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Radical Scavenging and Ion-Reducing Capacity of *Fumaria officinalis* Extracts Obtained by Traditional and Assisted Extraction Techniques †

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Abstract: The present research aimed to extract antioxidants from the fumitory aerial part in the flowering stage (containing leaves, stems, and flowers) by performing traditional and novel extraction procedures (maceration, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE)). The fumitory macerate showed significantly lower ABTS radical scavenging activity, expressed as a higher IC_{50} value (the concentration of extract required to neutralize 50% of radicals, 11.4 ± 0.1 mg/mL), in comparison to the other two extracts, whose IC_{50} values varied in a narrow range (8.6–9.5 mg/mL). In the DPPH assay, the trend was different: MAE (11.4 ± 0.3 mg/mL) ≥ UAE (12.0 ± 0.8 mg/mL) ≥ macerate (12.8 ± 0.1 mg/mL). In the CUPRAC assay, the UAE and MAE extracts (17.84 ± 0.85 and 18.05 ± 0.71 µmol Trolox equivalents (TE)/g, respectively) showed significantly higher antioxidant activity compared to the macerate (16.43 ± 0.45 µmol TE/g). Regarding the results of the FRAP method, there was no statistically significant difference in ferric ion reduction between the macerate, UAE, and MAE extracts (3.00–3.27 µmol Fe^{2+}/g). However, the extract prepared using MAE provided the highest antioxidant potential, as shown in all four tests used. Due to demonstrated extracts' antioxidant properties, additional research could address additional biological effects or the creation of delivery systems or encapsulates for the controlled delivery of fumitory bioactives.

Keywords: extraction; fumitory; antioxidant assays; polyphenols

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1. Introduction

Antioxidant compounds can protect cells and tissue via various mechanisms depending on the physicochemical properties of the employed antioxidants, such as the conversion of reactive oxygen species to non-radical species and eliminating the auto-oxidative chain reaction, as well as lowering the oxygen amount [1]. Antioxidants of natural plant origin are classified into three main groups, namely polyphenols, vitamins, and carotenoids [1,2]. Polyphenols are a large group of plant secondary metabolites that can be employed as preservatives, antioxidants, and additives. There is growing interest in extracting these

metabolites from plant sources to obtain a safe, natural, and low-cost alternative to synthetic compounds, among which some possess toxic and mutagenic effects [2,3]. Natural antioxidant compounds can be found in a wide range of herbal sources, including fruits, vegetables, seeds, spices, and herbs. Since the majority of these natural components may be derived from underutilized plant species and food by-products, the interest in them stems from both their biological and economic significance [2]. *Fumaria officinalis* L. (fumitory, Fumariaceae family) is a scrambling annual plant, distributed and cultivated throughout Europe, and several studies have shown its antimicrobial, antioxidant, antispasmodic, laxative, anthelmintic, anticoagulant, cholagogue, cytotoxic, and sedative potential [4–6]. The plant contains large amounts of phenolcarboxylic acids, tannins, and flavonoid compounds, as well as isoquinoline alkaloids, responsible for the above-mentioned extract potential [4,6]. Therefore, due to its chemical profile, in the present study, the fumitory was employed as a natural source of antioxidant components.

Additionally, traditional and modern extraction technologies can be employed with the aim of extracting/isolating target bioactives, such as antioxidants, from the herbal matrix. Their simple operation and cost-effectiveness are the main advantages of traditional extraction processes, while the prolonged period of extraction, large amounts of extraction solvents, and lower extraction yields represent their disadvantages [3,7]. On the other hand, novel extraction technologies, such as microwave and ultrasonic extraction, provide higher extraction yields with minimal solvent consumption and during a shortened extraction period [7]. Hence, the extraction technique significantly affects the extraction yield of target compounds, including antioxidants. Commonly recognized as safe, ethanol and its combinations with water have been effectively employed in environmentally friendly extraction processes of polyphenolics from a broad range of herbal sources. Large-scale technologies can use ethanol, which is regarded as a low-toxicity extraction solvent that yields a large amount of polyphenols [8]. Moreover, ethanol is approved as a generally recognized as safe (GRAS) substance by the Food and Drug Administration. Thus, in the present study, an ethanol–water mixture, as well as various extraction techniques, was employed.

In the present research, the antioxidant compounds of the fumitory were extracted by using traditional and assisted extraction procedures (maceration and ultrasound- and microwave-assisted extraction (UAE and MAE, respectively)). In addition, the antioxidant capacity of the obtained extracts was investigated in the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), cupric-ion-reducing antioxidant capacity (CUPRAC), and ferric-reducing antioxidant potential (FRAP) assays.

2. Materials and Methods

2.1. Chemicals

The aerial part of the fumitory (used in the extracts' preparation) was purchased in the pharmacy of the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, Serbia. Ethanol (96%), ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), DPPH, 2,4,6-tri-(2-pyridyl)-5-triazine, iron(III) chloride, iron(II) sulfate, and ammonium acetate were purchased from Sigma-Aldrich (Hamburg, Germany), and potassium persulfate was from Centrohem (Stara Pazova, Serbia). Iron (III) chloride, potassium ferricyanide, and iron (II) sulfate were from Sigma-Aldrich (Saint Louis, MO, USA); neocuproin was from Acros Organics (Geel, Antwerpen, Belgium); and cuprum chloride was from Fluka (Seelze, Germany).

2.2. Extraction Technologies

The liquid fumitory extracts were obtained using the ground aerial parts of the plant (1 g) and 70% *v*/*v* ethanol (30 mL), i.e., the solid-to-solvent ratio was 1:30. Due to preliminary screening, 70% *v*/*v* ethanol was selected as the most appropriate extraction medium to achieve the highest total polyphenol content. Maceration was performed at 25 °C, using the incubator shaker KS 4000i control (IKA, Staufen, Germany) for 60 min. The UAE process included an ultrasound probe (Sonopuls, Bandelin, Berlin, Germany), at an amplitude of 60%, for 15 min (the temperature was monitored and controlled due to the ice coating of the sample, 25–27 °C). MAE was performed at 100 °C in a Monowave 300 microwave reactor (Anton Paar GmbH, Graz, Austria) for 120 s under constant pressure in a closed vial. In the preliminary study, different temperatures during MAE were tested, from 60 to 160 °C. The highest concentration of polyphenols was obtained at 100 °C; thus, the mentioned temperature was used for further experiments. Subsequently, the extracts were filtered and subjected to an analysis of their antioxidant potential. The liquid extracts were kept in a refrigerator at 4 °C.

2.3. Determination of the Antioxidant Capacity

The anti-ABTS and anti-DPPH radical capacities of the three types of fumitory extracts were tested by employing spectrophotometric assays. The absorbance was measured using the UV spectrophotometer UV1800 from Shimadzu (Kyoto, Japan).

In the ABTS assay [9], the ABTS radicals were produced in the reaction of 7 mM ABTS in distilled water and 2.45 mM potassium persulfate in the dark at 25 °C for 18 h. The $ABTS^{\bullet+}$ working solution was diluted using ethanol (absorbance of ~0.700 at 734 nm). The $ABTS^{\bullet+}$ solution (2 mL) was mixed with diluted fumitory extracts (at different concentrations, 1–30 mg/mL, 20 µL). After 6 min of incubation in the dark at 25 °C, the absorbance was measured, and the radical scavenging activity of the extracts was calculated using the following equation:

$$\% \text{ inhibition} = (A_{0ABTS} - A_x) \times 100/A_{0ABTS} \quad (1)$$

where A_{0ABTS} was the absorbance of the control, and A_x was the absorbance of the $ABTS^{\bullet+}$ solution and extract. Trolox was used as a positive control. The data are shown as the extract concentration necessary to scavenge 50% of free radicals, IC_{50} (mg/mL). The IC_{50} values were calculated from the curve of the relationship between the percentage of neutralization and the concentration of the extract.

In the DPPH assay [10], the fumitory extracts (various concentrations, 1–30 mg/mL, 100 µL) were mixed with 2.8 mL of ethanol $DPPH^{\bullet}$ radical solution. The absorbance of $DPPH^{\bullet}$ radicals in ethanol was ~0.800 at 517 nm. The absorbance of the ethanol $DPPH^{\bullet}$ radical solution mixed with the extract was recorded after 20 min of incubation in the dark at 25 °C, and the percentage of inhibition was calculated using the following equation:

$$\% \text{ inhibition} = (A_{0DPPH} - A_x) \times 100/A_{0DPPH} \quad (2)$$

where A_{0DPPH} was the absorbance of the control, and A_x was the absorbance of the $DPPH^{\bullet}$ solution and extract. The data are shown as IC_{50} (mg/mL).

The CUPRAC of the fumitory extracts was examined using 0.8 mL of the extract, 1 mL of a solution of cupric (II) ions, 1.2 mL of ammonium–acetate buffer, and 1 mL of a solution of neocuproine [11]. The solution of cupric (II) ions (10^{-2} mol/mL) was prepared by dissolving 85.3 mg of copper (II) chloride dihydrate in 250 mL of distilled water. The 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 78 mg of neocuproine in 50 mL of methanol (7.5×10^{-3} mol/mL). The absorbance was read after 30 min at a wavelength of 450 nm. The calibration curve was obtained using Trolox. The data are shown as μmol Trolox equivalents (TE) per g of plant material.

The FRAP of the fumitory extracts was examined using 200 μL of distilled water, 40 μL of extract, and 1.8 mL of FRAP reagent [12]. The FRAP reagent was prepared using 2.5 mL of a 10 mmol/L 2,4,6-tri-(2-pyridyl)-5-triazine solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L $FeCl_3$, and 25 mL of 0.3 mol/L acetate buffer (pH 3.6) at 37 °C. The mixture was incubated at 37 °C for 10 min. The absorbance was read at a wavelength of 593 nm. The calibration curve was obtained using $FeSO_4$, and the data are shown as μmol Fe^{2+} equivalents per g of plant material.

2.4. Statistical Analysis

The statistical data processing was performed by one-way ANOVA and Duncan's post hoc test (STATISTICA 7.0). The differences were considered statistically significant at $p < 0.05$, and measurements were performed in triplicate.

3. Results and Discussion

The antioxidant activity of three different types of fumitory extract (macerate, UAE sample, and MAE sample) was investigated using four antioxidant methods (ABTS and DPPH assays, as well as CUPRAC and FRAP tests). The data are shown in Table 1, as mean values ± standard deviations, while different letters in the superscript show the presence of statistically significant differences.

Table 1. Antioxidant capacity of fumitory macerate and ultrasound (UAE) and microwave (MAE) extracts.

Sample	ABTS Assay (IC_{50}, mg/mL) [1]	DPPH Assay (IC_{50}, mg/mL)	CUPRAC Assay (μmol TE/g)	FRAP Assay (μmol Fe^{2+}/g)
Macerate	11.4 ± 0.1^{b}	12.8 ± 0.1^{b}	16.43 ± 0.45^{b}	3.00 ± 0.15^{a}
UAE extract	8.6 ± 0.4^{a}	12.0 ± 0.8^{ab}	17.84 ± 0.85^{a}	3.14 ± 0.21^{a}
MAE extract	9.5 ± 0.8^{a}	11.4 ± 0.3^{a}	18.05 ± 0.71^{a}	3.27 ± 0.18^{a}

[1] ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); TE, Trolox equivalents; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CUPRAC, cupric-ion-reducing antioxidant capacity; FRAP, ferric-reducing antioxidant potential; IC_{50}, concentration required to neutralize 50% of free radicals; values with the same letter in each column in the superscript 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 fumitory macerate showed significantly lower ABTS radical scavenging activity, expressed as a higher IC_{50} value (the concentration of extract required to neutralize 50% of radicals was 11.4 ± 0.1 mg/mL), in comparison to the other two extracts, whose IC_{50} values varied in a narrow range (8.6 and 9.5 mg/mL). However, in the DPPH assay, the trend of the antioxidant activity was different: MAE (11.4 ± 0.3 mg/mL) ≥ UAE (12.0 ± 0.8 mg/mL) ≥ macerate (12.8 ± 0.1 mg/mL) (Table 1). In the CUPRAC assay, the trend was as follows: UAE and MAE (17.84 ± 0.85 and 18.05 ± 0.71 μmol TE/g, respectively) > macerate (16.43 ± 0.45 μmol TE/g) (Table 1). Regarding the results of the FRAP method, there was no statistically significant difference in ferric ion reduction between the macerate, UAE, and MAE extracts (3.00–3.27 μmol Fe^{2+}/g) (Table 1).

MAE and UAE provided better antioxidant activity in the fumitory extracts (in the ABTS, DPPH, and CUPRAC assays) during a shortened extraction period compared to maceration, because of the occurrence of heat and mass transfer after the degradation of the plant cell walls under exposure to microwaves or ultrasound waves [3,13,14]. Namely, the degradation of the cell structure ensures a higher release rate of plant polyphenols, as the main antioxidant compounds, and, consequently, better antioxidant performance of the obtained extracts. Flavonoid compounds are responsible for the strong antioxidant

potential of the extracts. Moniruzzaman et al.'s study [15] reported that the flavonoid content in plant extracts was strongly correlated with the DPPH radical neutralization and ferric reducing potential. Hence, according to the literature data, fumitory extract formulations with high antioxidant activity contain high levels of phenolic and flavonoid components as well [16]. The presence or absence of significant differences among the fumitory extracts that showed the highest antioxidant potential in various employed tests can be explained by the fact that different secondary metabolites and their interactions can significantly affect the overall antioxidant activity of fumitory extracts [17]. Specifically, different plant-based antioxidants, such as thiols, D-ascorbic acid, sugars, sugar alcohols, and particularly phenolics, are responsible for cupric ion reduction [18]. In a previous study, the LC-MS analysis of fumitory extracts revealed the presence of antioxidant compounds, including protopine-type (protopine, oxo-, methyl, and/or acetyl protopine derivatives and cryptopine) and spirobenzylisoquinoline-type alkaloids (fumariline and fumarophycine). Additionally, chlorogenic and caffeoylmalic acids were also identified, as well as quercetin trihexoside, rutin, methylquercetin pentoside hexoside, isoquercitrin, quercetin, and kaempferol deoxyhexosylhexoside [19]. Since different reagents, conditions, mechanisms, and kinetics of the reaction occur during the measurements in various antioxidant assays, the differences observed in the comparison of the employed extraction techniques and the obtained data in the used antioxidant assays are not surprising.

4. Conclusions

In the present study, the antioxidant capacity of fumitory extracts prepared using maceration, UAE, and MAE was investigated. The results showed that the anti-ABTS, anti-DPPH, and cupric-ion-reducing capacities of the extracts were significantly affected by the employed extraction procedure. Additionally, novel extraction techniques provided fumitory extracts with significantly higher antioxidant capacities compared to the traditional maceration process. Hence, the extract prepared using the MAE technique showed the best results in all four employed antioxidant assays. However, in the case of the ferric-reducing capacity of the fumitory extracts, the extraction protocol did not significantly change the mentioned variable. Due to the shown antioxidant effects of fumitory extracts, further analyses can include the investigation of other biological effects, as well as the development of delivery systems or encapsulates for the controlled or prolonged delivery of fumitory bioactives and the physicochemical characterization of carriers with fumitory bioactives.

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Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl
CUPRAC	Cupric-ion-reducing antioxidant capacity
FRAP	Ferric-reducing antioxidant potential
IC_{50}	Concentration required to neutralize 50% of free radicals
TE	Trolox equivalents
UAE	Ultrasound-assisted extraction
MAE	Microwave-assisted extraction

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