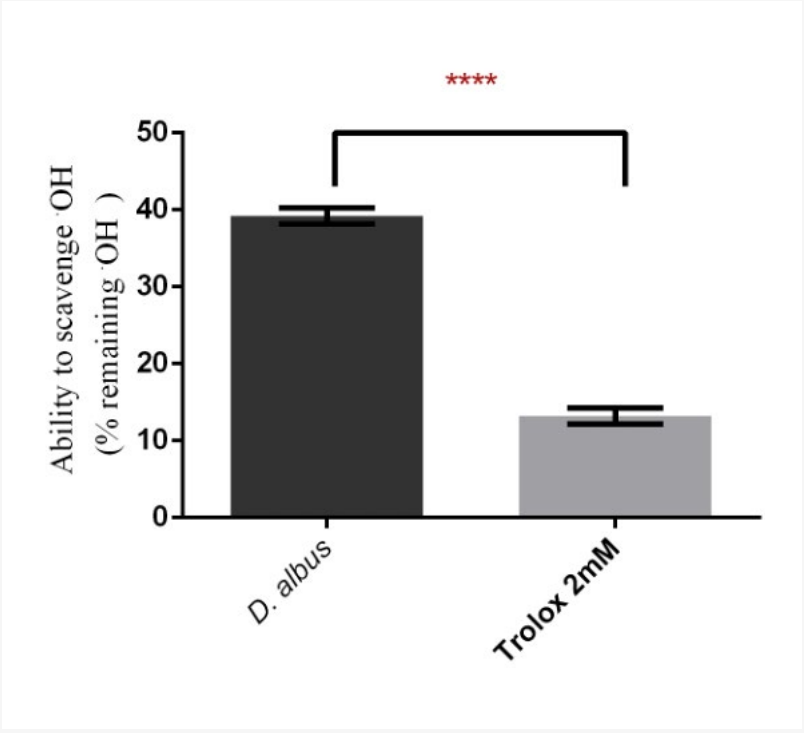


Figure 1. The ability of *Dictamnus albus* leaf extract to scavenge ·OH radicals. The ability was measured as a percentage of the residual ·OH radical after the addition of the extract and compared to the Trolox standard (2 mM). Results are expressed as mean value ± SD (n=3). Asterisks indicate statistical significance (p<0.0001).

Slika 1. Sposobnost izvlečka listov *Dictamnus albus*, da odstranjuje radikale ·OH. Sposobnost je bila izmerjena kot odstotek preostalega ·OH radikala po dodatku ekstrakta in primerjana s standardom Trolox (2 mM). Rezultati so izraženi kot povprečna vrednost ± SD (n=3). Zvezdice označujejo statistično pomembnost (p<0,0001).

wide (3.25-1225 µg/mL). Similarly, for 27 medicinal plants from the area of North Macedonia, the ability to reduce Cu^{2+} values was in the range of 53-1068 µmolTrolox/g_{DW} (Tusevski et al., 2014).

The extract of *Dictamnus albus* at a concentration of 300 µg/mL showed a good ability to chelate Fe with a value of 54±3% (Table 1). Gonçalves et al. (2014) showed that the ability to chelate Fe for different plants is very wide-ranging and that it depends on the plant species and the solvent used. Namely, for Mediterranean medicinal plants, it was shown that the ability to chelate Fe is in the range of 38-94% for a cold infusion and in the range of 40-67% for a hot infusion for a concentration of phenolic compound of 400 µg/mL. The antioxidant capacity of the extract is influenced by the plant's genetic potential, environmental factors in its natural habitat, the extraction process, and the choice of solvent used. Lipid peroxidation can be triggered by redox-active metals, particularly Fe. Gonçalves et al. (2014) demonstrated that the Fe^{2+}-chelating capacity of an extract plays a varying role in preventing lipid peroxidation, depending on the plant species. Consequently, the ability to chelate Fe emerges as a crucial property of the extract in combating lipid peroxidation.

This antioxidant property of medicinal plants evaluated by different methods usually correlates very well with the TPC, indicating that phenolic compounds contribute the most to the antioxidant potential of the extract (Petkov et al., 2022).

In the extract of *Dictamnus albus* leaves, we did not detect correlations between the concentration of phenolic compounds and the different antioxidant methods used in the research (Table 3). In contrast to our results, other authors have shown the existence of a correlation between the content of phenolic compounds and the antioxidant activity of plant extracts (Piluzza and Bullitta, 2011; Muflihah et al., 2021). Our results indicate that part of the antioxidative activity can be attributed to metabolites (terpenoids, ascorbate, glutathione). Among the antioxidant assays, a strong negative correlation (-0.998) with statistical significance ($p<0.036$) was observed between the Fe-reducing ability and H_2O_2 scavenging capacity, suggesting a shared neutralization mechanism in the *D. albus* extract.

Metal content

The importance of determining the metal content in medicinal plants is well documented in the literature (Kulhari et al., 2013; Vinogradova et al., 2023). The human body relies on various essential minerals, such as Fe, Cu, Zn, and Mn, for metabolic processes. These minerals are predominantly supplied by plants, which absorb them from the soil and transfer them into the food chain (Srivastava et al., 2017). However, plants are highly sensitive to environmental conditions and tend to accumulate metals, with the intensity of this uptake process directly affecting their overall elemental composition (Kulhari et al., 2013). This accumulation raises significant environmental concerns due to the potential adverse effects of metals on both animal and human health.

Metals, Fe, Cu, Mn, and Zn, are toxic only in higher concentrations (Nissar et al., 2020), but they can also cause serious consequences. For instance, Fe is one of the primary toxic substances contributing to deaths in children under six years old (Spanierman, 2011). Gastrointestinal toxicity can occur after ingestion of more than 20 mg/kg of Fe, while moderate intoxication is observed with elemental Fe ingestion exceeding 40 mg/kg. Serious toxicity, potentially fatal, arises at doses greater than 60 mg/kg (Spanierman, 2011). Nickel was identified as an allergen by the Contact Dermatitis Society of America in 2008, with its minimum risk level for inhalation set at 0.2 µg/m^3 for exposure durations of 15–364 days. However, no dietary limit for nickel has been established (Bhat et al., 2010; Kulhari et al., 2013).

The content of beneficial trace metals in medicinal plants generally decreases in the following order: Fe>Mn>Cu>Zn>Ni (Chizzola, 2012). Our results show that the content of micronutrients in *Dictamnus albus* leaves decreases in the order of Fe>Mn>Zn>Cu>Ni. Compared to the literature data, the Cu content was lower than Zn, while the Mn content was higher than the Zn content (Table 2). The Fe content in the leaves of *D. albus* was 44.16±0.685 mg/kg, Cu 6.06±0.253 mg/kg, Zn 21.64±0.571 mg/kg, Mn 22.01±0.413 mg/kg, and Ni 1.21±0.112 mg/kg (Table 2). The permissible limit for essential metals in medicinal plants is 50 mg/kg for Zn, 20 to 150 mg/kg for Cu, 200 mg/kg for Mn and Fe, and 5 mg/kg for Ni (WHO, 2007; Baba and Mohammed, 2021). The values of all measured metals in the leaves of *D. albus* were below the permissible doses for medicinal plants.

In the *Dictamnus albus* from the Kashmir Himalaya, the content of microelements in the leaves was as follows: Fe 1.130 mg/kg, Cu 0.023 mg/kg, Zn 0.055 mg/kg, and Ni 0.105 mg/kg (Nissar et al., 2020), which are significantly lower values for all metals compared to our results. The reason may be the specific conditions related to altitude

Table 2. Content of iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), and nickel (Ni) in *Dictamnus albus* leaves. Results are expressed as mean value ± SD (n=3).

Tabela 2. Vsebnost železa (Fe), bakra (Cu), mangana (Mn), cinka (Zn) in niklja (Ni) v listih *Dictamnus albus*. Rezultati so izraženi kot povprečna vrednost ± SD (n=3).

	(mg/kg_{DW})				
	Fe	**Cu**	**Zn**	**Mn**	**Ni**
D. albus leaves	44.16±0.680	6.06±0.253	21.64±0.572	22.01±0.413	1.21±0.112

and temperatures in the Kashmir Himalaya. Differences also may stem from soil composition and various abiotic and anthropogenic influences.

According to Kulhari et al. (2013), there are varying concentrations of Mn, Fe, and Zn in leaf samples of medicinal plants from various sites in northwestern India, which were influenced by environmental pollution and soil composition. The study reported the highest Fe content in *Withania somnifera* (L.) Dunal leaf samples (17.44±0.0202 mg/kg) and the lowest in *Salvadora oleoides* Decne. Leaves (11.03±0.0497 mg/kg), with all Fe concentrations falling within permissible limits. The highest Zn content was found in *Bacopa monnieri* (L.) Wettst. (4.80±0.0907 mg/kg), while the lowest was in *Commiphora wightii* (Arn.) Bhandari (2.10±0.0173 mg/kg). Mn content ranged from 2.39±0.0218 mg/kg in *Withania somnifera* to 5.22±0.1040 mg/kg in *Acacia nilotica* (L.) Willd. ex Delile and Ni content ranged from 0.19±0.0371 mg/kg in *Withania somnifera* to 0.59±0.0202 mg/kg in *Acacia nilotica* (Kulhari et al., 2013). In our *Dictamnus albus* samples, higher concentrations of Fe, Mn, Zn, and Ni (Table 2) were measured compared to the levels reported in the plant samples from northwestern India (Kulhari et al., 2013). On the other hand, the concentrations of the metals (mg/kg) in the leaves of medicinal plants from the local markets in Nigeria were in the range 5.08-23.67 for Zn, 1.28-13.45 for Cu, 0.96-1.95 for Ni, and 20.58-108.50 for Fe (Sulaiman et al., 2024). Metal concentrations in plants from Nigeria were slightly higher than in *D. albus* (Table 2). These differences may be attributed to species-specific characteristics, substrate composition, and ecological conditions in the collected plants' habitats. The concentrations range for essential metals in plants *Annona squamosa* L., *Psidium guajava* L., *Anacardium occidentale* L., and *Ficus sycomorus* L. from Nigeria were: Zn (0.109-0.658 mg/kg), Cu (0.026-0.079 mg/kg), Mn (0.039-1.269 mg/kg), Fe (0.019-0.107 mg/kg), and Ni (0.054-0.144 mg/kg) (Baba and Mohammed, 2021), which are significantly lower compared to the values measured in *D. albus* (Table 2).

The ability of plants to absorb and accumulate microelements is influenced by various factors, such as soil type, climate, industrial pollution, and agricultural practices. Although trace elements are generally insoluble, plants have developed strategies to enhance the uptake of essential metal ions. One such mechanism involves the release of organic molecules and metabolites, including phenolic acids and flavonoids, which form water-soluble chelates with transition metal ions (Petkov et al., 2022). These metal-phenolic chelates enhance solubility, facilitating their transport into plant cells and movement across cellular compartments. Consequently, plants act as vital intermediaries, transferring trace elements from the soil to animals and humans (Petkov et al., 2022).

No correlations were detected between the metal content (Fe, Cu, Mn, Zn, and Ni) and the concentration of phenolic compounds in the *Dictamnus albus* extract (Table 3). Theuma and Attard (2020) reported a positive correlation between phenolic compound content and the concentration of Cu and Mn, suggesting that increased levels of phenolic compounds are associated with higher concentrations of Cu and Mn. This relationship can be explained by the ability of phenolic compounds to chelate Cu, while Mn complexes with condensed polyphenols (Theuma and Attard, 2020). Our results revealed a strong positive correlation (0.999) with statistical significance ($p<0.02$) between Fe concentration and the DPPH radical scavenging activity of the *D. albus* extract. Additionally, high correlation values were observed for Cu and Mn concentrations with DPPH radical scavenging activity (0.996 and 0.993, respectively), nearing statistical significance ($p=0.058$ for Cu and $p=0.077$ for Mn). The correlations between TPC, DPPH capacity, and Fe and Cu content were previously reported by Dobrinas et al. (2021), who suggested that TPC may serve as a reliable indicator of DPPH capacity and the influence of antioxidant compounds on mineral bioavailability.

Table 6. Percentage mortality rates (MR), mortality time (MT), susceptibility status (S), knockdown rates (KR), and knockdown time (KT) of individual essential oil and their combinations against females *Anopheles* mosquito at 1 hour after exposure.

Tabela 6. Odstotki smrtnosti (MR), čas smrtnosti (MT), stanje občutljivosti (S), stopnja otrpnosti (KR) in čas otrpnosti (KT) posameznih eteričnih olj in njihovih kombinacij proti samicam komarja *Anopheles* 1 uro po izpostavljenosti.

		Phenolic compound	DPPH IC_{50}	H_2O_2 IC_{50}	Fe Chelation	Cu^{2+} Reduction	Fe^{3+} Reduction	Scavenge $^{\cdot}OH$	Fe (mg/kg_{DW})	Cu (mg/kg_{DW})	Mn (mg/kg_{DW})	Zn (mg/kg_{DW})	Ni (mg/kg_{DW})
Phenolic compound	Pearson's r	—											
	p-value	—											
DPPH IC_{50}	Pearson's r	0.527	—										
	p-value	0.646	—										
H_2O_2 IC_{50}	Pearson's r	-0.909	-0.833	—									
	p-value	0.273	0.373	—									
Fe Chelation	Pearson's r	-0.049	-0.875	0.460	—								
	p-value	0.969	0.322	0.695	—								
Cu^{2+} Reduction	Pearson's r	0.060	-0.817	0.361	0.994	—							
	p-value	0.962	0.392	0.765	0.069	—							
Fe^{3+} Reduction	Pearson's r	0.884	0.863	-0.998	-0.510	-0.413	—						
	p-value	0.309	0.337	0.036	0.659	0.729	—						
Scavenge $^{\cdot}OH$	Pearson's r	-0.959	-0.266	0.755	-0.235	-0.339	-0.717	—					
	p-value	0.182	0.829	0.455	0.849	0.780	0.491	—					
Fe (mg/kg_{DW})	Pearson's r	0.500	0.999	-0.815	-0.890	-0.835	0.846	-0.235	—				
	p-value	0.667	0.020	0.394	0.302	0.371	0.358	0.849	—				
Cu (mg/kg_{DW})	Pearson's r	0.448	0.996	-0.779	-0.915	-0.866	0.814	-0.177	0.998	—			
	p-value	0.704	0.058	0.431	0.264	0.334	0.395	0.886	0.037	—			
Mn (mg/kg_{DW})	Pearson's r	0.421	0.993	-0.760	-0.927	-0.880	0.796	-0.148	0.996	1.000	—		
	p-value	0.723	0.077	0.450	0.246	0.315	0.414	0.905	0.056	0.019	—		
Zn (mg/kg_{DW})	Pearson's r	0.247	0.954	-0.628	-0.980	-0.953	0.671	0.037	0.963	0.977	0.983	—	
	p-value	0.841	0.195	0.568	0.128	0.197	0.532	0.977	0.174	0.137	0.118	—	
Ni (mg/kg_{DW})	Pearson's r	0.924	0.811	-0.999	-0.426	-0.325	0.996	-0.779	0.792	0.755	0.735	0.598	—
	p-value	0.249	0.397	0.024	0.720	0.789	0.060	0.431	0.418	0.455	0.474	0.592	—

Conclusions

Our study demonstrated that the leaves of *Dictamnus albus* contain significant amounts of phenolic compounds and exhibit strong antioxidant activity, including the ability to scavenge DPPH radicals, H2O2, reduced Fe3+, Cu2+, and chelate Fe. For the first time, we have demonstrated that *D. albus* extract possesses significant hydroxyl radical scavenging capacity, emphasizing its potential role in mitigating oxidative stress. The concentrations of metals such as iron, copper, zinc, manganese, and nickel in the extract were comparable to those in other medicinal plants, affirming its safety as a source of essential elements for human health. The pronounced antioxidant activity of *D. albus* extract indicates its potential as an adjunct in therapies for oxidative stress-related diseases. Further studies on its bioactive properties could contribute to the development of innovative therapeutic agents.

Author Contributions

Conceptualization, S.Š. and B.K.; methodology, S.Š., M.M., and B.K.; formal analysis, T.T., D.M. and Đ.N.; investigation, S.Š., T.T., D.M., Đ.N., M.M. and B.K.; resources, M.M. and B.K.; data curation, D.M., Đ.N., M.M. and B.K.; writing—original draft preparation, S.Š., D.M., Đ.N., M.M. and B.K.; writing—review and editing, S.Š., D.M., Đ.N., M.M. and B.K.; visualization, B.K.; supervision, M.M. and B.K.; funding acquisition, M.M. and B.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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