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Application of *thyme* essential oil for the synthesis and *in silico* analysis of novel 2-substituted benzimidazolines

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The wide range of biological properties possessed by essential oils containing phenolic monoterpenoids makes them appealing for the development of new biologically active compounds. Thymol is the primary phenolic monoterpenoid found in essential oils of *Thymus vulgaris*. As an addition to its bioactive profile, thymol is a highly promising scaffold for future synthetic modifications toward new hybrid molecules with potential biological activities.

The present study aims at the regioselective α-amidoalkylation of thymol and thyme essential oil, thereby expanding the applicability of this synthetic approach. The reactions were carried out by *in situ* generated *N,N*-diacyliminium reagents formed from benzimidazole or 5,6-dimethylbenzimidazole with methyl or ethyl chloroformate in dichloromethane at room temperature. Four novel *N,N*-diacylated benzimidazoline-thymol hybrids were synthesized (4a-d) with yields in the range from 65% to 83%. The conducted *in silico* toxicity analysis revealed that the compound (4a) exhibited the lowest predicted toxicity (1298.76 mg/kg) among the other synthesized analogues. The structure of the synthesized compounds was characterized using ^{1}H-, ^{13}C-NMR, FTIR spectroscopic analyses and HRMS data.

Keywords: α-amidoalkylation, benzimidazole, monoterpenoid hybrids, thymol, thyme essential oil, *in silico* analysis

INTRODUCTION

The synthesis of novel 2-substituted benzimidazoles with a broad spectrum of biological activities such as antibacterial [1–3], antiproliferative [1, 4, 5], anticancer [2, 5], anthelmintic [2, 3, 5], antiviral [6, 7], antioxidant [8–10], photoprotective [8, 9], antispasmodic [11], among others, is crucial for the discovery of potentially active pharmacological agents [12]. Modern bioactive molecules design often involves the development of novel hybrid molecules [13]. Molecular hybridization is a strategic approach in medicinal chemistry aimed at the synthesis of novel molecules with an improved biological activity and therapeutic potential [13, 14]. α-Amidoalkylation reactions are important in organic synthesis for constructing a carbon–carbon (C-C) bond [15] and have found widespread application in the design and synthesis of molecular hybrids [16, 17]. Natural products, including components of essential oils such as thymol, known for its antibacterial [18], anti-inflammatory [19], antioxidant [20], and other properties, represent an important source for the development of novel bioactive compounds [21].

In a recently published study, we applied thyme and oregano essential oils for molecular hybridization. We successfully combined benzothiazole and natural monoterpenoids to obtain new bioactive compounds with promising UV-B and antimicrobial activity. As addition, a low toxicity was established through *in silico* analysis, and SPF values are comparable to the commercial filter PBSA [22]. In this context, a similar strategy could be applied for the synthesis of new benzimidazole derivatives containing a thymol fragment.

The synthetic group of Itoh *et al.* [23, 24] synthesized 2-substituted compounds by activation of the benzimidazole ring with ethyl chloroformate. Such types of syntheses are applied in the development of bioactive molecules. For the first time, an adduct of benzimidazole and ethyl chloroformate was isolated and spectrally characterized by Venkov and Statkova [25]. The authors established that the adduct is sufficiently stable to be isolated. Later, the same authors synthesized a series of 1,2,3-trisubstituted 2-(2-oxoalkyl)-2,3-dihydrobenzoimidazoles and investigated their antiproliferative activity. One of the products obtained via amidoalkylation of benzalacetone (Figure 1), exhibited pronounced selective antiproliferative activity against human metastatic melanoma (A2058) at a concentration of 10^{-4} M [4].

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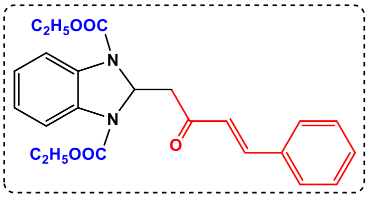


Figure 1. Benzimidazoline-benzalacetone hybrid.

The literature data and the presented results provide a solid basis for conducting experimental studies targeted to the synthesis and evaluation of new compounds that combine a benzimidazole core with another natural fragment. In this regard, the aim of the current study was the pplication of thyme essential oil for the synthesis of novel benzimidazoline-thymol hybrids by reaction of α-amidoalkylation and their subsequent *in silico* analysis.

EXPERIMENTAL

General information

Commercial solvents and reagents, such as benzimidazole, 5,6-dimethylbenzimidazole, alkyl chloroformates, thymol and thyme essential oil were purchased from Sigma-Aldrich (Merck EAD, Sofia, Bulgaria). Melting points were measured on a Boetius PHMKO5 hot-stage apparatus (Carl Zeiss Jena, Germany). FTIR spectra were taken in KBr tablets on a Perkin Elmer 1750 Fourier Transform spectrometer. For HRMS analysis a HRMS Q-Exactive Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) spectrometer was successfully applied. ^{1}H-, ^{13}C-NMR spectra were recorded on Bruker Avance AV600 spectrometer (Bruker, Billerica, MA, USA) at IOCCP-BAS, Sofia, in DMSO-d$_6$ as a solvent. To average out the rotamers reaching adequate assignment of peaks and structure determination, the spectra were measured at 80 °C. TLC was done on precoated 0.2 mm Merck silica gel 60 plates with eluent mixture of petroleum ether:diethyl ether 2:1. All pure samples were isolated by recrystallization from petroleum ether:diethyl ether in various ratios described in the general procedures.

General procedure for amidoalkylation of thymol using adducts derived from benzimidazole or 5,6-dimethylbenzimidazole with alkyl chloroformate

To a solution of 1 mmol benzimidazole or 5,6-dimethylbenzimidazole in 10 ml dichloromethane, 1 mmol of triethylamine (Et_3N) is added as a hydrogen chloride (HCl) scavenger, followed by slow dropwise addition of the acid chloride – either 2.4 mmol ethyl chloroformate or 2.8 mmol methyl chloroformate. Immediately afterward, 1 mmol of thymol is added. The reaction mixture is stirred with a magnetic stirrer for 30 min at 0 °C, then left at room temperature for 24 h. The product is isolated by extraction with 3×20 ml of CH_2Cl_2, sequentially with 30 ml of hydrochloric acid:water (1:4) solution and 50 ml of water. The resulting organic extract is dried over anhydrous sodium sulfate (Na_2SO_4), and the solvent is evaporated. The products (4a-d) are isolated as white powders by recrystallization from petroleum ether:diethyl ether 4:1 with increasing polarity to 1:1.

General procedure for the synthesis of 2-substituted benzimidazoles by efficient One-pot reaction with thyme essential oil

To benzimidazole or 5,6-dimethylbenzimidazole (2 mmol) dissolved in 10 ml of dry dichloromethane, 2 mmol of triethylamine (Et_3N), alkyl chloroformate – 2.5 mmol ethyl chloroformate or 3 mmol methyl chloroformate are successively added and the reaction mixture is left for 20 min in an ice bath (-4–0 °C). After 20 min a second equivalent of alkyl chloroformate is added to the reaction mixture, and adduct formation follows at room temperature 25 °C for 20 min. The third stage of the reaction proceeds successfully without the need of isolating the acyliminium reagents by adding 1.2 g (containing at least 2.9 mmol of thymol) of essential oil to the same reaction vessel. Then, the reactions proceed for 24 h at room temperature. The products (4a-d) are isolated according to the abovementioned successful procedure by recrystallization from petroleum ether:diethyl ether 8:1 with increasing polarity to 4:1. The obtained yields are calculated based on benzimidazole. In these experiments, it was found that the acyliminium reagents were stable in the presence of the essential oil over the course of 24 h.

Spectral characterization of the obtained compounds

Dimethyl 2-(4-hydroxy-5-isopropyl-2-methylphenyl)-1H-benzo[d]imidazole-1,3(2H)-dicarboxylate (4a); Yield: (65 %); Melting point: 200–202 °C; ^{1}H-NMR (600 MHz, 80 °C DMSO-d_6, δ ppm, J Hz): 1.15 (d, J=7.04 Hz, 6 H), 2.50 (s, 3 H), 3.18-3.23 (m, 1 H), 3.86 (s, 6 H), 6.73 (s, 1 H), 7.02 (s, 1 H), 7.13 (s, 1 H, CH), 7.22-7.25 (m, 2 H), 7.78 (br. s., 2 H), 9.05 (br. s., 1 H, OH); ^{13}C-NMR (150 MHz, 80 °C, DMSO-d_6, δ ppm, J Hz) 18.2, 22.7, 26.7, 53.2, 74.6, 114.1, 117.2, 123.7, 124.00, 128.8, 132.7, 134.6, 151.8, 155.1; FTIR (KBr, cm^{-1}): 3426, 3364, 2956, 1716, 1690, 1622, 1596, 1503, 1445, 1385, 1313, 1280, 1197, 1136, 1025, 958, 859, 827, 749, 572; HRMS m/z (ESI): calcd for $C_{21}H_{24}N_2NaO_5^+$ $[M+Na]^+$ 407.1577, found

407.1579; calcd for $C_{21}H_{23}N_2O_5^-$ [M-H]$^-$ 383.1612, found 383.1610;

Diethyl 2-(4-hydroxy-5-isopropyl-2-methylphenyl)-1H-benzo[d]imidazole-1,3(2H)-dicarboxylate (4b); Yield: (83 %); Melting point: 191–193 °C; ^{1}H-NMR (600 MHz, 80 °C DMSO-d_6, δ ppm, J Hz): 1.19 (d, J=7.04 Hz, 6 H), 1.32 (t, J=6.46 Hz, 6 H), 2.50 (s, 3 H), 3.22-3.26 (m, 1 H), 4.32 (q, J=7.04 Hz, 4 H), 6.75 (s, 1 H), 7.07 (s, 1 H, CH), 7.13 (s, 1 H, CH), 7.25 (d, J=2.93 Hz, 2 H), 7.84 (br. s., 2 H), 9.19 (br. s., 1 H, OH); ^{13}C-NMR (150 MHz, 80 °C, DMSO-d_6, δ ppm, J Hz) 14.4, 18.3, 22.7, 26.8, 62.1, 75.0, 114.0, 117.1, 123.6, 124.5, 128.9, 132.6, 132.9, 134.6, 151.3, 155.0; FTIR (KBr, cm^{-1}): 3405, 2966, 1719, 1678, 1621, 1590, 1501, 1409, 1378, 1378, 1281, 1275, 1190, 1035, 871, 851, 759, 740, 576; HRMS *m/z* (ESI): calcd for $C_{23}H_{28}N_2NaO_5^+$ [M+Na]$^+$ 435.1890, found 435.1887; calcd for $C_{23}H_{27}N_2O_5^-$ [M-H]$^-$ 411.1925, found 411.1928;

Dimethyl 2-(4-hydroxy-5-isopropyl-2-methylphenyl)-5,6-dimethyl-1H-benzo-[d]imidazole-1,3(2H)-dicarboxylate (4c); Yield: (73 %); Melting point: 215–216 °C; ^{1}H-NMR (600 MHz, 80 °C DMSO-d_6, δ ppm, J Hz): 1.20 (d, J=6.46 Hz, 6 H), 2.44 (s, 6 H), 2.50 (s, 3 H), 3.21-3.25 (m, 1H), 3.86 (s, 6 H), 6.74 (s, 1 H), 7.04 (s, 1 H), 7.08 (s, 1 H, CH), 7.60 (br. s., 2 H), 9.27 (br. s., 1 H, OH); ^{13}C-NMR (150 MHz, 80 °C, DMSO-d_6, δ ppm, J Hz) 18.2, 20.5, 22.7, 26.9, 52.3, 74.8, 115.3, 117.1, 124.3, 128.8, 131.5, 132.6, 134.6, 143.4, 151.7, 155.0; FTIR (KBr, cm^{-1}): 3417, 3191, 2957, 2930, 2868, 1722, 1699, 1514, 1447, 1384, 1198, 1124, 984, 869, 755, 570; HRMS *m/z* (ESI): calcd for $C_{23}H_{28}N_2NaO_5^+$ [M+Na]$^+$ 435.1890, found 435.1892; calcd for $C_{23}H_{27}N_2O_5^-$ [M-H]$^-$ 411.1925, found 411.1923;

Diethyl 2-(4-hydroxy-5-isopropyl-2-methylphenyl)-5,6-dimethyl-1H-benzo-[d]imidazole-1,3(2H)-dicarboxylate (4d); Yield: (68 %); Melting point: 203–206 °C; ^{1}H-NMR (600 MHz, 80 °C DMSO-d_6, δ ppm, J Hz): 1.24 (d, J=6.46 Hz, 6 H), 1.33 (t, J=6.75 Hz, 6 H), 2.46 (s, 6 H), 2.50 (s, 3 H), 3.25-3.29 (m, 1H), 4.32 (q, J=7.04 Hz, 4 H), 6.77 (s, 1 H), 7.09 (d, J=2.93 Hz, 2 H), 7.67 (br. s., 2 H), 9.22 (br. s., 1 H, OH); ^{13}C-NMR (150 MHz, 80 °C, DMSO-d_6, δ ppm, J Hz) 14.4, 18.3, 19.8, 22.7, 26.9, 61.9, 75.2, 115.2, 117.1, 124.8, 129.0, 130.8, 132.5, 134.6, 151.2, 155.0; FTIR (KBr, cm^{-1}): 3408, 2970, 2930, 2868, 1716, 1678, 1514, 1407, 1289, 1127, 1018, 879, 759, 575; HRMS *m/z* (ESI): calcd for $C_{25}H_{32}N_2NaO_5^+$ [M+Na]$^+$ 463.2203, found 463.2205; calcd for $C_{25}H_{31}N_2O_5^-$ [M-H]$^-$ 439.2238, found 439.2241.

In silico T.E.S.T. analysis

To predict selected properties using *in silico* methods, computational analysis was applied in this study using freely available software - T.E.S.T. (version 5.1.2) [28]. Table 3 presents data on the properties of the newly synthesized compounds (4a-d). The T.E.S.T. software, developed by the U.S. Environmental Protection Agency (EPA, Washington, DC), was used to evaluate oral rat LD_{50} (mg/kg), mutagenicity and water solubility (mg/l) at 25 °C, employing the nearest neighbour machine learning method. The toxicological profile and physicochemical properties of the newly synthesized compounds were assessed. The obtained results are presented in Table 3.

RESULTS AND DISCUSSION

The present study applies the concept of molecular hybridization by combining a benzimidazole fragment with a natural, biologically active monoterpenoid phenol - thymol. As the main synthetic approach, a multicomponent α-amidoalkylation reaction was employed for the synthesis of the target *N*,*N*-diacylated benzimidazoline–thymol hybrids (4a-d) (Scheme 1).

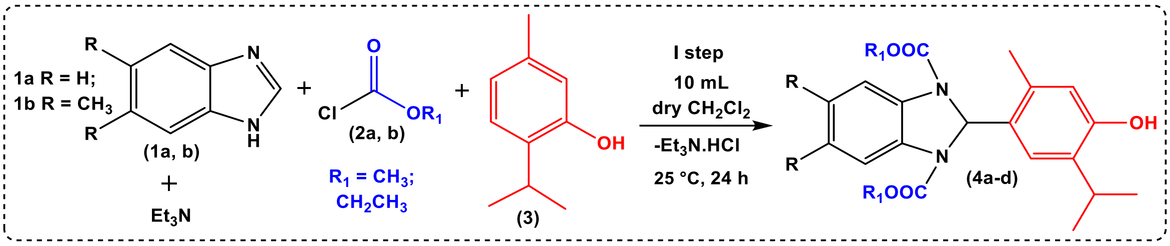
The reaction conditions and yields of thymol-containing hybrid molecules are presented in Table 1.

Table 1. Yields of thymol hybrids (4a-d) obtained according to Scheme 1.

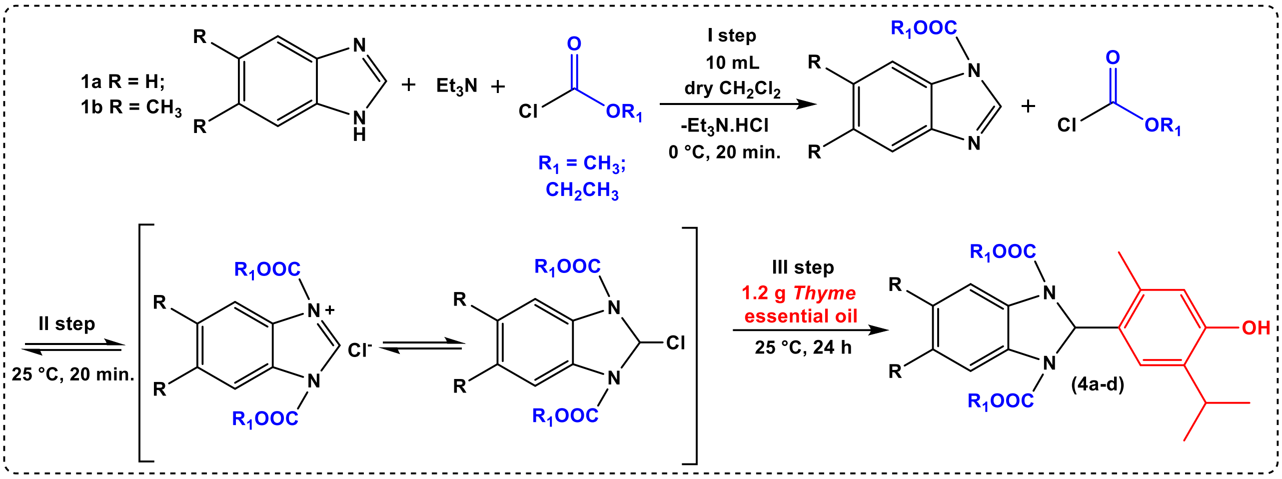
Compound (4)	R	R_1	Yield, %
a	H	Me	65
b	H	Et	83
c	CH_3	Me	73
d	CH_3	Et	68

The obtained experimental data indicate that under these reaction conditions, the substitution predominantly occurs at the *para*-position of the aromatic ring. The observed regioselectivity of the amidoalkylation reaction provides a rationale approach for extending the study by applying the same synthetic approach to more complex natural matrices, such as essential oils.

Building on previous work and the recently published study on the amidoalkylation of thyme essential oil (thymol chemotype) with acyliminium reagents derived from benzothiazole and alkyl chlorformates [22], the investigations were extended using benzimidazole, 5,6-dimethylbenzothiazole, methyl, and ethyl chloroformates to obtain new hybrid molecules.



Scheme 1. α-Amidoalkylation reaction for the synthesis of thymol hybrids (4a-d).



Scheme 2. Application of thyme essential oil in α-amidoalkylation reaction for the synthesis of thymol hybrids (4a-d).

In the present study, one-pot synthetic strategy was successfully applied, in which all reaction steps proceeded sequentially in a single reaction vessel without the need for isolation and purification of the intermediates. The key steps of the synthetic pathway, including the used reagents and the reaction conditions, are presented in Scheme 2. Essential oil (EO) was purchased from a commercial source, with thyme oil characterized by the 'Standard Winter' chemotype. According to the corresponding ISO specifications, this chemotype should contain thymol within the range of 35% to 55% [26]. To verify the chemical composition of the commercial samples, gas chromatography–tandem mass spectrometry (GC-MS/MS) was conducted in accordance with a validated analytical method [27]. The results confirmed that the composition of the analysed EO met the criteria outlined in the applicable ISO standard. The amount of thyme oil used was deliberately selected (1.2 g) to correspond to the lowest permissible thymol content (35%) according to the ISO standard 19817:2017 [26]. Under these reaction conditions, the amidoalkylation reaction proceeded successfully. The reaction progress and the formation of the target products were monitored by thin-layer chromatography (TLC), using the corresponding reference compounds (4a-d) for comparative analysis. The target compounds synthesized using thyme essential oil, were isolated by recrystallization with yields ranging from 66% to 70% and are presented in Table 2.

Table 2. Yields of thymol hybrids (4a-d) obtained from thyme essential oil according to Scheme 2.

Compounds (4)	R	R_1	Yield, %
a	H	Me	69
b	H	Et	70
c	CH_3	Me	66
d	CH_3	Et	67

As a result of the conducted syntheses, four novel crystalline compounds were obtained with good yields ranging from 65% to 83% (Tables 1, 2). They were isolated by recrystallization. The synthesized compounds were characterized using ^{1}H- and ^{13}C-NMR, FTIR spectroscopy, and high-resolution mass spectrometry (HRMS), which unequivocally confirmed their structures.

In comparison with the previously obtained benzothiazole–thymol hybrid molecules [22], the adducts of benzimidazole with alkyl chloroformates synthesized in this study demonstrated improved stability. As a result, the reactions were successfully carried out using equimolar ratios of the *in situ* generated *N*,*N*-diacyliminium reagents and thymol, leading to the formation of the target products with high yields. Unlike the cited study, the reaction was not successful when using carvacrol, likely due to steric hindrance caused by the presence of two bulky alkoxycarbonyl substituents.

As with the previously reported benzothiazole-containing compounds [22], the newly synthesized benzimidazole–thymol hybrids (4a–d) were also predicted to exhibit low toxicity (Table 3).

Bhoi *et al.* conducted an *in silico* toxicological analysis of the obtained hybrid compounds containing thymol (Figure 2) using the online tool preADMET, which predicts key toxicological parameters such as mutagenicity, carcinogenicity, and other important properties [11].

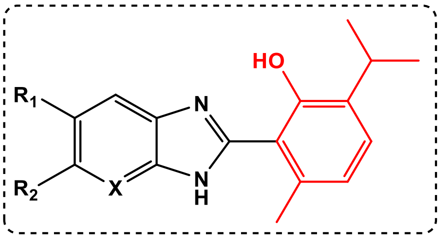


Figure 2. Benzimidazole-thymol hybrids.

The *in silico* modeling-based approach enables a preliminary assessment of the safety and pharmacokinetic properties of novel synthetic compounds. For a more in-depth evaluation of the synthesized benzimidazole derivatives, using the *in silico* machine learning method based on the nearest neighbour approach, implemented in the software system Toxicity Estimation Software Tool (T.E.S.T.), version 5.1.2 [28], the following properties of the compounds presented in Table 3 were successfully predicted: oral rat LD_{50}, mutagenicity and water solubility at 25 °C.

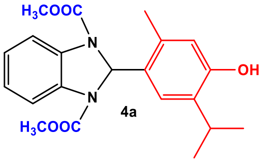
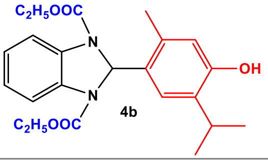
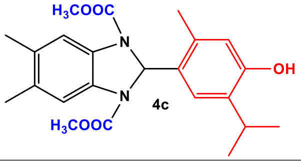
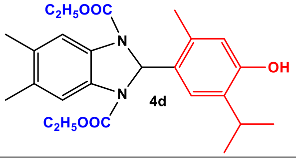
Among the investigated compounds (4a-d), the compound 4a exhibited the highest predicted LD_{50} value (1298.76 mg/kg), suggesting the lowest acute oral toxicity. All compounds were predicted to be non-mutagenic and demonstrated water solubility at 25 °C in the range of 49.19 to 56.37 mg/l.

CONCLUSIONS

In the present study, the optimal reaction conditions for *α*-amidoalkylation of thymol using benzimidazole, 5,6-dimethylbenzimidazole, and acid chlorides, were successfully established. An efficient one-step method was developed for the synthesis of novel hybrid molecules, which was successfully applied using thymol and commercially available thyme essential oil. It was found that the acyliminium reagents retain their stability in the presence of thyme essential oil, allowing the reaction to proceed without formation of undesired side products.

The successfully synthesized novel *N*,*N*-diacylated benzimidazoline–thymol hybrids (4a-d) were evaluated using *in silico* methods. All compounds exhibited the predicted low oral toxicity in rats (high LD_{50} values), negative mutagenicity results, and good water solubility at 25 °C, making them promising candidates for further pharmacological investigations (Table 3).

Table 3. *In silico* toxicity analysis by software system T.E.S.T., version 5.1.2.

Compound (4a-d)	MW, g/mol	Oral rat LD_{50} mg/kg	Mutagenicity	Water solubility at 25 °C mg/l
 4a	384.43	1298.76	Mutagenicity Negative	49.19
 4b	412.49	827.54	Mutagenicity Negative	52.78
 4c	412.49	827.54	Mutagenicity Negative	52.78
 4d	440.54	883.83	Mutagenicity Negative	56.37

Among them, compound 4a showed the highest predicted LD_{50} value (1298.76 mg/kg). Although the present study does not include biological testing, the newly synthesized compounds may serve as a good lead for further pharmacological studies and screening for potential biological activity.

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Decontamination of waste waters containing azo dye β-naphthol orange with residues from essential oil industry – common sage (*Salvia officinalis* L.) and yellow hornpoppy (*Glaucium flavum* Crantz)

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Waste waters containing the azo dye β-naphthol orange (βNO) were subjected to purification by adsorption using common sage (CS) and yellow hornpoppy (YH) residues from essential oil industry. The preliminary characteristic of the biomasses suggested that they contain 71.85±1.74% (CS) and 64.06±0.46% (YH) total dietary fibers (DF), and 6.59±0.28% and 5.44±0.86% soluble DF, respectively. The adsorption equilibrium was reached around the 20th min (1 g adsorbent; 20 mL, 1 mg/mL dye in water), and the amount of adsorbed azo dye was 19.58±0.12 mg (0.64 mg/mL from 1 mg/mL – 63.52% efficiency) for CS and 19.55±0.24 mg/mL (0.71 mg/mL from 1 mg/mL – 71.24% efficiency) for YH. Preliminary washing of the residues with 0.1 N HCl increased their decontamination abilities. At pH 1.1 the βNO removal was above 99±0.1% for both CS and YH. Comparison of the removal efficiency of CS and YH with aluminum oxide, silica gel, activated carbon, *Rosa damascena* Mill. (RD), *Lavandula angustifolia* L. (lavender, L), *Melissa officinalis* L. (melissa (M)) and *Achillea millefolium* L. (yarrow (Y)) wastes, suggested similar decontamination abilities for RD, L, M and Y (55-79%), while activated carbon showed 90% adsorption. The present study suggests that CS and YH wastes are able to remove βNO from water solutions with 56.75% and 64.16% efficiency, respectively.

Keywords: β-naphthol orange, azo dye, common sage (*Salvia officinalis* L.), yellow hornpoppy (*Glaucium flavum* Crantz), bio-adsorption.

INTRODUCTION

Azo dyes are among the most widely used organic pigments with wide applications in textile and food industries [1]. Some of the azo dyes might be toxic to living organisms. Exposure to light, mild temperatures and microorganisms' action brake down their molecules at a slow pace or not at all, making the azo dyes hazardous substances. Therefore, developing and applying functional techniques for decontamination of wastewaters containing azo dyes is a must [2].

β-Naphthol orange (βNO) is used for dyeing silk, polyamide and wool textile, paper products, toner preparations, leather goods, hairs cosmetics, and in staining for scientific purposes [3]. Numerous processes, such as microbial decay, exposure to oxidizing agents (including light), and adsorption [4] could be found in the literature and in practice. The latter method could be considered as the most used for βNO removal. Copious compounds and matters were investigated as adsorbents [4-6] as waste materials from various industries (electricity generation, agriculture, timber, food, etc.) showed promising potential [7-11]. However, in the literature, to the best of our knowledge, no data could be found on the application of common sage (CS) and yellow hornpoppy (YH) industrial wastes for the purification of waters contaminated with azo dyes, such as βNO. For this reason, the present study focused on the utilization of CS and YH biomasses as adsorbents for βNO removal from its water solutions.

MATERIALS AND METHODS

Materials

The common sage (*Salvia officinalis* L.) biomass, resulting from industrial steam distillation of bio-certified fresh plants, was supplied by Mirkovo distillery (Mirkovo, Sofia region, Bulgaria, 2024 harvest). The yellow hornpoppy (*Glaucium flavum* Crantz) residue (after extraction of the raw material with methanol containing 35% H_2SO_4 for obtaining of glaucine) was donated by Zelenikovo distillery (Zelenikovo, Brezovo region, Bulgaria, 2024). βNO (4-(2-hydroxy-1-naphthylazo) benzenesulfonic acid sodium salt) was obtained from Merck (Germany).

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Methods

The CS and YH biomasses were roughly chopped using a garden shredder and then finely milled with a laboratory milling machine (average particle size of 483.9±2.6 μm). The milled residues were dried in a laboratory thermostatic drier Diterm (Robotika, Velingrad, Bulgaria) at 60 °C for 48 h. The biomasses were washed with deionized water, acetone, 0.1 N HCl, and 0.1 N NaOH, as described in [12].

1) *Contact time of the adsorbent with βNO*. The βNO removal was carried out as described in [12]. After filtration of the solutions the adsorption of the non-absorbed dye was measured at 500 nm (LLG-uniSPEC 2, LLG Labware, Germany). The concentration of the unabsorbed *βNO* was determined using a calibration curve, plotted with dye's aqueous solutions with specified concentrations.

2) *Solutions' pH sway on βNO removal.* Solutions of βNO (1 mg/L) in several solvents were prepared: 1. *pH 1*.10 – 0.1 M sulfuric acid 2. *pH 3.55* – deionized water; 3. *pH 5.30* – 50 mM citrate buffer; 4. *pH 8*.10 – 50 mM phosphate buffer; 5. *pH 12*.90 – 0.1 N sodium hydroxide. The experiments were performed as described above (point 1). The conditions were: time 40 min, βNO concentration: 1 mg/mL (20 mL), adsorbent mass: 1 g; room temperature (20 °C), and shaking rate – 100 rpm. After the end of the specified time, filtration and determinations were performed as described in point 1).

3) *Temperature influence on βNO removal.* Temperature effect on βNO removal was investigated at 0, 20, 40, 60, 80 and 100 °C. The conditions were: time 40 min, βNO concentration: 1 mg/mL (20 mL), adsorbent mass: 1 g; pH: 3.55; shaking rate – 100 rpm. After the end of the specified time, filtration and determinations were performed as described in point 1).

4*) Initial washing / adsorbent influence on βNO removal*. The impact of the initial washing of the raw material or adsorbent on βNO removal was performed with activated carbon (Himtex Ltd., Dimitrovgrad, Bulgaria), Al_2O_3 (Merck, Germany) and silica gel (Reanal, Budapest, Hungary), biomasses from lavender (*Lavandula angustifolia* L.), melissa (*Melissa officinalis* L.), and yarrow (*Achillea millefolium* L.), resulting after industrial steam distillation (denoted as L, M and Y, respectively); *Rosa damascena* Mill. biomass, resulting from steam-water distillation (RD); L, M, Y and RD biomasses were obtained from Zelenikovo distillery (2022 harvests). The conditions were: time 40 min, βNO concentration: 1 mg/mL (20 mL), room temperature (20 °C), adsorbent mass: 1 g; pH: 3.55; shaking rate – 100 rpm. After the end of the specified time, filtration and determinations were performed as described in point 1).

5) *βNO concentration influence*. Solutions of βNO in water with 0.125, 0.25, 0.5, 1.0 and 2.0 mg/mL concentrations were subjected to adsorption with CS and YH: time 40 min, 20 °C, adsorbent mass: 1 g; pH: 3.55; shaking rate – 100 rpm. After the end of the specified time, filtration and determinations were performed as described in point 1).

The moisture content of the dried CS and YH residues was determined using analytical balance Kern DAB 100-3 (Kern&Sohn Gmbh, Balingen, Germany). The protein quantities were determined by the Kjeldahl method (MultiKjel K-365 – Büchi, Switzerland) using 6.25 nitrogen multiplication factor. The PUC and DE of the biomasses were determined as described in [12]. The dietary fibers analyses were performed with K-TDFR-100A (Megazyme, Ireland).

Statistical analysis

The experimental data (three replicates) are presented as mean value ± standard deviation. For analysis one-way ANOVA test (Tukey's post hoc test; $p < 0.05$) was used with Microsoft Excel 2013 (additional XL Toolbox NG module installed).

RESULTS AND DISCUSSION

Preliminary characteristics of CS and YH residues

The analyses of the CS and YH residues revealed that they were rich in lignocellulosic material (Table 1). The amounts of TDF determined for CS and YH were 64.06±0.46% and 71.85±1.74%, respectively and the IDF fraction was predominating – 57.47±0.46% for CS and 66.41±1.74% for YH. The protein amount determined for CS was 12.34±1.14% and 8.66±0.92% for YH biomass. The CS residue (8.03±0.40%) had two times higher ash content than YH (3.31±0.16%). One possible explanation could be the difference in their processing – the YH raw material was extracted with methanol containing 35% sulfuric acid and this extractant could remove a large part of the inorganic matter. The moisture content of the biomass was 10.11±0.15% and 10.95±0.27% for YH and CS, respectively. The PUC, commonly referring to pectins in the plant matrix, differed significantly for both residues – 10.62±0.20% for YH and 3.83±0.20% for CS.

Table 1. General characteristics of sage and yellow poppy residues: soluble (SDF), insoluble (IDF) and total dietary fiber (TDF), protein, ash, moisture, degree of esterification (DE) and polyuronide content (PUC)

	DF			Protein, %	Ash, %	Moisture, %	DE, %	PUC, %
	TDF, %	IDF, %	SDF, %					
CS	64.06±0.46^b	57.47±0.46^b	6.59±0.28^a	12.34±1.14^a	8.03±0.40^a	10.31±0.12^a	69.89±0.16^a	3.83±0.20^b
YH	71.85±1.74^a	66.41±1.74^a	5.44±0.86^a	8.66±0.92^b	3.31±0.16^b	9.47±0.14^a	62.33±1.31^b	10.62±0.20^a

Results are presented as the mean of three measurements; ^{a,b} Different letters in columns indicate statistically different values (Tuckey's HSD test, $p < 0.05$).

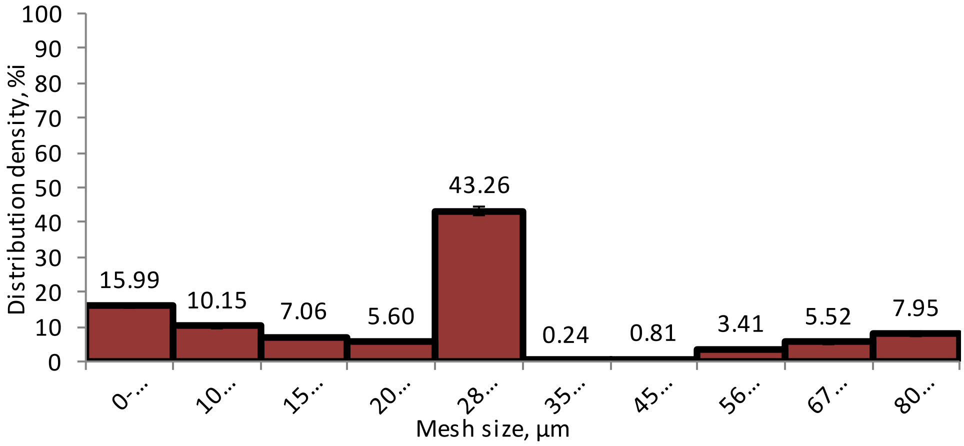

Figure 1. Distribution density pattern of the CS and YH residues

This could also be explained with the different industrial processing of the plant material, as it is known that alcoholic solutions preserve extraction of polysaccharides to a large extent. Figure 1 presents information from the mesh size analyses of CS and YH. The average particle size of the residues was 483.9±2.6 µm and the dominating fraction was 280-355 µm (43.26%).

Contact time influence on βNO adsorption

The CS and YH residues were pretreated with four solvents: deionized water, 0.1 N HCl acetone and 0.1 N NaOH. These washings intended removal of low-molecular organic and inorganic matters from the residues and also activation / deactivation of the functional groups of compounds present in the plant materials – mostly carboxyl (pectins and proteins) and amino groups (proteins).

Adsorption kinetics of βNO removal by the residues was initially explored. The experimental observations are presented in Figure 2 for CS and Figure 3 for YH residues. Adsorption is based on physical and chemical interactions of the adsorbent with the substances in the solution. The predominating functional groups in the adsorbent and the compounds adsorbed play a vital role and determine the effectiveness of the process. These interactions are stronger and adsorption is faster in the beginning because of the availability of a lot of unoccupied groups on the surface of the adsorbent [9].

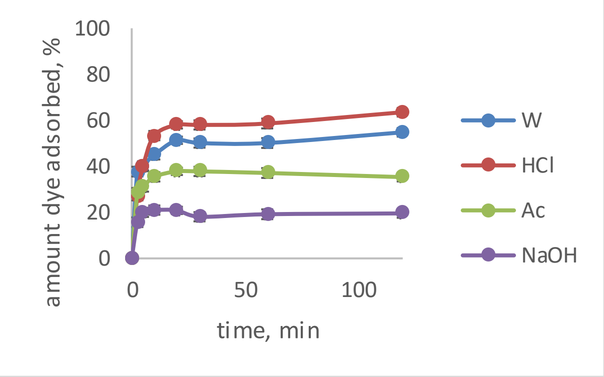

Figure 2. Contact time influence on βNO adsorption by CS residue washed with deionized water (W), 0.1 M HCl (HCl), acetone (Ac) and 0.1 M NaOH (NaOH)

Equilibrium is established when adsorbent's groups are taken by the adsorbed compounds and equilibrium concentration is reached. Our observations suggested that equilibrium for CS and YH was reached between 10th and 20th min. For CS residue washed with 0.1 N HCl effectiveness of removal around 60% was observed while for other the adsorbent it was lower. For YH residues washed with deionized water (W), 0.1 M HCl (HCl) and acetone (Ac) the removal effectiveness exceeded 60%. For both residues washed with 0.1 N NaOH the effectiveness was lower: between 15-20%.

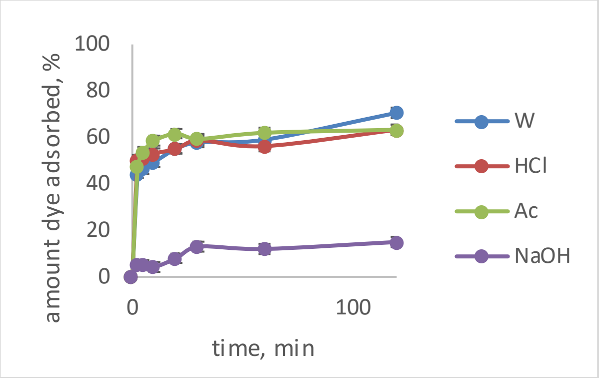

Figure 3. Contact time influence on βNO adsorption by YH residue washed with deionized water (W), 0.1 M HCl (HCl), acetone (Ac) and 0.1 M NaOH (NaOH)

Influence of pH on βNO removal

In the next experiments, because of the observations from previous section, the pH influence on βNO removal was explored. The adsorption process was investigated with five solutions having from high acidity – 1.10 to highly basic pH – 12.90 (Figure 4 – CS residue; Figure 5 – YH residue). The data clearly showed that lower pH (5 or less) increases adsorption effectiveness for both residues. The highest percentage of removed βNO was observed at pH 1.10 – almost 90% for CS and around 80% for YH.

In any cases, above pH 7 adsorption effectiveness of the CS and YH residues dropped down below 50% and for YH pretreated with NaOH it was the lowest one. Similar observations and conclusions were made in the studies [9, 13, 14] focusing on βNO removal using rose and lavender (residues from industry for essential oil production), brewery spent yeasts, and melissa and yarrow wastes (also from industry for essential oil production), respectively. Because βNO (as a salt) is a charged compound, lowering the pH would result in less electrostatic repulsion between the dye and plant matrices (pectins and proteins could be regarded as the biopolymers having the highest amount of charged groups – carboxyl and amino).

Temperature influence on βNO removal

The best results for βNO adsorption were observed at lower temperatures: 20 or below. At 40°C or above a trend for lowering the capacity was clearly observed (Figures 6 and 7). CS residues washed with water and 0.1 N HCl showed the highest effectiveness at 20 °C and for YH the residue pretreated with acetone showed the highest effectiveness at 20 °C (above 80%) and at 4 °C (above 70%). Comparably, Marovska *et al.* [9] and Hambarliyska *et al.* [14] showed that temperature increase lowered the adsorption rate of βNO on rose and lavender (steam-water and steam distilled) residues and melissa and yarrow biomass (steam distilled), respectively.

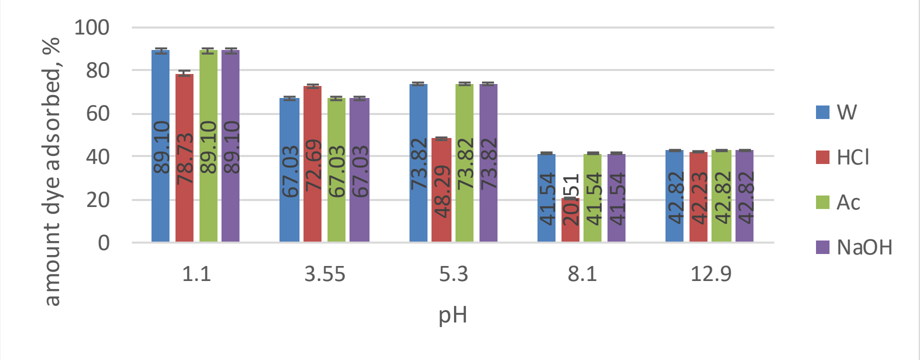

Figure 4. pH influence on βNO removal by CS residue

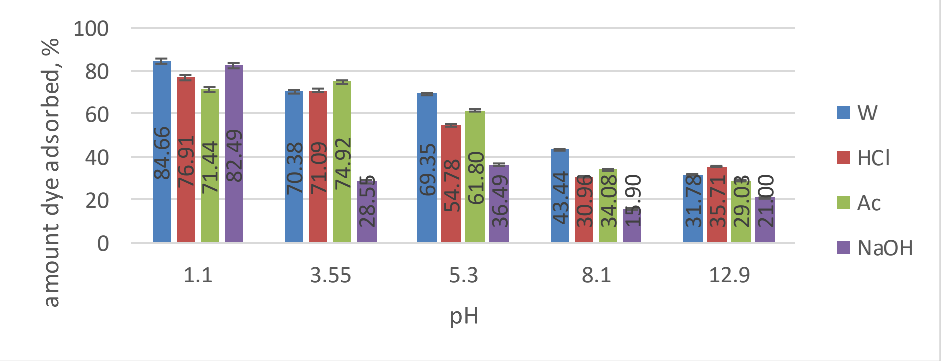

Figure 5. pH influence on βNO removal by YH residue

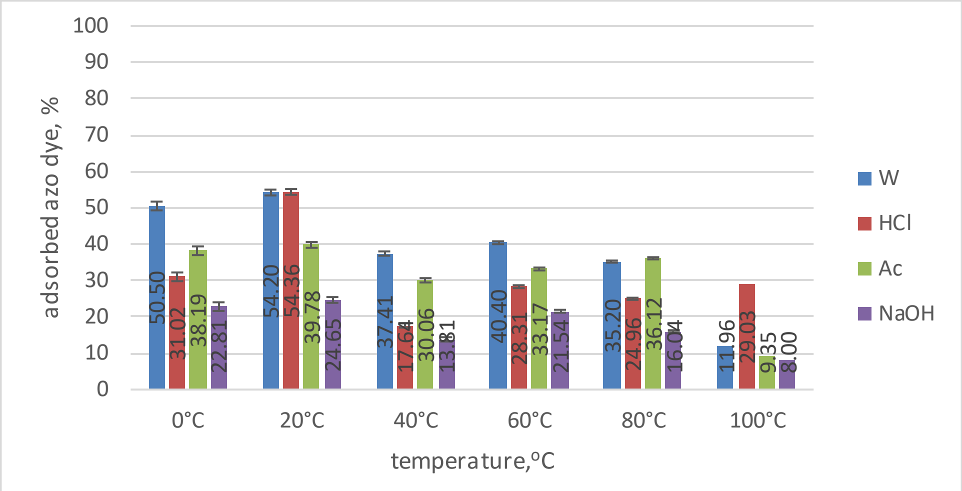

Figure 6. Temperature influence on βNO removal by CS residue

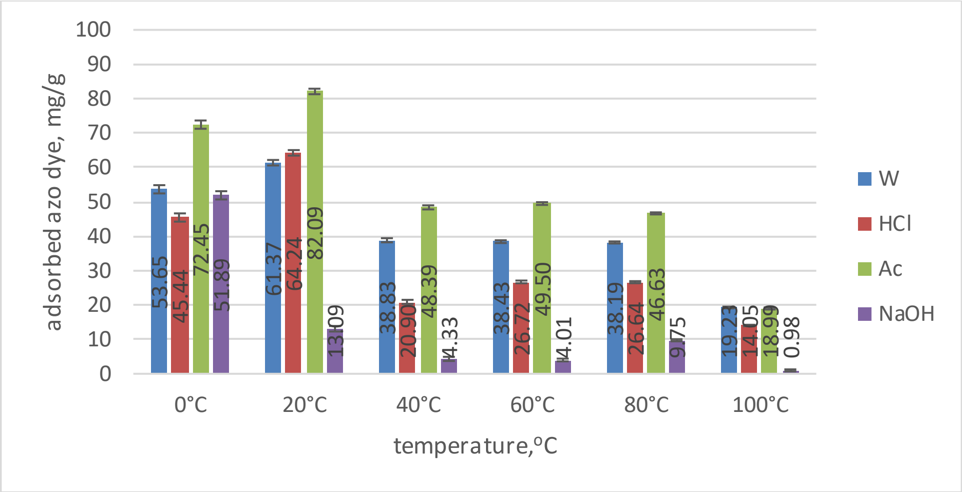

Figure 7. Temperature influence on βNO removal by YH residue

This trend for unfavorable influence and decrease in adsorption rate might be explained with increasing the desorption rate when increasing the temperature.

Initial treatment / adsorbent influence on βNO removal

Furthermore, juxtaposition of the adsorption of βNO by CS and YH (residues washed with water) and other materials (three substances used industrially: Al_2O_3, silica gel (chromatographic), activated carbon, and biomasses from industrial distillation of rose (RD), lavender (L), melissa (M), and yarrow (Y) was carried out (Figure 8). The data suggest that YH (water washed) residue showed a potent effectiveness for βNO removal (around 65%). Only activated carbon, M and Y residues (pretreated with 0.1 N HCl) showed better performance (almost 90%, 80% and 75%, respectively). Juxtaposition of effectiveness of common sage with rose and lavender (all pretreated with water) revealed similar adsorption capacity with slightly better performance by CS_W. Close results were demonstrated by Hambarliyska *et al.* [14] investigating the removal of βNO with residues from melissa and yarrow and Marovska *et al.* [9] researching adsorption of βNO with rose and lavender post-distillation biomasses.

Influence of βNO initial concentration on removal effectiveness

Further, the removal of βNO from its solutions with different concentrations was explored (Figure 9). For all investigated concentrations of βNO (2, 1, 0.5, 0.25, and 0.125 mg/mL) the CS and YH residues (initially pretreated with water) were able to remove aboe 50% of the azo dye. The highest effectiveness was observed for 0.125 and 0.25 mg/mL concentrations – 80% and almost 70% removal, respectively. Both adsorbents showed similar adsorption effectiveness, the negligible differences being within the limit of statistical insignificance.

Confronting the results of the present study with those obtained by Marovska and Slavov [12] who investigated the removal of βNO with rose and lavender biomass, it could be concluded that CS and YH residues showed slightly lower adsorption effectiveness.

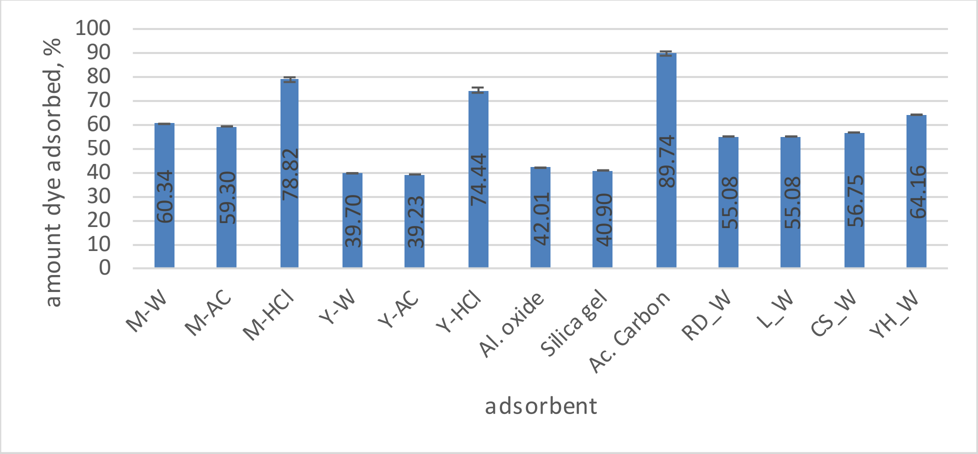

Figure 8. Juxtaposition of initial treatment / adsorbent influence on the βNO adsorption

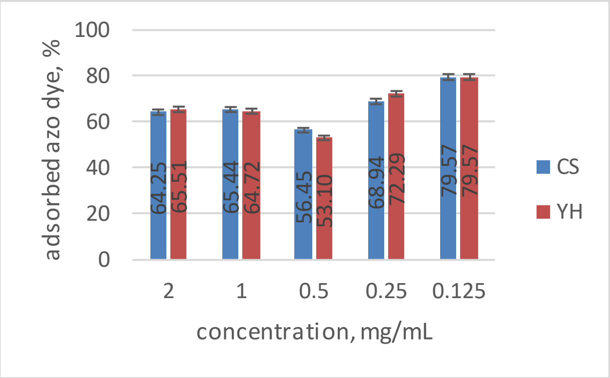

Figure 9. Influence of initial concentration of βNO on its removal by CS and YH. βNO below 0.5 mg/mL.

CONCLUSIONS

The present study focused on the adsorption of azo dye βNO with two residues from the industry of essential oil production, namely common sage and yellow hornpoppy (initially pretreated with four solvents), which showed a promising potential for waste waters purification. Residues washed with 0.1 N HCl demonstrated the highest adsorption capacity for βNO removal. The equipoise state of adsorption was reached between 10th and 20th min. The pH was the predominating factor affecting the adsorption rate and below pH 5 higher efficiency was observed. Juxtaposition of the effectiveness of CS and YH residues (water-washed) with that of several adsorbents (Al_2O_3, silica gel, activated carbon, and rose, lavender, melissa and yarrow post-distillation biomasses) lead to the conclusion that YH showed better adsorption effectiveness than CS. The optimal conditions for βNO removal could be summarized as follows: pH below 3, initial pretreatment of the residues with dilute acids, temperatures below 20 °C, contact time 30 min, and initial concentration of βNO below 0.5 mg/mL.

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Phytonutrients, polyphenols and triterpenes in *Taraxacum officinale* Weber ex F.H.Wigg flowers

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Dandelion or *Taraxacum officinale* Weber ex F.H. Wigg is a well-known medical and edible plant that is a source of various nutrients and biologically active substances. Considering the lack of studies of the dandelion flowers justifying their use as food, the present study was done to emphasize the nutritional level for dry and fresh flowers. The chemical composition for 100 g of dry matter was: crude proteins 13.90±0.06% dw (2.17±0.01% fw); crude fat 7.57% dw (1.18±0.01% fw); total sugars 5.99±0.01% dw (0.93±0.00% fw) (fructose, glucose and sucrose); cellulose 11.50±0.12% dw (1.79±0.02% fw); pectin 3.35±0.05% dw (0.52±0.01% fw). The amounts of the yellow-colored pigments total carotenoids (2.51±0.01% dw and 0.39±0.01% fw, respectively) were determined. The amino acid composition of the protein was determined - the results show that dandelion flowers are a potentially good source of the amino acids histidine and lysine. Six phenolic acids were found in dandelion flowers, with chicory acid, protocatechuic acid and chlorogenic acid being in the largest quantities. The triterpene composition of the edible dry and fresh dandelion flowers was determined, α-amyrin, β-amyrin and lupeol were found, and the amount of the phytosterol β-sitosterol was also determined. The results of this study show the nutritional potential of *Taraxacum officinale* flowers as a suitable resource for the preparation of fresh salad and culinary processed foods that are the product of native wild and cultivated plants.

Keywords: dandelion, *Taraxacum officinale* Weber ex F.H. Wigg, nutrients, edible flowers

INTRODUCTION

Herbs have accompanied humanity for centuries, serving important roles in medicine, cosmetology, and culinary practices. In recent years, there has been a growing consumer interest in the wholesomeness of food, with a particular focus on products that are not only nutritious but also biologically active. For millennia, plants have been a rich source of chemical compounds exhibiting various biological activities, including potent antioxidant properties [1].

Dandelion (*Taraxacum officinale* Weber ex F.H. Wigg) is an herbaceous perennial belonging to the *Asteraceae* family. It is non-toxic, often considered a weed, and is widely distributed across the Northern hemisphere [2]. The therapeutic use of dandelion has been mentioned by Arabian, Native American, Chinese, and Ayurvedic medicine [3].

The name of the genus *Taraxacum* means bitter herb in Arabic, while in Greek, it may be derived from the words taraxia (eye disorder) and akeomai (to cure). The common name ‘dandelion’ probably comes from the French (dent de lion), meaning teeth of the lion, referring to the tooth-like edges of the leaves [3, 4].

In Russia, India, and China, the dandelion has been used in ethnopharmacology as a traditional folk medicine due to its hepatic and hypoglycemic effects [5]. Another interesting aspect reported in ethnopharmacological studies is that immigrants continue their traditional practices for natural medicine even once inserted in highly industrialized countries, indicating the traditional importance of herbal medicine [6].

Dandelion is often consumed as a food (mainly as a salad ingredient), as it is a rich source of micronutrients such as minerals and vitamins [7]. The young leaves are placed in many dishes, and the inulin-rich roots are used as substitutes for coffee or tea [8]. Additionally, the *Taraxacum* leaf extract can be used as a flavoring agent for various foods, including alcoholic and soft drinks, frozen dairy desserts, candies, baked goods, puddings, and cheese [9,10].

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Various dandelion plant parts have been studied both chemically and nutritionally. Apart from being used as a remedy for illness, dandelion petals, leaves, and roots are processed into various food products. The beneficial effects of dandelion are dependent on the chemical compounds contained in the plant [10]. These include sesquiterpene lactones, which have been found to have anti-inflammatory and antibacterial effects, as well as triterpenes or phytosterols, which possess anti-atherosclerotic properties. In addition, dandelions have high levels of phenolic compounds, including phenolic acids with antioxidant properties and coumarins with anticancer, anti-inflammatory, antibacterial, and antithrombotic effects. The roots are also rich in inulin, which has a probiotic, hypoglycemic, and immune-boosting effect [10].

The traditional uses of dandelion that are mentioned in the literature concern its use as a remedy for kidney diseases, diabetes, bacterial infections, diuretic, liver, kidney, and spleen disorders, and anti-inflammatory factors. On the other hand, dandelion parts are used as food, mainly as a salad ingredient, young leaves are placed in many dishes, and the inulin-rich roots are used as substitutes for coffee or tea [8].

Modern research has demonstrated that the therapeutic effects of raw dandelion were ascribed to the bioactivity's constituents, including polyphenols, tocopherols, cinnamic acid derivatives, flavonoids, triterpenoids, polysaccharides, and riboflavin [11, 12].

Even though these food components are not vital for the metabolism of the body, they improve the overall health of consumers by enhancing their physiological activity [13]. For this reason, studies on the secondary metabolites of plants have grown exponentially over the past years, and these compounds are considered potent substances for improving human health.

Taraxacum officinale has been extensively characterized in terms of its biochemical profile and nutritional composition, primarily due to its global distribution and economic importance as both food and feed. Many available data focus on phytonutrients, polyphenols, and triterpenes in the roots and leaves [14], whereas considerably less information is available for the flowers.

The medicinal properties of plants like antidiabetic, antimicrobial, antidiuretic, antioxidant, etc. are mainly dependent on the type, nature and concentration of secondary metabolites present. Reports have shown that some of the most important bioactive compounds are phytochemicals such as alkaloids, saponins, flavonoids, tannins, sterols, and phenolic compounds [14-16]

On this basis, the aim of the present study was to determine the nutritional (macro- and micronutrient) composition of dandelion flowers (carbohydrates, fats and proteins), phenolic acid composition, pentacyclic triterpenes and antioxidant activity, both fresh and dried flowers.

EXPERIMENTAL

Plant materials

Aerial parts (flowers) of a wild-growing population of dandelion (*T. officinale* Weber ex F.H.Wigg.) in Plovdiv region, Bulgaria were randomly collected during flowering stages (on May, 2023). The samples were dried in the shadow at room temperature for 10 days and finely ground in a laboratory homogenizer. Fresh flowers were frozen and stored at -20 °C.

Analysis of moisture content

For the determination of moisture content, the milled sample (~1.5 g) was dried in an automated moisture analyzer (KERN DLB, Germany) at 105 °C until constant weight. Ash content was determined as the pulverized sample (0.5 g) was placed in a crucible and ignited in a muffle furnace at 550 °C until there was no change in the mass of the sample.

Analysis of crude lipid content

For the estimation of crude lipid content, the ground sample (10.0 g) was packed in a cellulose thimble and subjected to an exhaustive extraction with n-hexane (200 ml) for 8 h in a Soxhlet extractor. The obtained crude extract was dried under vacuum, and its weight was used for the calculation of the lipid content.

Analysis of crude protein content

The crude protein content was evaluated by the Kjeldahl method (N × 6.25). The total carbohydrate content of the fruits was analyzed by the phenol–sulfuric acid method [17].

Amino acid composition

For the estimation of amino acid composition, the sample (300 mg) was hydrolyzed (5 ml, 6N HCl) in a sealed glass ampule at 105 ◦C for 24 h. The sample was vacuum-dried, reconstituted in 10 ml of 20 mM HCl, and filtered. A total of 20 μL of the collected filtrate was derivatized using an AccQ Fluor kit (WATO52880, Waters Corp., Milford, NH, USA) according to the manufacturer's instruction manual. The resulting derivatives were separated on an ELITE LaChrom HPLC system (VWR™ Hitachi, Tokyo, Japan) equipped with a diode array detector and a reversed-phase column C18 AccQ Tag (3.9 mm × 150 mm) operating at 37 ◦C. The volume of the injected sample was 20 µL. The elution was performed at a flow rate of 1.0 ml/min [18]. The different amino acid derivatives were detected at 254 nm.

Total carotenoid and chlorophyll content

The total content of chlorophylls and carotenoids was determined by using 80% acetone as a solvent. Absorbance was measured at three different wavelengths: 662, 644, and 470 nm using Camspec M107 VIS spectrophotometer (Spectronic-Camspec Ltd., Leeds, UK) according to Lichtenthaler and Wellburn [19].

Pectin and cellulose

For the estimation of the pectin content of dandelion flowers the method described by Ivanov *et al.* [20] was used. The quantitative estimation of cellulose was performed gravimetrically. Briefly, a sample (0.5 g) was gently boiled (30 min) with 25 ml of acetic acid-HNO_3 reagent (acetic acid: H_2O: HNO_3 8:2:1 v/v/v) in a round-bottom flask fitted with a reflux condenser. After cooling, the insoluble residue was filtered through a sintered glass filter (G3) under vacuum, washed with deionized water to neutral pH, then with ethanol (96% v/v), and finally with an excess of petroleum ether. The obtained residue was dried in a laboratory oven at 50 °C to a constant weight. The resulting cellulose was corrected for its ash content [17].

High-performance liquid chromatography analysis of available carbohydrates, phenolic acids, pentacyclic triterpenes and phytosterols.

In a plastic centrifuge tube, about five g of dry dandelion flowers were weighed out. To this, distilled water (25 ml) for sugars; 50% ethanol for phenolic acids; and n-hexane for terpenes was then added. The tube was placed in an ultrasonic bath (Siel UST 5.7-150, Gabrovo, Bulgaria), and ultrasound-assisted extraction was performed with a frequency of 35 kHz (300 W) at 40 °C. After heating for 20 min, the tube was cooled in running water, then filtered successively through a paper and a PTFE filter (0.45 µm). The quantitative chromatographic separation of free sugars was carried out on a Shodex® Sugar SP0810 (300 mm × 8.0 mm i.d.) column having Pb^{2+} as a counter ion and a Shodex SP-G guard column (5 µm, 6 mm × 50 mm) (Shodex Co., Tokyo, Japan) according to method described by Ognyanov *et al.* [17]. The quantitative chromatographic separation of phenolic acids was carried out on a Supelco Discovery® HS C18 column (5 µm, 250 mm × 4.6 mm) according to the method described by Stoyanova *et al.* [18]. The quantitative chromatographic separation of pentacyclic triterpenes and phytosterols was carried out on a Supelco Discovery® HS C18 column (5 µm, 250 mm × 4.6 mm) according to the method described by Vrancheva *et al.* [21].

Total phenolic content and total flavonoid content

The total phenolic content was determined according to the method with Folin–Ciocalteu's reagent [17]. Gallic acid (10–200 µg/ml) was employed as a calibration standard ($Y=12.557X - 0.0871$, $R^2=0.9983$). The total flavonoid content was determined according to the method of Vrancheva *et al.* [21]. The calibration curve was constructed with quercetin dihydrate (10–200 mg/L, $Y=0.0119X - 0.0467$, $R^2=0.9895$).

In vitro antioxidant activity assays

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging ability and cupric ion reducing antioxidant capacity (CuPRAC) were determined. The preparation of extracts was carried out as described by Ivanov *et al.* [22].

Statistical analysis

The experimental data (three replicates, n=3) are presented as mean value ± standard deviation. For analysis Microsoft Excel 2013 was used.

RESULTS AND DISCUSSION

Traditional medicinal plants exert their therapeutic effects due to the bioactive compounds they contain. *Taraxacum officinale* (dandelion) is highly valued for its unique biological properties and significant pharmacological activity [23]. Modern research has demonstrated that the therapeutic effects of raw dandelion are attributed to its bioactive constituents, including tocopherols, cinnamic acid derivatives, flavonoids, triterpenoids, polysaccharides, and fatty acids [24-27].

In our study, both fresh and dried dandelion flower samples were analyzed for their chemical composition, based on 100 g of dry matter (dw) and 100 g of fresh weight (fw), as presented in Table 1. The comparative nutritional analysis of fresh and dried *T. officinale* flowers revealed notable differences. The results showed that dried flower samples were nutritionally superior to their fresh counterparts. The moisture content in fresh flowers was 84.39 ± 0.05 g/100 g, which decreased to 11.25±0.02 g/100 g dw after drying, representing an 86.7% reduction. The ash content increased from 0.15±0.02 g/100 g in fresh flowers to 7.01 g/100 g in dried samples, reflecting an increase of approximately 40 times. Similarly, the total carbohydrate content increased from 11.00±0.02 g/100 g in fresh flowers to 70.52±0.14 g/100 g in dried flowers, corresponding to an increase over 5 times. The results show that there are no significant nutrient losses during drying.

Taraxacum officinale herbs and root are rich sources of polysaccharides, which consist of monosaccharides such as arabinose, glucose, galactose, fructose, xylose, mannose, and their glycoside and inulin [27, 28]. In dandelion flowers only glucose, fructose and sucrose were identified in concentrations of total sugars 5.99±0.01 g/100 g dw, glucose 1.91±0.01 g/100 g dw fructose, 2.39±0.01 g/100 g dw and sucrose 1.58±0.01 g/100 g dw. The fresh flowers contain about 5 times less sugar than dried flowers (Table 1). In comparison, in other parts of the dandelion, such as the leaves, similar sugars were found in different concentrations as well - glucose 30.9 µg/g, fructose, rhamnose 5.9 µg/g, and sucrose 8.2 µg/g [29].

The content of crude protein in dandelion flowers (2.17±0.01 g/100 g fw and 13.90±0.06 g/100 g dw) (Table 1) is much lower as compared to that identified in leaves (3.82 g/100 g fw and 16.01 g/100 g dw) [30]. Although there is more protein in the leaves (about 25%) than in the flowers, the amount of lipids is higher in the flowers (about 43%, 7.57±0.07 g/100 g dw) compared to their amount in the leaves (4.29 g/100 g dw) [30]. In comparison, dandelion flowers are a good source of the nutritional components crude protein, crude lipids and carbohydrates compared to other known edible flowers (*Calendula officinalis*, *Etlingera elatior*, *Hedychium forrestii*, *Helianthus annuus*, *Hibiscus rosa-sinensis*, *Rhododendron arboretum*, *Rosa* spp., *Spilanthes oleracea*, *Tagetes erecta*, *Tropaeolum majus*). The amounts of these components are in the upper range in dandelion for crude proteins (from 1.20% to 2.38% fw); for crude fat (from 0.2% to 1.52% fw) and carbohydrates (from 2.15% to 14.15% fw) [32].

The content of chlorophyll A and B in the dried flowers was 34.12 mg/100 g dw and 17.16 mg/100 g dw, respectively. These values are higher than those previously reported for the flowers and roots (4.72±0.05 mg/100 g dw) [31] but lower compared to the leaves and stem (239.51 ± 0.015 mg/100 g dw) [30].

HPLC qualitative and quantitative analysis of phenolic compounds

Phenolic compounds (polyphenols) are secondary metabolites synthesized in plants and possess one or more phenolic rings with one or more attached hydroxyl groups.

Table 1. Nutritional values of dandelion flowers.

Compounds	Dry dandelion flowers, g/100g dw	Fresh dandelion flowers, g/100g fw
Moisture	11.25±0.02	84.39±0.05
Crude fat	7.57±0.07	1.18±0.01
Crude protein	13.90±0.06	2.17±0.01
Ash	7.01 ±0.11	0.15±0.02
Carbohydrates	70.52 ±0.14	11.00±0.02
Cellulose	11.50±0.12	1.79±0.02
Pectin	3.35±0.05	0.52±0.01
Total sugars	5.99±0.01	0.93±0.00
Glucose	1.91±0.01	0.29±0.00
Fructose	2.39±0.01	0.37±0.00
Sucrose	1.58±0.01	0.24±0.00
Sweetness index	9.54±0.01	1.46±0.00
Chlorophyll A (mg/100g)	34.12 ± 1.01	5.3 ±0.01
Chlorophyll B (mg/100g)	17.16 ± 1.04	2.6 ±0.01
Total carotenoids	2.51 ± 0.01	0.39±0.01

All data are presented as mean value ± standard deviation (n=3)

Generally, more than half of the phenolic compounds have antibacterial, antifungal, anti-inflammatory, and anti-tumor properties [33].

Phenolic acids contribute to the overall health improvement, primarily because of antioxidant and anti-inflammatory actions [34] which help in the prevention of cardiovascular diseases and various cancers [35], protect against oxidative damage diseases; and exhibit antimicrobial, antimutagenic, hypoglycemic, and anti-platelet aggregating activities [36]. More than 30 phenolic compounds have been identified and isolated in the different dandelion plant parts [37].

The contents of five organic acids - protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, and chicoric acid in dandelion (*Taraxacum officinale*) flower samples were quantified using HPLC (Table 2). Among the identified compounds, chicoric acid was present in the highest concentration, 575.14±0.31 mg/100 g dw. It was followed by protocatechuic acid and chlorogenic acid, with concentrations of 452.21±0.13 mg/100 g dw and 377.45±0.21 mg/100 g dw, respectively. Our results are in good agreement with a previous report showing that chicoric acid and chlorogenic acid (120.0 ± 0.50 mg/ 100 g dw) [38] were the predominant compounds in different parts of the dandelion. A similar trend was observed in fresh flower samples, with chicoric acid being the most abundant, followed by protocatechuic acid and chlorogenic acid. Chen *et al.* [39] reported that the extracts obtained from dandelion flower grown in China contained from 75 to 98 mg/100 g dw of chicoric acid and from 10 to 12 mg/100 g dw of chlorogenic acid. These concentrations are comparatively lower (over 4 times) than those found in the flower samples analyzed in our study. Ferulic acid was detected at a moderate level in our study, similar to the concentration in other authors' studies on flowers [40]. Caffeic acid was found at a concentration of 31.24±0.05 mg/100 g dry weight, which is about 3 times higher than previously reported concentrations of dandelion extracts obtained from flowers (9.47±0.29 mg/100 g dry weight) [39,40].

Table 2. Phenolic acids, total polyphenols, total flavonoids and antioxidant activities of dandelion (*T. officinale*) flowers

Compounds	Dry dandelion flowers, mg/ 100 g dw	Fresh dandelion flowers, mg/ 100 g fw
Phenolic acids		
Protocatechuic acid	452.21±0.13	70.59±0.02
Chlorogenic acid	377.45±0.21	58.92±0.03
Caffeic acid	31.24±0.05	2.87±0.01
Ferulic acid	140.69±0.12	21.96±0.02
Chicoric acid	575.14±0.31	89.77±0.05
p-Coumaric acid	262.23±0.11	40.93±0.02
Total polyphenols	2346.21±0.93	366.24±0.14
Total flavonoids	982.36±0.33	153.34±0.05
Antioxidant activity		
DPPH method	14521.07±78.18	2266.74±12.20
CuPRAC method	28808.30±127.41	4496.97±19.88

All data are presented as mean value ± standard deviation (n=3).

The total polyphenol content in dandelion flowers in our study was 2346.21±0.93 mg GAE/100 g dw and 366.24±0.14 mg GAE/100 g fw, while the total flavonoid content was 982.36±0.33 mg QE/100 g dw and 153.34±0.05 mg QE/100 g fw (Table 2). The antioxidant capacity of dandelion (*T. officinale*) flowers was assessed using two established methods - CuPRAC and DPPH radical scavenging method. The results are presented in Table 2. Thus, the high antioxidant activity observed in the flower extract might be a result of the high amount of chicoric acid, chlorogenic acid and other phenolic acids in the flower extract (Table 2). As shown in the table, the CuPRAC method revealed the highest antioxidant activity in dandelion flowers, with values approximately twice higher than those obtained by the DPPH method. According to the research of another author [31], in different parts of the plants (leaves, flowers, roots) the results indicated that total phenolics, DPPH and CuPRAC values were higher in *Taraxacum officinale* flowers: 2346.21 mg GAE/100 g dw, 14521.07 mM TE/100 g dw (DPPH method), and 28808.30 mM TE/100 g dw (CuPRAC method). The antioxidant capacity of the different parts of the plant positively correlated with their phenolic content.

Amino acid composition

The amino acid composition can determine the nutritional quality of foods. Amino acids are building blocks of proteins in muscle fibers and other structures in the body. They help to transport nutrients, prevent illness, and perform other functions. Their deficiency can result in decreased immunity, digestive problems, depression, slowed growth in children, and many other health issues [41].

The identified amino acids contribute to the overall pharmacological effects of this type of medicinal plant material. However, the existing literature lacks sufficient studies on the individual amino acid composition in *Taraxacum officinale* flowers. In our study, seventeen amino acids were identified and quantified. The composition and content of essential, semi-essential and nonessential amino acids in our sample are presented in Table 3.

The content of essential amino acids is significantly higher for four specific acids, which have relatively close values. The highest content was found for L-histidine (19.83±0.01 mg/g dw), followed by L-lysine (13.73±0.01 mg/g dw), L-isoleucine (10.93±0.01 mg/g dw). The isoleucine value corresponds to the value reported for the dandelion root [44]. Histidine facilitates growth, creation of blood cells, and tissue repair. It also helps to maintain the special covering over nerve cells, which is called myelin sheath [41]. Isoleucine is of great interest as a nutritional and dietary supplement, as well as for enteral and parenteral protein nutrition. It affects the replenishment of the deficit of proteins, carbohydrates, and amino acids, and has an antitoxic effect [45, 46]. The third most predominant amino acid was L-phenylalanine

(8.66±0.01 mg/g dw), followed by L-valine (8.51±0.01 mg/g dw). Phenylalanine helps the body to use other amino acids, as well as proteins and enzymes. It is needed in treating brain disorders and for normal functioning of the central nervous system. Its deficiency can lead to poor weight gain [47]. The nonessential amino acid proline is present in *Taraxacum officinale* flowers in the greatest amount (18.79±0.01 mg/g dw). Proline accumulation is a common physiological response to salinity and osmotic stress in many plant species [43].

Table 3. Amino acids content in *Taraxacum officinale* flowers

Amino acid	Concentration, mg/g dw	Ref. protein mg/g [42]	AAS* % [41]
Essential amino acids			
L-Valine, Val	8.51±0.01	15	56.7
L-Leucine, Leu	1.61±0.01	21	7.7
L-Isoleucine, Ile	10.93±0.01	15	72.8
L-Methionine, Met	1.05±0.01	20	6.2
L-Threonine, Thr	0.77±0.01	11	7.0
L-Lysine, Lys	13.73±0.01	18	76.3
L-Phenylalanine, Phe	8.66±0.01	21	62.4
Semi essential amino acids			
L-Histidine, His	19.83±0.01	15	132.2
L-Arginine, Arg	6.06±0.01	-	-
Nonessential amino acids			
Glycine, Gly	2.59±0.01	-	-
L-Alanine, Ala	14.36±0.01	-	-
L-Serine, Ser	7.59±0.01	-	-
L-Aspartic acid, Asp	10.75±0.01	-	-
L-Glutamic acid, Glu	8.85±0.01	-	-
L-Cysteine, Cys	0.20±0.01	-	-
L-Tyrosine, Tyr	4.46±0.01	-	-
L-Proline, Pro	18.79±0.01	-	-

All data are presented as mean value ± standard deviation (n=3); * AAS - Amino acid score

In dandelion, pentacyclic triterpenoids and phytosterols are of many types such as gigantursenol A, taraxasterol, β-sitosterol, β-sitosterol-3-*O*-β-D-glucoside, stigmasterol, and β-sigmasterol-3-*O*-β-D-glucoside. lupane-, bauerane-, and euphane-type triterpenoids were isolated from the roots and leaves [24, 26, 48]. In dandelion flowers, beta-sitosterol predominates, and of the triterpenes, alpha- and beta-amyrin and lupeol have also been determined in concentrations of about 100 µg/100g dw (Table 4). In dandelions, triterpenoids and sterols exhibit remarkable anti-oxidative and anti-inflammatory activities [48].

Table 4. Pentacyclic triterpenes and phytosterols in dandelion flowers.

Compounds	Dry dandelion flowers, µg/100 g dw	Fresh dandelion flowers, µg/100 g fw
α-Amyrin	176.20 ±0.09	27.50±0.02
β-Amyrin	105.37±0.05	16.45±0.01
Lupeol	100.22±0.07	15.64±0.02
β-Sitosterol, mg/100 g	16.29±0.10	2.54±0.02

CONCLUSION

Dandelion flowers combine macronutrient value (proteins, fibers, healthy fats) with exceptionally high levels of polyphenols, triterpenes, and phytosterols - making them one of the most nutritionally potent and health-promoting commonly eaten floral foods. Dandelion flowers are a suitable source of the essential amino acids histidine and lysine, as well as polyphenolic compounds (chicoric acid and protocatechuic acid) with high antioxidant potential. Their regular intake (fresh or processed) can bolster antioxidant defenses, support cardiovascular and liver function, aid digestion, and contribute to overall dietary variety [30, 32, 34].

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Ecological benefits and antioxidant activity of the green macroalga *Cladophora glomerata*

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This study evaluated the ecological and industrial potential of *Cladophora glomerata* from the Aytoska River. The species was analyzed for bioactive compounds, including phenolic acids and flavonoids, and for antioxidant activity using five complementary methods. Additionally, the adsorption properties of *C. glomerata* were assessed for potential use in bioremediation of water pollutants. The content of phenolic acids and flavonoids in extracts obtained with different solvents did not exceed 2.8 mg GAE/g and 2.2 mg RE/g, respectively. These findings highlight the dual potential of *C. glomerata* as a natural source of antioxidants and as a sustainable material for water purification applications. Low antioxidant activity was determined by DPPH, ABTS, H_2O_2, FRAP and CUPRAC analysis. *C. glomerata* demonstrates potential for the removal of nitrates and nitrites from water.

Key words: Aquatic ecosystem, *Cladophora glomerata*, total phenolic content, total flavonoid content, antioxidant activity

INTRODUCTION

Macroalgae have attracted the attention of many researchers as a source of compounds with different biological activities, including both complex organic compounds and primary and secondary metabolites. These include lipids, peptides, enzymes, carbohydrates, tannins, phenolic compounds, terpenoids, polyunsaturated fatty acids (PUFAs), phytopigments, and others. Thus, algae are a viable and economical biomass source of valuable compounds with potential applications in nutraceutical, pharmaceutical, chemical, food and cosmetic industries due to their biologically active and regenerative properties [1-4]. In general, macroalgae are classified as red (*Rhodophyta*), brown (*Phaeophyta*) and green (*Chlorophyta*) species according to their chemical composition and mainly due to the presence of specific pigments (phycobilins, fucoxanthin and chlorophyll, respectively) [5]. The *Chlorophyta* make up the majority of green algae and together with the *Streptophyta* belong to the *Viridiplantae* [6]. They are the most diverse group of algae worldwide today in terms of number of species (at least 7000), organization of the plant body (unicellular to multicellular) and habitat (from the snow surface to various symbiotic relationships) [7]. *Chlorophyta* can be effective phyco-mediators. Their presence in water can reduce the contents of dyes [8], heavy metals [9, 10], and nutrients nitrogen and phosphorus from different types of waste water [11-13] with the possibility of using the residual biomass as biofuel [14, 15], as biofertilizer by recovering nutrients from wastewater and converting them to biochar [16], and producing bioplastics and additives as plasticizers to enhance the quality of the final product [17]. The genus *Cladophora* belongs to *Chlorophyta* and has different forms and many species that are widely distributed in brackish and freshwater environments. It adapts well to a wide range of environmental conditions, such as different temperature, salinity and nutrient concentrations [18]. The most common in freshwater ecosystems are the species of the genus *Cladophora* Kützing [19]. This genus is rich in phytochemical compounds that can be used to maintain the health of both humans and animals. Due to the variety of secondary metabolites, species of this group possess antioxidant, antidiabetic, antihypertensive, antiparasitic, antimicrobial, anticancer, and cytotoxic properties [20]. *Cladophora glomerata* is a representative of the green filamentous algae belonging to the family *Chlorophyceae*, and is one of the most common algae, both in freshwater and marine environments [21]. The identification of filamentous algae is often a complex task. Several taxonomic characters are used in the genus *Cladophora:* thallus color, types of branching, cell design and size, general structure of the plant, basal cells, and shape of zoospores [22]. Populations of *C. glomerata* occurring in water bodies in Bulgaria

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are described as forming dense green cotton-like tufts or long "tails" up to 50 cm long. The thallus is attached to stones, concrete, or wood by a disc-shaped holdfast of short-branched filaments. The filaments are single-row, cylindrical with cells most often 50-90 µm wide and up to four times their width long. The lateral branches are formed just below the apical cell at an angle of about 40-70 °. The branches are thinner than the main axis and often intertwine, forming a dense mat. Each cell contains a large reticulate chloroplast with several clearly distinguishable pyrenoids. The cells are multinucleated and have a relatively thick, sometimes layered cell wall. Reproduction is most often asexual by tetraflagellate zoospores, and sexual stages are rare. The mass development of the algae takes place in spring and early summer in nutrient-rich rivers and reservoirs. During the summer, the tuffs break up into fragments overwinter attached to the substrate [23]. The species is reported to be a rich source of saturated and unsaturated fatty acids which have antibacterial activity [24]. A study evaluating the biochemical profile of freshwater *C. glomerata* reported that the algae contain high levels of quercetin, rutin, and lutein, along with tyrosinase inhibition corresponding to an SPF of approximately 12, suggesting that the algae are a viable natural UV-protector and antioxidant for the production of creams and lotions in skin care [25]. With improved extraction methods, *C. glomerata* proves to be an important source of phenols, tocopherols, carotenoids and chlorophyll pigments, which makes it suitable for use as a functional food and dermal antioxidant [26]. In a study evaluating the biosorption properties of *C. glomerata*, the algae achieved 57% Pb removal, 49% Cd, 39% chemical oxygen demand (COD), and 31% total nitrogen removal from industrial wastewater [27]. Another study on a pilot wetland dominated by *C. glomerata* found 81% elimination of total nitrogen, with the species reported to have a preference for NH_4^+ [28].

The aim of this article is to evaluate the benefits of *C. glomerata* investigating the content of bioactive compounds such as phenolic acids and flavonoids with potential applications in the food, cosmetic and pharmaceutical industries, and to evaluate the antioxidant activity of the algae. Additionally, preliminary experiments were conducted to test *C. glomerata* as a biosorbent for the removal of nitrogen compounds (nitrate, nitrite and ammonium) in polluted waters.

MATERIALS AND METHODS

Sampling of waters and C. glomerata

Water sampling was carried out from 5 points: 1 - Aytoska River - estuary N 42.5086 E 27.3372; 2 - Aytoska River - after Kameno N 42.5712 E 27.3088; 3-Burgas Lake-west N 42.4931 E 27.3442,4-Burgas Lake - center N 42.5105 E 27.3676; 5- Chakarliyska River - estuary N 42.4933 E 27.3439. Algae samples were taken from May to October 2024, from the mouth of the Aytoska river (point 1), just before the flow into the Burgas lake - the largest natural lake in Bulgaria (Figure 1).

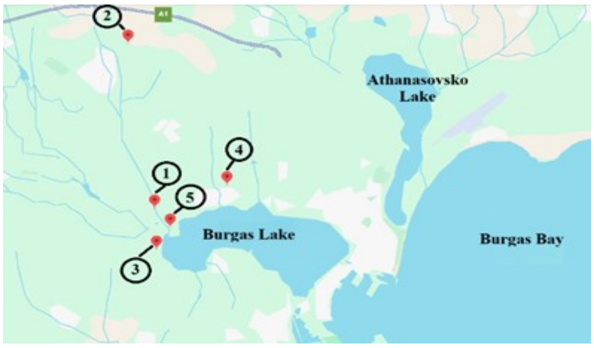

Figure 1. Location of monitoring points.

Sample preparation

Fresh *C. glomerata* biomass was collected from the sampling site, thoroughly washed with tap water to remove sand particles, epiphytes, shells, and other debris, and left on filter paper to reduce excess surface water. For further experiments, the algal biomass was dried under sunlight for 5 days (ambient temperature 25–30 °C, relative humidity 50–60%) or in an oven at 40–45 °C in order to preserve thermolabile compounds [29].

For the experiments on the antioxidant activity of macrophytes, the plant material was ground in a grinder to obtain a uniform powder, which was sieved through a 2-mm sieve [20, 21, 30, 31]. A portion of the dried algae was chopped and separated into particles with an average size of 1-5 mm, for the adsorption experiments [32].

Reagents and instruments

All chemicals were of analytical grade quality and were purchased from Honeywell and Sigma Aldrich (USA). The main physicochemical parameters (pH, dissolved oxygen, temperature, salinity, total dissolved solids (TDS), resistivity, and conductivity were measured with a WTW Multi 3630 IDS portable multimeter. The indicators $N\text{-}NO_2$ - nitrite nitrogen, NO_2 - nitrite, were measured with ready-to-use Hach® LCK cuvette tests with DR

3900 spectrophotometer (Hach, Loveland, CO, USA).

Assays for total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (by DPPH, ABTS, HPSA, FRAP, CUPRAC assay) were performed with Spectroquant Pharo 300, UV/Vis (Merck, USA).

Determination of the biosorption capacity, total phenolic content, total flavonoid content and antioxidant activity

- *Biosorption capacity.* Water samples collected from five monitoring points were pre-filtered using 0.45 μm syringe filters to remove suspended solids. A series of batch adsorption experiments was then prepared, consisting of 125 mL aliquots of filtered water and 0.25 mg of the adsorbent material. The adsorbent (*C. glomerata* biomass) was placed in permeable mesh bags (dimensions: 4.5×6 cm; mesh thickness: 20–40 μm) to allow free exchange with the water phase. The bags were immersed in the prepared water samples, and the optimal contact time was set at 120 min based on previously reported studies [33, 34].
- *Extraction method.* To determine the total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity, the same extraction was performed. Dry leaves and stems of *C. glomerata* were powdered and extracted with 60% methanol, 40% ethanol and distilled water using a mechanical shaker. The extracts were filtered and used for further analyses.
- *Total phenolic content (TPC) measured by the Folin-Ciocalteu (FC) assay.* The TPC of the extracts was assessed according to the method described by Kirkova *et al.* [35] using the FC assay: 0.1 mL of plant extracts (60% methanolic, 40% ethanolic or aqueous extract), 6 mL of water and 0.5 mL of 0.2 M FC reagent were placed into the test tube. After 4 min 3.4 mL of 7.5% Na_2CO_3 was added. Samples and blank were stored in the dark for 2 h and then were measured at 765 nm against the blank sample using a spectrophotometer (Spectroquant Pharo 300, Merck, USA). The concentration of the phenolic compounds in the extracts was calculated using gallic acid as standard, and the results were expressed as milligrams gallic acid equivalents per gram of extract (mg GAE/g) [35].
- *Determination of total flavonoid content (TFC).* The TFC of extracts was determined as follows: 0.8 mL of the extract was mixed with 1.6 mL of 1% $AlCl_3$ and 1.2 ml of 1 M CH_3COOK. Samples and blank were stored in the dark for 2.5 h after which the absorbance was measured at 440 nm against the blank sample on a spectrophotometer (Spectroquant Pharo 300, Merck, USA) [36].
- *Determination of antioxidant activity. DPPH assay.* The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity of the 60% methanolic and 40 ethanolic extracts was measured following the method described by Kirkova *et al.* [35]. This method assesses the reaction of the antioxidants with stable DPPH free radicals. The absorbance was measured at 515 nm against a methanol blank using a spectrophotometer (Spectroquant Pharo 300, Merck, USA) [35].
- *ABTS assay.* The 2,2′- azino-bis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) scavenging activity of the extracts was evaluated according to the original method of Re *et al.* [37] with slight modifications from our previously study [35]. The $ABTS^{\bullet+}$ was generated by reacting ABTS (7 mM) in H_2O and $K_2S_2O_8$ (2.45 mM) at room temperature (25 °C) in the dark for 14-16 h. The absorbance at 734 nm was measured against a methanol blank using a spectrophotometer (Spectroquant Pharo 300, Merck, USA) [35].
- *H_2O_2 assay.* Hydrogen peroxide scavenging activity (HPSA) of the extracts was measured using 0.2 M phosphate buffer (PB, pH = 7.4) and H_2O_2 (2 mM dissolved in PB). A mixture of 0.1 mL plant extract, 0.6 mL of H_2O_2 and 3.3 mL of PB was incubated in dark for 10 min. The absorbance was measured at 230 nm using a spectrophotometer Spectroquant Pharo 300 (Merck, USA) [35].
- *FRAP assay.* The ferric reducing antioxidant power (FRAP) of the extracts was determined using the method of Benzie [19], with modifications as described by Docheva *et al.* [38]. This method is based on the reduction of Fe^{3+} to Fe^{2+}. Absorbance was measured at 593 nm using the same spectrophotometer.
- *CUPRAC assay.* The cupric ion reducing antioxidant capacity (CUPRAC) was assessed according to Apak *et al.* [39], with slight modifications [35]. The reaction mixture consists of 1 mL of $CuCl_2$ solution (10 mM), 1 mL of neocuproine ethanolic solution (7.5 mM) and 1 mL of CH_3COONH_4 buffer solution (1 M, pH=7). Then 0.1 mL of extract and 1 mL of deionized H_2O were added to a final volume of 4.1 mL. The absorbance was measured at 450 nm after 30-min incubation in dark using the same spectrophotometer [35].

For all antioxidant assays Trolox was used as a standard and results were expressed as millimoles of Trolox equivalents per gram of extract (mM TE/g).

Statistical analysis

All experimental measurements were carried out in triplicate. Data represent mean ± standard deviation (SD) of three independent experiments. Statistical analysis was conducted using Microsoft Excel 2020. The percentage removal of nitrate ($N-NO_3$, NO_3), ammonium ($N-NH_4$, NH_4) and nitrite ($N-NO_2$, NO_2) from water samples was calculated in agreement with the formula given in [40].

RESULTS AND DISCUSSION

In this study *C. glomerata* showed potential for water purification, as a reduction of $N-NO_3$ from 4.54 mg/L to 3.3 mg/L, and NO_3 from 20.1 mg/L to 14.6 mg/L was observed in samples from sampling point 2. The percent removal was 27.31% for $N-NO_3$, and 27.36% for NO_3. The results of water samples from sampling points 3, 4, and 5 showed reduction of $N-NO_2$ and removal of 16.22%, 66.67%, and 73.81%, respectively.

A reduction in NO_2 was observed in samples from sampling points 4 and 5 of 67.44% and 73.19%, respectively, and results are presented in Figure 2.

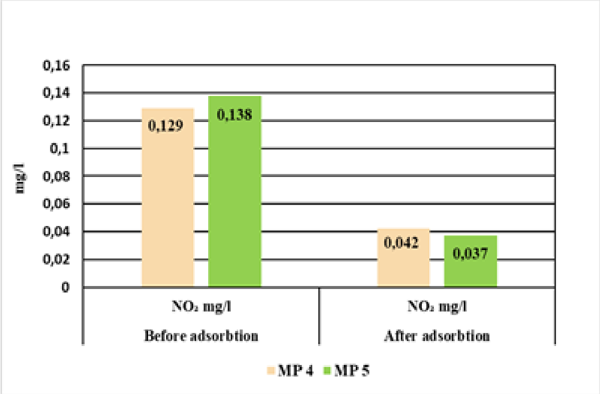

Figure 2. NO_2 before and after adsorption

Only in samples from sampling point 4 a decrease of $N-NH_4$ and NH_4 was observed and the percent removal was 58.23% and 58.03%, respectively. Basic physicochemical parameters were measured before and after adsorption in all samples from the 5 sampling points. The results showed pH values varying from 8.09 to 8.54 before adsorption and from 7.9 to 8.9 after adsorption. This suggests that the parameter does not change drastically and remains in the alkaline zone. pH is an important parameter in adsorption processes as it creates a suitable environment for retaining certain ions on the surface of the biosorbent [41]. For example, Cu^{2+} and Pb^{2+} are adsorbed significantly better by *C. glomerata* at pH 4-5 (and below) due to the increased availability of negatively charged functional groups [42]. For dissolved oxygen (O_2 mg/L), the initial concentration was between 8.44 mg/L to 8.63 mg/L, and after adsorption it was 8.02 – 8.26 mg/L. A slight decrease in dissolved oxygen values may indicate possible metabolic activity in the processes of oxidation, reduction and active ion transport, which may further assist the purification process [43]. The temperature conditions remained almost unchanged: before adsorption - an average of 22.7 °C, and after adsorption 22.4 °C. This might suggest a stable process without need for parameter adjustment. Temperatures in the order of 25 °C are used as a standard for optimal conditions that increase the adsorption capacity of algae [44].

Total phenolic and total flavonoid content

Numerous factors determine the chemical composition of algal biomass and the level of biologically active compounds, including taxa, habitat, climate conditions, environmental stressors, biomass collection time, and techniques. In general, algae are a well-known natural raw material that contains phenolic compounds. One of the most significant and most discussed features of phenolic and flavonoid compounds is their antioxidant activity. Its primary function is to bind, stabilize and inactivate free radicals [45]. The total phenolic content (TPC) of various *C. glomerata* extracts is presented in Table 1.

Table 1. Total phenolic and total flavonoid content of *C. glomerata* extracts

Extract Solvent	Total phenolic content, (mg GAE/g) *	Total flavonoid content, (mg RE/g) **
60% Methanol	0.9 ± 0.04	0.6 ± 0.03
40% Ethanol	2.8 ± 0.07	0.5 ± 0.02
Distilled water	1.2 ± 0.05	2.2 ± 0.03

* GAE: Gallic acid equivalent ** RE: Rutin equivalent

The highest TPC content was measured in the extract obtained with 40% ethanol (2.8±0.07 mg GAE/g). The content of phenolic acids detected in the aqueous extracts was approximately two times lower (1.2±0.05 mg GAE/g), while in the 60% methanolic extracts three times lower (0.9±0.04 mg GAE/g) compared to the 40% ethanolic extract. The relatively low TPC in *C. glomerata* may be attributed to the presence of only three phenolic acids – gallic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid. In previous research it was found that the variation in phenolic acids content among different algal biomasses is relatively low [44]. Our results are consistent with those reported by

Nutautaitė *et al.* [45] who investigated the TPC in *C. glomerata* using acidified aqueous-methanol extraction and reported values ranging from 0.22 mg GAE/g DM to 1.32 mg GAE/g DM. In a study conducted by Ruiz-Medina *et al.* [1] *C. glomerata* samples collected from the Canary Islands and extracted with 80% methanol yielded a significantly higher TPC: 5.90±0.83 mg GAE/g DM. These values are notably higher than those observed in our study, in which samples collected from the Aytoska River were used. The highest flavonoid content in our study was obtained using distilled water (2.2±0.03 mg RE/g). No significant differences were found between the extracts obtained with 60% methanol (0.6±0.03 mg RE/g) and 40% ethanol (0.5±0.02 mg RE/g). The results are summarized in Table 1. Ruiz-Medina *et al.* [1] reported a TFC of 3.04±0.24 mg CAE/g DM in *C. glomerata* extracts from the Canary Islands, obtained by extraction with 80% methanol. This value is similar to that found in the aqueous extracts of *C. glomerata* collected from the Aytoska River.

J. Fabrowska *et al.* suggested that low or decreasing levels of phenolic acids or flavonoids may be due to aging algal populations [46].

Antioxidant activity

Algae are a natural material with a diverse and complicated chemical composition. The methods for assessing antioxidant activity have proven that it is not only due to the existence of phenolic compounds, but also to the presence of additional antioxidants [45]. In this regard the antioxidant activity of *C. glomerata* was evaluated using five different methods: DPPH, ABTS, HPSA, FRAP, and CUPRAC. Higher values indicate stronger antioxidant activity of the extracts (Figure 3).

The antioxidant activity of *C. glomerata* determined in this study is in line with previous reports, although some variations are observed depending on the extraction method and drying conditions. For instance, Lithuanian samples showed relatively low DPPH radical scavenging activity (8.22–11.09%), while other authors reported much higher values, up to 65.2% for methanolic extracts from Thailand [47].

The highest antioxidant activity of *C. glomerata* was reported by the HPSA method - 38.4 mM TE/g in the aqueous extracts, 30.5 mM TE/g - in 60% methanol extracts, and 27.9 mM TE/g - in 40% ethanol extracts.

The antioxidant activity reported by the CUPRAC method was weaker than by the HPSA method, varying from 22.4 mM TE/g (40% ethanolic extracts) to 14.9 mM TE/g (60% methanolic extracts). The extracts from *C. glomerata* exhibited the lowest activity, determined by the ABTS method, with no difference observed between the extracts (from 5.5 mM TE/g in 40% ethanol extracts to 5.0 mM TE/g in 60% methanol extracts).

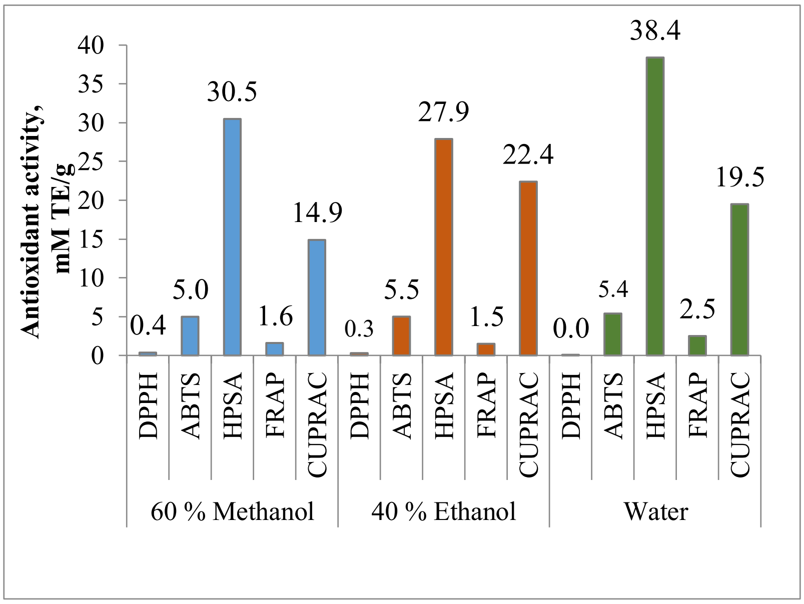

Figure 3. Antioxidant activity of 60% methanolic, 40% ethanolic and aqueous extracts

Our data are in accordance with the studies of Sáez-González *et al.* [48] who investigated *C. glomerata* extracts obtained with different solvents and found that the antioxidant activity of the extracts, determined by ABTS, varies on average about 30 mg T/g for aqueous and ethanolic extracts (equivalent to 5.2 mM TE/g). Our studies indicate a higher activity of the extracts, determined by the FRAP method, and a lower activity by the DPPH method compared to the studies of Sáez-González *et al.* [48].

CONCLUSION

This study is among the first ones to simultaneously evaluate the bioremediation capacity and antioxidant profile of *C. glomerata*, highlighting its dual functionality. While previous studies have focused primarily on its ecological role or on its bioactive compounds, the present work demonstrates its integrated potential both as a sustainable biosorbent for water quality improvement and as a valuable natural source of phenolic and flavonoid antioxidants. Among the tested samples, the aqueous extracts showed the highest activity measured by five different methods (HPSA, CUPRAC, ABTS, FRAP, and DPPH), which can be attributed to their higher content of water-soluble flavonoids. This combined perspective provides a new framework for the future use of *C. glomerata* in circular bioeconomy strategies, bridging environmental and industrial applications.

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Evaluation of antioxidant activity of *Stuckenia pectinata* L.

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The aim of this study was to determine the antioxidant activity of *Stuckenia pectinata* L. Dried leaves and stems of *Stuckenia pectinata* L. were extracted with 60% methanol, 40% ethanol, and water. Total phenolic content, total flavonoid content, and antioxidant activity were evaluated using CUPRAC, H_2O_2, ABTS, FRAP and DPPH methods. The relative antioxidant capacity index (RACI) was applied for a more comprehensive comparison. The phenolic and flavonoid contents were found to depend on the extraction solvent. Both aqueous and 40 % ethanolic extracts had lower levels of phenolic acids and flavonoids compared to the 60 % methanolic extracts. The 40% ethanolic extracts showed the highest antioxidant activity by CUPRAC, H_2O_2, ABTS, and FRAP methods. The highest activity was recorded in CUPRAC assay, followed by ABTS assay and H_2O_2 assay. The DPPH method showed very low activity. No significant differences were observed between leaf and stem extracts. *Stuckenia pectinata* as a biosorbent for the removal of inorganic pollutants from aquatic environments significantly increased the reduction of nitrogen compounds concentration (85.71% reduction of N-NO_2 and 83.87% of NO_2) when adding leaves of the plant. This confirms the applicability of the plant to be used as a natural adsorbent.

Keywords: Aquatic ecosystem, *Stuckenia pectinata* L., total phenolic content, total flavonoid content, antioxidant activity

INTRODUCTION

Reactive oxygen species (ROS) is a collective term used for oxygen-containing free radicals, depending on their reactivity and oxidizing ability [1]. ROS are involved in many physiological processes. As a result, there is a need to explore substances with free radical scavenging and antioxidant activity [2]. Antioxidants are employed to protect biomolecules from the damaging effects of ROS [1]. At present, there are keen interests and wide-spread research on exogenous antioxidants from natural sources, perhaps, due to the fact that they are less expensive, readily available and believed to have lesser side effects when compared to their synthetic counterparts [2]. Phenolic acids and flavonoids are polyphenolic compounds that may have beneficial health effects because of their antioxidant properties and their inhibitory role in various stages of tumor development in animal studies [3]. There are various methods for determining antioxidant activity, which are generally classified into 2 assays based on their mode of action mechanisms, namely electron transfer mechanism (ET) and hydrogen atom transfer mechanism (HAT) assays [4, 5]. In addition to their role in environmental remediation, aquatic macrophytes have also attracted interest for their potential as sources of bioactive compounds with antioxidant properties. Aquatic plants have been the subject of considerable research in phytoremediation as they have demonstrated the ability to remove a wide range of contaminants in water bodies [6]. Furthermore, the abundance of macrophyte species and the beneficial substances they possess have been widely documented and effectively applied as raw materials for biofertilizer, animal feed or therapeutic agents [7]. *Stuckenia pectinata* L., a synonym of *Potamogeton pectinatus* L., is a submersed perennial macrophyte also called sago pond grass, fennel and ribbon grass [8]. The species is cosmopolitan and is a food source for many species of waterfowl [9], as well as shelter for amphibians, reptiles, fish, and mammals [10], but can also create problems in irrigation canals and recreational areas [11,12]. *Stuckenia pectinata* L., possesses a variety of beneficial physiological characteristics - its ability to grow in oligotrophic to eutrophic waters, and its ability to improve water quality by removing nutrients and heavy metals, makes it an important tool for the purification of aquatic ecosystems [13, 14]. In a study related to the accumulation of As, Pb, Zn by different macrophyte species, *Stuckenia pectinata* L., demonstrated the easiest accumulation of these elements from the river substrate, whereby the authors proved that the species can help to restore areas exposed to metal and metalloid pollution [15]. Macrophyte possesses natural antioxidant properties, and is suitable for use as an additive in the food and pharmaceutical industries.

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Furthermore, phytochemical screening of this species indicates the presence of important medicinal and active substances such as flavonoids, saponins, alkaloids, terpenes and glycosides in *Stuckenia pectinata* L. which can be used as natural preservative in the processing of food raw materials [16].

The aim of this study was to determine the total phenolic content, total flavonoid content and antioxidant activity of *Stuckenia pectinata* L. collected from Burgas Lake, Bulgaria. Also, a preliminary study of macrophyte adsorption was conducted to reduce the concentration of various pollutants in water samples from the lake. The species *Stuckenia pectinata* L. was selected due to its high tolerance to pollutants and adverse environmental conditions, as well as the content of potentially useful bioactive compounds of high value.

MATERIALS AND METHODS

Sampling of Stuckenia pectinata L.

Samples of *Stuckenia pectinata* L. were collected in May-October 2024 from three points in Burgas Lake (1. Burgas Lake - east N 42.483257 E 27.438163; 2. Burgas Lake - west N 42.493123 E 27.344237; 3. Burgas Lake - center N 42.51058 E 27.367608). The collected samples were pre-rinsed several times with tap water to remove any dirt, sand particles and epiphyton. The samples were left on filter paper and dried at 25°C in the dark for 15 days [16]. The dried plant material was divided into parts, leaves and stems, and then cut into different fractions in the range of 1-5 mm for the adsorption experiments. The water samples from Burgas Lake were pre-filtered through 0.45 µm syringe filters to eliminate solid particles and ensure sample homogeneity [17]. The adsorption experiment was carried out in 250 ml Erlenmeyer flasks, the adsorbent (leaves and stems of *Stuckenia pectinata*) was prepared in an amount of 0.25 mg per 125 ml of water sample and was added to water-permeable bags measuring 4.5 × 6 cm and about 20-40 µm thick [18, 19], which were immersed in the water samples. The adsorption process was carried out at an optimal contact time of 120 min, based on previous studies showing that during this period the adsorption reached significant values without any signs of saturation of the adsorbent [20]. The samples were analyzed before and after adsorption for selected physicochemical parameters. For the experiments on the antioxidant activity of macrophytes, the plant material was ground in a grinder to obtain a uniform powder, which was passed through a 2 mm sieve [21, 22]. After that, the powdered dried leaves and stems of *Stuckenia pectinata* L. were extracted using 60% methanol, 40% ethanol, and distilled water under the following extraction conditions: 0.2 g of *Stuckenia pectinata* L. powder was mixed with 10 mL of solvent. The extraction was carried out for 30 min at room temperature on an orbital mechanical shaker at 60 revolutions per minute (rpm). The extracts (60 % methanolic, 40 % ethanolic and aqueous) were filtered by a 0.45 µm syringe filter and were used for further analysis – determination of total phenolic content, total flavonoid content and antioxidant activity by DPPH-assay, ABTS-assay, H_2O_2-assay, FRAP-assay and CUPRAC-assay.

Reagents and instruments

All chemicals were of analytical grade quality and were purchased from Honeywell and Sigma Aldrich (USA). The main physicochemical parameters (pH, dissolved oxygen, temperature, salinity, TDS (total dissolved solids), conductivity), were measured with a WTW Multi 3630 IDS portable multimeter, the indicators N-NO_2 - nitrite nitrogen, NO_2 - nitrite, were measured with ready-to-use Hach® LCK cuvette tests with DR 3900 spectrophotometer (Hach). Assays for total phenolic content, total flavonoid content and antioxidant activity were performed with spectrophotometer Spectroquant Pharo 300, UV/Vis (Merck, USA).

Determination of total phenolic content (TPC) and total flavonoid content (TFC)

The TPC of the extracts was assessed using the Folin-Ciocalteu method (FC method). Absorbance was measured at 765 nm. The concentration of the phenolic compounds in the extracts was calculated using gallic acid as standard with linear regression of gallic acid calibration curve: $y=0.0349 +0.10095x$, with $r^2=0.9892$. The results were expressed as mg gallic acid equivalents per g extract (mg GAE/g) TFC was assessed using the method described by Kirkova *et al.* [23]. Absorbance was measured at 440 nm against the blank – methanol. The concentration of the flavonoids in the extracts was calculated using rutin as a standard with linear regression of calibration curve: $y=0.0722+0.02564x$ and $r^2=0.9993$. The results were expressed as mg rutin equivalents per g extract (mg RE/g) [23].

DPPH assay

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging capabilities of the extracts were evaluated using the free radical method described by Kirkova *et al*. [23]. The absorbance was measured at 515 nm against methanol as a blank [23].

ABTS assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) scavenging activities of the extracts were evaluated according to the original method of Re *et al.*, 1999 with slight modifications [25]. The absorbance at 734 nm was measured for each sample relative to methanol [25].

H_2O_2 assay

The hydrogen peroxide scavenging activity (HPSA) of the extracts was determined using 0.2 M phosphate buffer (PB, pH = 7.4) and H_2O_2 (2 mM dissolved in PB). A reaction mixture containing 0.1 mL of plant extract, 0.6 mL of H_2O_2 and 3.3 mL of PB was prepared in a test tube. After incubation in the dark for 10 min, the absorbance was measured at 230 nm for each sample [24].

FRAP assay

The ferric reducing antioxidant power (FRAP) of the extracts was measured using the method of Benzie with a slight modification reported by Docheva *et al.*, 2020 [26, 27]. Absorbance was measured at 593 nm against a blank sample which consisted of 0.05 ml of the respective extraction solvent and 0.15 ml of distilled water mixed with 1.5 ml of FRAP reagent.

CUPRAC assay

The cupric ions (Cu^{2+}) reducing power of the extracts was determined using method described by Apak *et al.*, 2006 [28] with slight modifications. The absorbance was measured at 450 nm after incubation in the dark for 30 min [23, 27].

The antioxidant activity was determined using Trolox as a standard, and the results were expressed as mM TE/g. The linear regression equations for the Trolox standard curve were as follows:

CUPRAC $y=0.0349+0.10095x$, with $r^2=0.9892$,
H_2O_2 $y=0.0349+0.10095x$, with $r^2=0.9892$,
ABTS $y=0.0349+0.10095x$, with $r^2=0.9892$,
FRAP $y=0.0349+0.10095x$, with $r^2=0.9892$,
DPPH $y=0.0349 +0.10095x$, with $r^2=0.9892$.

Relative antioxidant capacity index (RACI)

RACI was calculated by assigning equal weights to all applied assays, according to Petrovic *et al.*, 2016 [28]:

$$RACI=(sum-ave)/stdv, \qquad (1)$$

where: *sum* - sum of all applied assays (CUPRAC, FRAP, H_2O_2, ABTS); *ave* - average of all applied assays (CUPRAC, FRAP, H_2O_2, ABTS); *stdv* – standard deviation of all applied assays (CUPRAC, FRAP, H_2O_2, ABTS).

Statistical analysis and quantitative calculations

All observations were taken in triplicate and the data were expressed in mean ± standard deviation. One-way ANOVA and Duncan`s test were employed to identify differences in the means of each group. Statistics were analyzed using SPSS Statistics (version 25, IBM Corp.) and significance was declared at $p<0.05$. Correlation was performed using MS Excel correlation data analysis. Calculation of percent removal of nitrate ($N\text{-}NO_3$, NO_3), ammonium ($N\text{-}NH_4$, NH_4) and nitrite ($N\text{-}NO_2$, NO_2) from water samples was performed in agreement with formula 2 according to [29].

$$\%\ Removal = \frac{(C_i - C_e)}{C_i} \times 100 \qquad (2)$$

where C_i and C_e are the initial and final concentrations of the contaminants in the sample.

RESULTS AND DISCUSSION

- *Total phenolic and total flavonoid content.* Plants produce diverse phytochemicals such as lignans, tannins, phenolic acids, stilbenes, and flavonoids at different concentrations in leaves, stem, flowers, fruits and roots [30, 31]. The yield of extracted phenolic acids and flavonoids depends on the solubility of the compounds in the extraction solvents [30]. High solubility of phenolic acids and flavonoids in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents [32]. In this study 60 % methanolic, 40 % ethanolic and aqueous extracts of *Stuckenia pectinata* L. leaves and stems were prepared to examine the TPC, TFC and antioxidant activity (Table 1). The highest TPC was measured in 60 % methanolic extracts of *Stuckenia pectinata* L. (18.1±0.6 mg GAE/g – leaves extract and 12.4±0.4 mg GAE/g – stems extracts), followed by 40 % ethanolic extracts – 13.9±0.4 mg GAE/g – leaves extract and 13.0±0.4 mg GAE/g – stems extracts. The lowest TPC was detected in aqueous extracts of leaves and stems - 9.7±0.3 mg GAE/g and 9.9±0.3 mg GAE/g, respectively – Table 1.

To date, no studies have been found in the scientific literature regarding the content of phenolic acids, flavonoids, and antioxidant activity of extracts from *Stuckenia pectinata* L. (also known as *Potamogeton pectinatus* L.).

Table 1. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of *Stuckenia pectinata* L. extracts

Stuckenia pectinata L.	TPC*, mg GAE/g	TFC*, mg RE/g	Antioxidant activity**, mM TE/g				
			CUPRAC	FRAP	H_2O_2	ABTS	DPPH
60% Methanol							
Leaves	18.1±0.6	9.1±0.2	403.4±28.1^{d}	95.0±3.8^{ab}	135.0±9.9^{cd}	118.9±8.7^{b}	2.3±0.03^{a}
Stems	12.4±0.4	4.3±0.1	405.1±28.1 ^{d}	93.0±3.8^{a}	88.4±6.5^{a}	115.1±8.7^{b}	2.6±0.03^{a}
40% Ethanol							
Leaves	13.9±0.4	10.6±0.3	548.1±38.2^{f}	105.9±4.1^{ab}	135.6±9.9^{cd}	134.2±9.8^{cd}	2.2±0.03^{a}
Stems	13.0±0.4	5.5±0.1	546.4±38.2^{f}	90.6±3.8^{ab}	112.4±8.3^{c}	124.9±9.1^{c}	2.4±0.03^{a}
Distilled water							
Leaves	9.7±0.3	10.2±0.3	187.7±13.0^{cd}	58.3±2.4^{a}	118.9±8.35^{b}	96.1±6.9^{ab}	n.d.
Stems	9.9±0.3	7.9±0.2	254.0±15.3^{cd}	47.5±2.1 ^{a}	103.4±8.2^{b}	87.9±6.7^{a}	n.d.

*Each value is a mean ± SD (n = 3). **Values in the same column followed by a different letter (a-f) are significantly different ($p< 0.05$). n.d. - not determined.

Haroon and Daboor (2009) [33] investigated the TPC and TFC in the Egyptian macrophyte species *Potamogeton nodosus*. They found the highest TPC in the methanol extract (19.313 mg GA/g) while the lowest content was observed in the chloroform extract (2.271 mg GA/g). The TFC in the methanol extract was up to 17.885 mg RU/g. In addition, Jelena *et al.* (2014) found the highest values of TPC and TFC in ethyl acetate extract (28.45 mg GA/g and 102.09 mg RU/g respectively) and the lowest in ethanol extract [33, 34]. Some of these results were higher than those, observed in our study. This could be attributed to various factors, such as plant growth form, location where the plant was growing, time of samples collection, environmental conditions in the sampling area. Results of the present study showed that among all extracts, the aqueous methanol and aqueous ethanol extracts had the highest TPC. This may be due to the fact that phenolics are often extracted in higher amounts in polar solvents such as aqueous methanol/ethanol [35]. Amounts of TFC in all leaves extracts were higher compared to the stem extracts, regardless of the solvent used. The TFC in leaves varied between 9.1±0.2 mg RE/g (60 % methanol) and 10.6±0.3 mg RE/g (40 % ethanol), while in steams - 4.3±0.1 RE/g (60 % methanol) and 7.9±0.2 mg RE/g (water). The differences in the extract yields of the tested materials in the present analysis might be ascribed to the different availability of extractable components, resulting from the varied chemical composition of plants [35].

- *Antioxidant activity (AOA):* The antioxidant activity of *Stuckenia pectinata* L. extracts was evaluated using five methods employing different mechanisms of action: H_2O_2, ABTS, and DPPH assays employ a hydrogen atom transfer mechanism (HAT), while CUPRAC, FRAP assays utilize an electron transfer mechanism (ET). These methods were selected, because they are rapid, robust, and accurate for systematical assessing of the total antioxidant capacity of extracts from *Stuckenia pectinata* L. on a large scale [36]. The combination of these methods allows a more complete characterization of the antioxidant properties of the *Stuckenia pectinata* L. extracts due to the different mechanisms involved in radicals - ABTS$^{\bullet+}$, DPPH$^{\bullet}$ and H_2O_2, and the ability of these extracts to reduce Fe^{+3} to Fe^{+2} (FRAP method) and Cu^{+2} to Cu^{+} (CUPRAC method). A post-hoc statistical test (Duncan`s test) was applied to assess the differences between the mean values of each extract in the group and to determine their statistical similarity [37, 38]. The antioxidant activity of *Stuckenia pectinata* L. extract including 60 % methanolic, 40 % ethanolic and distilled water is presented in Table 1. It was found that the extracts exhibited a greater ability to reduce Cu^{+2} to Cu^{+} than Fe^{+3} to Fe^{+2}. The 40% ethanolic extract showed the highest antioxidant activity across both methods, followed by the 60% methanolic extract. Distilled water extracts of *Stuckenia pectinata* L. exhibited the lowest antioxidant activity (Table 1). The antioxidant activity of the extracts of *Stuckenia pectinata* L. determined by H_2O_2 assay ranged between 88.4±6.5 mM TE/g for the 60 % methanolic extract (from steam) to 135.6±9.9 mM TE/g for the 40 % ethanolic extract (from leaves). The antioxidant activity measured by the ABTS assay was similar, ranging from 87.9±6.7 mM TE/g (water extract from leaves) to 134.2±9.8 mM TE/g (40% ethanolic extract from leaves). The extracts of *Stuckenia pectinata* L. showed no significant activity against DPPH assay – Table 1.

- *Relative antioxidant capacity index (RACI) and phenolic antioxidant coefficients (PAC):* Relative antioxidant capacity index (RACI), calculated by assigning equal weight to all applied assays, was used to achieve more comprehensive comparison between analyzed samples, as well as applied assays [27, 39]. Due to the low values of the DPPH analysis, it is not included in the RACI calculations. The highest values of RACI were ascribed to the distilled water extract of *Stuckenia pectinata* L. leaves – 6.35 and *Stuckenia pectinata* L. stems - 4.09, which is associated with the smaller standard deviation in the values for antioxidant activity obtained by the different methods (Figure 1). The 60% methanol extracts had RACI of 3.90 (leaves) and 3.42 (stems). Despite the highest antioxidant activity of the 40% ethanol extracts obtained by the different methods, the RACI values were the lowest, which can be explained by the large standard deviation obtained for the values of the individual methods.

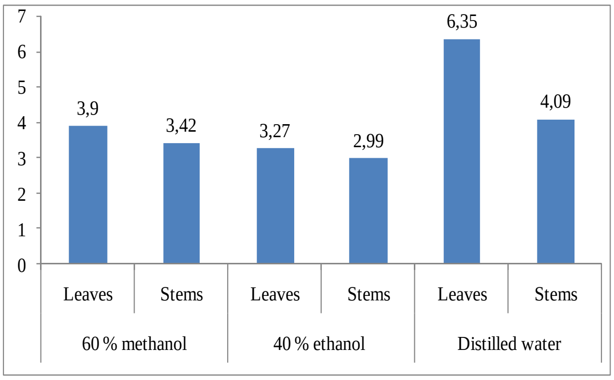

Fig. 1. RACI values

The *Stuckenia pectinata* L. extracts have higher RACI values compared to germander (1.57 and 1.33), sage (0.85), mentha (0.73), and mountain germander (0.77) [30].

- *Correlation between TPC / AOA and TFC / AOA:* The correlations between TPC / AOA and TFC / AOA of *Stuckenia pectinata* L. extracts are presented in Table 2. It is noteworthy that there is a higher correlation between TPC and AOA compared to TFC and AOA. The highest correlation was reported between TPC and FRAP assay (R^2=0.554) and TPC and ABTS assay (R^2=0.447). There were weak correlations between TPC and CUPRAC assay and TPC and H_2O_2 assay - R^2 = 0.321 and R^2 = 0.320, respectively. The best correlation was established between TFC and H_2O_2 assay - R^2 = 0.657. No correlation between TFC and CUPRAC assay, TFC and FRAP assay and TFC and ABTS assay was observed – Table 2.

Table 2. Linear regression between TPC and AOA and TFC and AOA by CUPRAC assay, FRAP assay, H_2O_2 assay and ABTS assay of extracts of *Stuckenia pectinata* L.

	Methods of antioxidant activity determination			
	CUPRAC	FRAP	H_2O_2	ABTS
TPC	0.321	0.554	0.320	0.447
TFC	0.057	0.013	0.657	0.020

- *Adsorption study with Stuckenia pectinata L.:* Before and after adsorption, the main physicochemical parameters of the samples were measured with leaves and stems of *Stuckenia pectinata* L. as follows: pH from 8.1 to 7.82 (leaves) and 7.98 (stems); Salinity, ‰ from 8.5 to 8.7 (leaves) and 8.6 (stems); Conductivity, µS/cm from 4670 to 4900 (leaves) and 4740 (stems). The leaves of *Stuckenia pectinata* L. as adsorbent offered the most significant percent removal of N-NO_2 mg/l and NO_2 mg/l 85.71% and 83.87%, respectively, and with stems 28.57% and 30.11%, respectively.

CONCLUSION

This is the very first study of *Stuckenia pectinata* L. extracts from leaves and stems, collected in Burgas Lake, Bulgaria. It may be concluded that *Stuckenia pectinata* L. is a rich source of phenolic acids (TPC) and flavonoids (TFC). The AOA of *Stuckenia pectinata* L. extracts decreased in the following order: CUPRAC assay, ABTS-assay, H_2O_2 assay, FRAP assay and DPPH assay. It is found that 40 % ethanolic extracts had the highest antioxidant activity, followed by 60 % methanolic extracts and aqueous extracts. No difference in the total phenolic content, total flavonoid content and antioxidant activity between leaf extracts and stem extracts of *Stuckenia pectinata* L. was observed. There were higher correlations between TPC and AOA compared to TFC and AOA. The aquatic macrophyte *Stuckenia pectinata* L. was used in this preliminary adsorption study. Satisfactory results were obtained in static adsorption with macrophyte leaves and the reduction of N-NO_2 mg/l and NO_2 mg/l was 85.71% and 83.87% respectively.

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Stage filtration of wastewater from rose processing

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Membrane processes are applied in different industrial sectors, including treating wastewater to restore its quality. Ultrafiltration polyacrylonitrile membranes with molecular weight cut-off of 1 kDa (UF1) and 25 kDa (UF25) were used individually in a single-stage process and sequentially in a two-stage ultrafiltration process, with the aim to purify wastewater from hydrodistillation of oil-bearing roses. Wastewater contains mainly non-volatile phenolic compounds and is considered as a biopollutant.

In a single-stage operation the permeability of the UF1 membrane was 13 l/m^2.h, and that of the UF25 was 20 l/m^2.h with a rejection of 58% on the total polyphenols, decreasing from 11.3 mg GAE/ml to 6.6 mg GAE/ml. In a two-stage process, the UF25 membrane retains the values, while the permeability of UF1 increases to 28 l/m^2.h and reaches a rejection of the total polyphenols up to 91%, with their concentration decreasing to 1.02 mg GAE/ml.

The use of membranes in a two-stage ultrafiltration mode increases the efficiency of the process. The concentrated and separated polyphenols in the retentate and permeate from the second stage were-microbiologically purified, which provided an opportunity for their direct application in food, pharmaceutical, cosmetic, etc. products and additives.

Keywords: membrane processes, oil-bearing roses, industrial wastewater, recovery of bio-resources

INTRODUCTION

The modern trends in the implementation of a circular economy promote the development of technological models that rely on the reuse of waste products and the extraction of resource components [1]. Membrane technologies have shown effectiveness in water purification and extracting of biologically active components, making them a potential solution for this approach [2]. The agro-industrial sector's rose processing on a large volume generates significant amounts of by-products, including solid and liquid waste streams [3, 4].

The treatment of wastewater from the hydrodistillation of oil-bearing rose flowers is important for protecting ecosystems and preventing water pollution, as it reduces the risk of eutrophication [5]. Purifying and restoring the quality of wastewater is primarily achieved through conventional methods [6-10]. According to the principles of sustainable development and the goals of bioeconomics, it is necessary not only to recycle wastewater but also to extract biologically active components with high potential for added value [11, 12]. Recovering these resources will reduce waste-related costs and generate additional income. Membrane processes such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) effectively purify wastewater from various sources, retaining substances that act as pollutants, including microorganisms, organic matter, poly- and oligomeric compounds. High concentrations of these substances can lead to reversible and irreversible membrane fouling negatively impacting permeability and operational longevity [13, 14]. These undesirable effects can be overcome by utilizing various membranes and processes in combination or hybrid form, revealing their potential [15-17]. In ultrafiltration, porous membranes are predominantly used, typically polymeric, with various structures and morphologies [18]. These membranes determine the molecular weight limits that can be retained by the membrane [19, 20]. The most commonly available polymer membrane brands are produced using the phase inversion method [21, 22].

When selecting a membrane, the user considers the practical assessment of its performance based on operating characteristics such as permeability and selectivity [23-25]. These values are interdependent and involve a compromise. Modifying membranes through changes in parameters like phase inversion can enhance their properties [26]. Choosing a

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membrane can be challenging because two membranes with the same molecular weight cut-off (MWCO) from different manufacturers may have different pore sizes and operating characteristics. Additionally, membranes exhibit varying capabilities and behaviors based on the qualitative and quantitative composition of the fluid [27].

This study aims to investigate the ultrafiltration treatment of wastewater after hydrodistillation of oil-bearing roses using polyacrylonitrile membranes with molecular weight cut-offs of 1 kDa (UF1) and 25 kDa (UF25). These membranes will be used separately in a single-stage process and sequentially in a two-stage ultrafiltration process.

Implementing these processes can lead to more efficient resource utilization and enhance environmental sustainability.

EXPERIMENTAL

The wastes were collected after distillation of fresh rose flowers from *Rosa damascena Mill. f. trigintipetala Dieck* (R.D.). The process was used for obtaining essential oil at semi-industrial processing line at the Institute of Roses and Aromatic Plants (IRAP) in Kazanlak, Bulgaria. The plantations were grown according to established technology in the experimental field of the Institute. The liquid waste was stored at a temperature of -20°C until its membrane filtration and was pre-filtered through standard filter paper.

PAN membranes were formed from a solution of polyacrylonitrile with MWCO of 25 kDa using a solvent – N,N-dimethylformamide (DMF) and with a MWCO of 1 kDa using as solvents dimethyl sulfoxide (DMSO) and DMF, products of Fluka, Germany. The homogeneous polymer solutions were filtered through a textile filter and after deaeration were drawn as a film onto a calendered polyester substrate attached to a glass plate. The polyester mat brand FO-2403, manufactured by Viledon Filter, Germany, has a density of 100 ± 5 g/m^2 and a thickness of 2 ± 0.1 nm. The plate was immersed in a laboratory bath with distilled water at a temperature of 25 ± 1°C for non-solvent induced phase separation (NIPS). During NIPS, PAN coagulates to form a porous membrane, which is then intensively washed with water until the solvent is completely removed. The washed membrane is then subjected to heat treatment in distilled water in the temperature range of 60 – 90°C.

The membranes were tested in a laboratory installation with a cylindrical module for horizontally installing of round flat membranes. The volume of the membrane filtration module with perpendicular pressure supply is 500 ml. The tests were conducted with a filtration volume of wastewater of 400 ml resulting in up to a 75% reduction.

The values of the permeability (J, l/m^2.h) and rejection (R, %) of the PAN membrane at different pressures were calculated by the following equations:

$$\mathrm{J} = \mathrm{V}/(S.\tau), \mathrm{l/m^2.h} \tag{1}$$

where: J - flux permeate through the membrane, l/m^2.h; V - volume of permeated flux, l; S - effective area of tested samples, m^2; τ - record time, h;

$$\mathrm{R} = \frac{C_2 - C_1}{C_2}.100,\ \% \tag{2}$$

where: R - rejection of the membrane, %; C_2 - concentration of retained matter in the feel, g/l; C_1 - concentration in the permeate, g/l.

The total phenolic content (TPC) was determined by the Folin-Ciocalteu assay [28]. The results were evaluated as gallic acid equivalent (mg GAE/ml) by linear regression equation of the calibration curve – $y = 2.5x+0.108$ and $R^2 = 0.9893$ at 765 nm. The calibration curve was constructed using various concentrations of gallic acid ranging from 0.02 to 0.10 mg/ml.

RESULTS AND DISCUSSION

The formed membranes are a composite structure consisting of an asymmetric porous PAN layer supported by a polyester backing [29]. This structure contributes to the mechanical resistance of the membrane, with its specific morphology designed to ensure the passage rate of liquid matches the driving force and selectively retains the composition of the filtered liquid based on the MWCO.

The membranes were used separately in a barofiltration process, with pressure applied above the wastewater and on the membrane, respectively. The results for the permeability of the membranes versus filtration volume are presented in Figure 1, demonstrating better functionality in terms of permeability for the UF25 membrane compared to UF1.

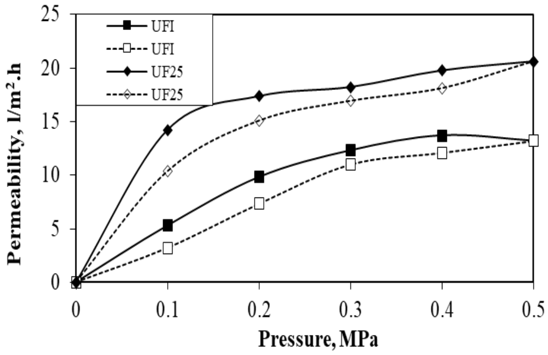

Fig. 1. Hysteresis curves of wastewater flux permeability in a single-stage filtration process

The permeability of UF25 sharply increases to 14.21 l/m^2.h at 0.1 MPa, then smoothly reaches 20.60 l/m^2.h at the maximum applied pressure of 0.5 MPa. For UF1, the permeability smoothly increases from 5.28 l/m^2.h at 0.1 MPa to 13.7 l/m^2.h at 0.4 MPa, with a slight decrease at 0.5 MPa. This decrease suggests a challenging flow, likely due to increase in the concentration of retained components in the interface with the membrane surface [30, 31].

Therefore, we can assume that 0.4 MPa is the effective limit working pressure for this membrane. The different behavior of the membranes is determined by the specifics of the structural morphology, which is a result of the thermodynamic conditions during their formation [32]. The course of the curves depends on the pores actively occupied during the filtration process. Even at low pressure, pores with a larger opening diameter are permeable, while pores with a smaller diameter gradually become active as pressure increases. The linear dependence of permeability on pressure shows a hysteresis area, which corresponds to the deformation reaction of the membrane structure during operation. The hysteresis area is visually similar for both membranes at the same mechanical load magnitude. However, the area between 0.1 and 0.3 MPa is noticeably larger for UF25 due to the greater asymmetry in the pore structure of the membrane [33].

Individually applied in a single-stage wastewater filtration process, the rejection values of UF1 and UF25 are shown in Figure 2. The values for the two membranes vary based on the applied pressure and molecular weight limit of retention, while maintaining the same qualitative and quantitative composition of the filtered material. The retention of the membranes was assessed against the determined analytical total polyphenol content of 11.3 mg GAE/ml in the wastewater.

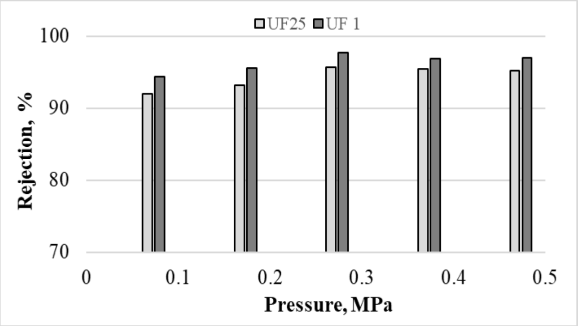

Fig. 2. Membrane rejection of TPC from the wastewater

The rejection of UF1 was consistently higher than that of UF25 at all pressures. Both membranes showed peak rejection values for polyphenolic components at 0.3 MPa. At this pressure, UF25 had a rejection rate of 95.75%, while UF1 had a rejection rate of 97.7% (Fig. 2). This translates to polyphenols passing through the UF25 membrane at 0.48 mg GAE/ml and only 0.25 mg GAE/ml through UF1. Consequently, 10.82 mg GAE/ml were eliminated from the wastewater using the UF25 membrane, and 11.05 mg GAE/ml with the UF1 membrane. The membrane rejection percentage values in the two-stage process are outlined in Table 1.

Table 1. Rejection of the two-stage ultrafiltration process at 0.3 MPa

Membrane type	Stage R, %	Final R, %
UF 25	95.75	99.02
UF 1	77.08	

The results were obtained at a system pressure of 0.3 MPa, which was determined to be optimal for both membranes in a single-stage filtration. In the two-stage filtration, the rejection of UF25 in the first stage is identical to the values obtained in the single-stage filtration (Fig. 2). However, during the second stage of filtration with UF1, a rejection value of 77.08% is obtained, which is lower compared to both the rejection value obtained with single-stage filtration using UF1 and that obtained with UF25. This is due to the lower concentration of TPC after the first stage, which was used in the calculations according to equation 2. As a result of the achieved rejection in the joint operation of the membranes in the two-stage filtration, the final rejection value increases to 99.02%, with only 0.11 mg GAE/ml of polyphenols passing through to the final permeate. The permeability of the UF1 membrane also increases, and its structural deformation decreases, as seen in the decreasing hysteresis area in Figure 3.

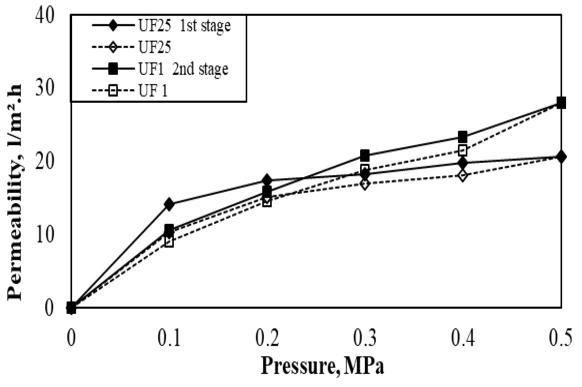

Fig. 3. Hysteresis curves of wastewater flux permeability in a two-stage filtration process

The composition of P1 is lightened in terms of the amount of TPC, which we believe is one of the main reasons for the almost twofold increase in permeability of the UF1 membrane, reaching 28 l/m^2.h at a pressure of 0.5 MPa. By conducting membrane filtration as a two-stage process with the sequential passage of wastewater flow through the UF25 and UF1 membranes, optimization of the process is achieved by utilizing the potential of UF1 nd improving the quality of the wastewater in terms of polyphenol composition.

CONCLUSIONS

The use of a two-stage membrane filtration approach, in which wastewater passes sequentially through UF25 and UF1, allows for maximum utilization of rejection of UF1 and results in the restoration of the polyphenol profile of the treated waters. The performance characteristics of the membranes were determined, and it was established that when applied sequentially in a two-stage ultrafiltration process and depending on the mode conditions, the permeability of the UF1 membrane increased from 13.2 to 28 l/m^2.h, while its structural deformation decreased.

It was found that more effective purification of industrial wastewater (up to 99.02%) is achieved in the two-stage ultrafiltration process with TPC rejection. The TPC in the purified water decreases from 11.3 mg GAE/ml to 0.11 mg GAE/ml, providing an opportunity for their valorization through the resulting retentate.

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Removal of Acid orange 7 by industrial lemon balm and yarrow solid wastes in dynamic conditions

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Melissa officinalis L. (M) and *Achillea millefolium* L. (Y) residues of industrial steam distillation were used as cheap, abundant and eco-friendly adsorbents for removal of the azo dye Acid orange 7 (AO 7) from aqueous solutions in dynamic conditions. The biomasses had 75.26±0.98% and 80.39±1.37% of dietary fibers (DF) for M and Y, respectively, and the amounts of soluble DF were 4.59±0.57% and 5.04±0.43%, respectively. The polyuronic acid content for both plant materials was statistically non-distinguishable: 14.98±0.86% for M and 15.48±1.04% for Y. The dynamic adsorption of AO 7 was investigated at five different pH values: 1.21 (0.1 N H_2SO_4), 3.63 (deionized H_2O), 5.49 (50 mM citrate buffer), 8.30 (50 mM phosphate buffer), and 12.80 (0.05 N sodium hydroxide). Preliminary washing of M and Y biomasses with 0.1 N HCl increased their removal efficiency compared with water- and acetone-washed biomasses. Within the pH range from 1.21 to 5.49 the AO 7 removal was above 93% for M and 94% for Y biomass. The present work demonstrated that residual agricultural biomasses (M and Y) are able to remove the azo dye AO 7 in dynamic conditions (column adsorption) from aqueous solutions with an efficiency of 93.2±0.3% and 94.3±0.5%, respectively.

Keywords: Acid orange 7, azo dye, lemon balm, yarrow, column adsorption, dynamic adsorption.

INTRODUCTION

The production of essential oils in Bulgaria is well developed and main products are world-known and recognized. Aromatic and medicinal species (belonging to families of *Rosaceae, Lamiaceae, Asteraceae*, etc.) had been grown industrially for centuries. Although the main essential oil plants are rose (*Rosa damascena* Mill.) and lavender (*Lavandula angustifolia* Mill.) [1, 2], a great number of aromatic species are grown besides them, such as lemon balm, chamomile, yarrow, hyssop, thyme, etc. Due to the low content of essential oil in the plants their industrial processing generates huge amounts of residual biomass. Generally, the producers cut out the biomass nearby distilleries, where it slowly degrades [2].

The essential oils of lemon balm (*M. officinalis*) and yarrow (*A. millefolium*) have been well known for their therapeutic benefits. Lemon balm (M) possesses calming and anti-inflammatory effects, while yarrow (Y) is valued for its antimicrobial and wound-healing properties [3]. During the extraction of the essential oils, significant amounts of by-products are generated, which are rich in fibrous materials. These residues may have potential applications as adsorbents for pollutants, such as synthetic dyes. While previous research has investigated utilization of agricultural by-products like tea waste, sawdust, and peanut shells for dye adsorption, the potential of essential oil industry residues from lemon balm and yarrow remains largely unexplored [4].

Dye pollution is a pervasive environmental issue with significant impacts on both ecosystems and human health. Most synthetic dyes used in industries such as textiles, food, and leather have aromatic structures and contain chromophore functional groups (as azo group (-N=N-)), which enhance their stability and give them intense coloration. Owing to their complex molecular structures, these dyes persist in water bodies for prolonged periods, making biodegradation difficult [5]. Many synthetic dyes are harmful to aquatic organisms. They disrupt ecosystems by lowering oxygen levels, inhibiting the photosynthetic activity of aquatic plants, and reducing biodiversity [6]. Azo dyes like Acid orange 7 (AO 7) are particularly hazardous due to their persistent nature and toxic effects on aquatic species [7].

The chemical structure of AO7 features an aromatic ring attached to a charged sulfonic acid group and an azo linkage, making it highly water-soluble and resistant to natural degradation. Its chemical formula is $C_{16}H_{11}N_2NaO_4S$, and it is commonly present in industrial effluents, including its sodium salt form [7]. The azo group is particularly concerning due to its potential to break down under certain environmental conditions, forming aromatic amines, which are highly carcinogenic and mutagenic [5]. For these reasons

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waste waters containing azo dyes have to be purified before entering the water cycle.

Adsorption is among the most utilized methods for pollutant removal. Numerous materials were used as adsorbents [8, 9] and wastes or by-products (resulting from various industries) showed promising potential [10-14]. Static adsorption runs in batch mode and is easier to carry out compared to dynamic adsorption. Dynamic adsorption (column mode) could be operated continuously with the restriction of regularly changing the adsorbent. Lemon balm and yarrow biomass was employed for organic pollutant removal in static conditions [15]. The present work aimed at the utilization of these industrial residues as adsorbents for AO 7 removal in dynamic conditions (column adsorption).

MATERIALS AND METHODS

Materials

The lemon balm (*Melissa officinalis*) and yarrow (*Achillea millefolium*) residues were kindly provided by the distillery in Zelenikovo (Brezovo, Bulgaria, 2022; Galen-N Ltd.). Rose (R) (after steam-water distillation) and lavender (L) by-products (after steam distillation) were obtained from Galen-N (Zelenikovo distillery, region of Plovdiv, Bulgaria, 2022 harvest). AO 7 (4-(2-hydroxy-1-naphthylazo) benzenesulfonic acid sodium salt) was purchased from Merck (Germany). Activated carbon was obtained from Himtex Ltd. (Dimitrovgrad, Bulgaria). A chromatography glass column with support and PTFE stopcock (20×400 mm, NS 29/32; Isolab, Germany) was employed for dynamic adsorption.

Methods

The residues were crushed with a garden shredder and further milled with a grain milling machine. The biomasses were washed with deionized water, acetone and 0.1 N HCl as described [16]. The AO 7 solutions (1 mg/mL) at different pH were prepared according to Wu *et al.* [17].

Dynamic adsorption was carried out in a 400 × 20 mm column, NS 29/32 (Isolab, Germany), equipped with a crucible filter at the lower end and a Teflon stopcock. 5 g of adsorbent were placed in the column and around 2 g of cotton were used at the upper end to prevent layer disruption during the addition of the liquid phase. 100 mL of the azo dye solution were gently poured, and after 30 min, 4 mL-fractions were collected in 5 mL Eppendorf tubes. Each fraction was filtered using filter paper and subsequently syringe filter (CA 0.45 μm, Isolab, Germany). The adsorption was assessed using an LLG-uniSPEC 2 UV-Vis spectrophotometer at 500 nm (LLG Labware, Germany). The concentration of AO 7 was calculated using a standard curve prepared with solutions of the azo dye of known concentrations.

The proteins' amount was assessed by the method of Kjeldahl (MultiKjel K-365 – Büchi, Switzerland). The PUC and DE of the biomasses were determined as described in [16]. The moisture content was determined with KERN DAB 100-3 analytical balance (KERN&SOHN GmbH, Germany). The total, insoluble and soluble dietary fibers (TDF, IDF and SDF, respectively) were determined using K-TDFR-100A (Megazyme, Ireland), according to AOAC method 991.43 "Total, soluble and insoluble dietary fibers in foods" (First action 1991) and AACC method 32-07.01 "Determination of soluble, insoluble and total dietary fibers in foods and food products" (Final approval 10-16-91). The ash was determined by igniting 1 g sample placed in crucible in a muffle furnace at 605 °C until constant weight.

Statistical analysis

The experimental data (three replicates) are presented as mean value ± standard deviation. For analysis one-way ANOVA test (Tukey's post hoc test; $p < 0.05$) was used with Microsoft Excel 2013 (additional XL Toolbox NG module installed).

RESULTS AND DISCUSSION

Chemical and physicochemical characteristics of M and Y

The amounts of total dietary fibers (TDF) in M and Y residues were 75.26±0.98 % and 80.39±1.37 %, respectively, while the amounts of soluble dietary fibers (SDF) were relatively low: 4.59±0.57 % (M) and 5.04±0.43 % (Y). The insoluble dietary fibers (IDF) fraction predominates (68.50±0.88 % for M and 74.87±1.19 % for Y) and it includes mostly cellulose, hemicelluloses and lignin. The protein content in both biomasses was statistically not significant: 6.69±0.24 % (M) and 6.33±0.38 % (Y). The polyuronic acid content (PUC), commonly referred to acidic polyuronic biopolymers in the plant matrix, was around 15 %, and the degree of esterification (DE) – above 85 % (Table 1). Figure 1 presents information from the mesh size analyses of the biomasses. The predominating fractions were in the 0-150 μm and 450-670 μm size range for M and Y residue, respectively.

Table 1. Chemical characteristics of M and Y biomasses

	TDF, %	IDF, %	SDF, %	DE, %	PUC, %	Ash, %	Protein, %
M	75.26 ± 0.98^{b}	68.50 ± 0.88^{b}	4.59 ± 0.57^{a}	85.60 ± 3.56^{a}	14.98 ± 0.86^{a}	8.66 ± 0.38^{a}	6.69 ± 0.24^{a}
Y	80.39 ± 1.37^{a}	74.87 ± 1.19^{a}	5.04 ± 0.43^{a}	87.20 ± 2.47^{a}	15.48 ± 1.04^{a}	5.96 ± 0.06^{b}	6.33 ± 0.38^{a}

Results are presented as the mean of three measurements; ^{a,b} Different letters in columns indicate statistically different values (One way ANOVA with post-hoc Tuckey's HSD test, $p < 0.05$).

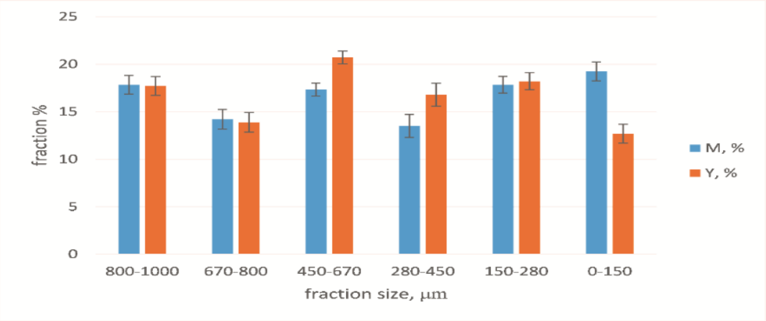

Figure 1. Particle size analysis of M and Y

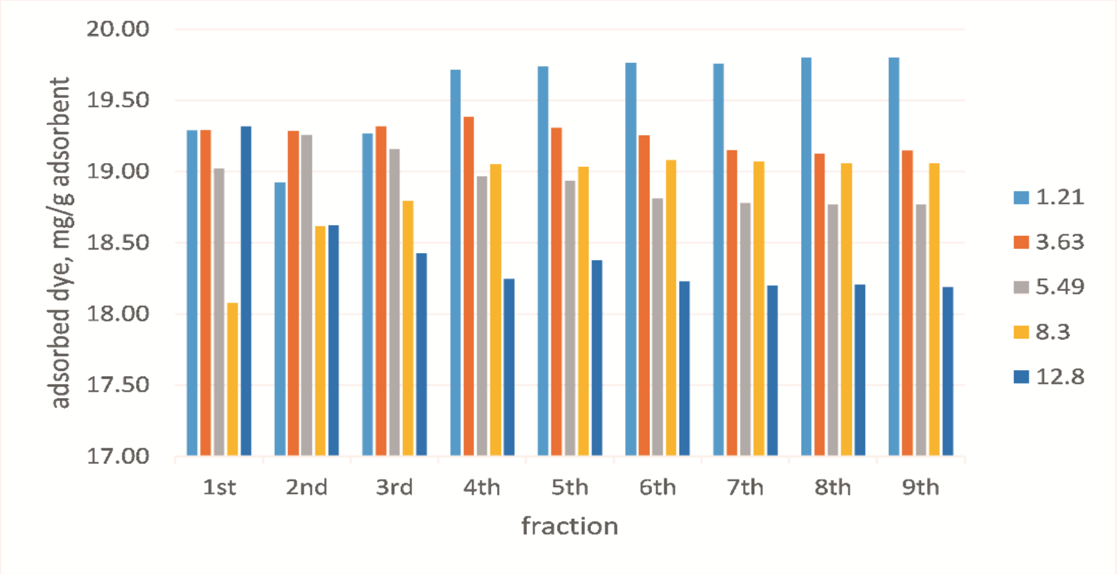

Figure 2. Adsorption of AO 7 at five different pH values on M residue washed with water

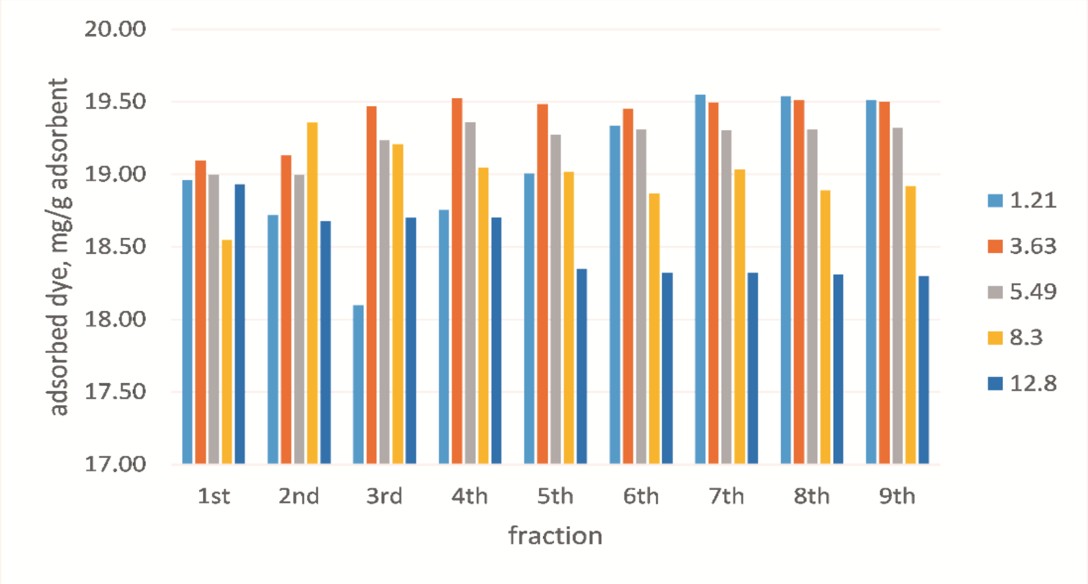

Figure 3. Adsorption of AO 7 at five different pH values on M residue washed with acetone

Contact time and pH influence on AO 7 dynamic adsorption

The residues were pretreated with deionized H_2O, acetone and 0.1 N HCl. These pretreatments were made to extract low-molecular substances from the biomasses. Washing with 0.1 N HCl led to changing the functional groups' charge: carboxylic groups will be neutralized while amino groups will get positive charge. It is expected that this positive charge will contribute significantly to the adsorption having in mind that AO 7 is bearing negative charge.

Adsorption of the dye was performed at five different pHs (from strongly acidic – pH 1.21 to basic – pH 12.80 conditions). The experimental results for utilization of M residues as adsorbent are presented in Figures 2-4. The process relies on physical and chemical interactions between biomasses and dye. The predominating functional groups in the adsorbent and the compounds adsorbed play vital role and determined the effectiveness of the process. These interactions are stronger and adsorption is fast in the beginning (first collected fractions) because the unoccupied groups on the surface of the adsorbent predominates [12]. The data presented suggested that lower pH (5 or less) favors adsorption effectiveness (Figures 3 and 4). The highest percentage of removed AO 7 was observed at lower pH (1.21) with acid-pretreated M residue: above 93% of removed dye. The water-washed M biomass showed better removal at pH 1.21. At higher pH (12.80) the effectiveness dropped and this tendency was observed for all pretreated M residues.

In the next experiments the Y residue was explored as adsorbent in dynamic conditions for AO 7 removal (Figures 5 to 7). Similar conclusions as for lemon balm residues could be drawn. The water-washed Y biomass exhibited the highest adsorption efficiency at lower pH till the 5th fraction collected while hydrochloric acid-washed Y showed slightly better performance at pH 3.63. In all cases, above pH 8.3 the effectiveness was low. Above this pH the percentage of adsorbed AO 7 was the lowest. Comparable findings and assumptions were made by Marovska *et al.* [12], Wu *et al.* [17] and Hambarliyska *et al.* [15] investigating the removal of β-naphthol orange (static conditions) employing rose and lavender (residues from industry for essential oil production), brewery spent yeasts, and lemon balm and yarrow wastes. Collection of fractions beyond 5th and 6th for both adsorbents showed that the processes of adsorption/desorption are entering equilibrium and it is difficult to achieve better removal of the AO 7.

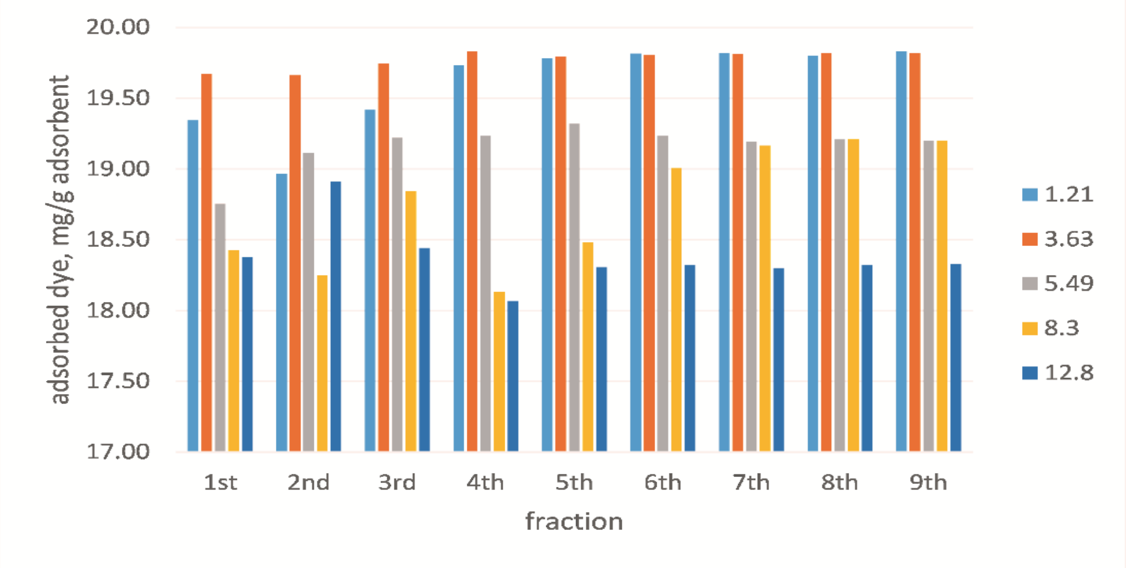

Figure 4. Adsorption of AO 7 at five different pH values on M residue washed with 0.1 N HCl

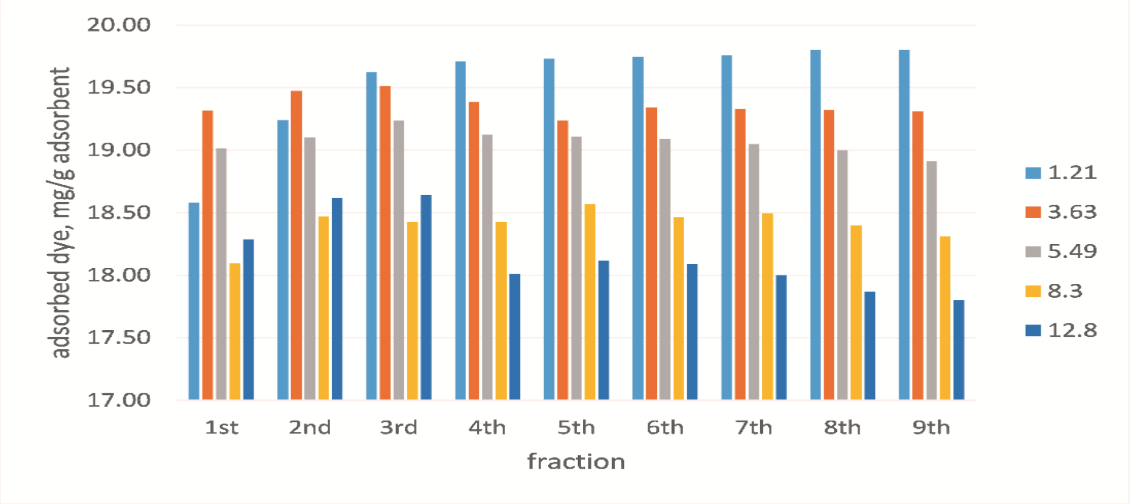

Figure 5. Adsorption of AO 7 at five different pH values on Y residue washed with water

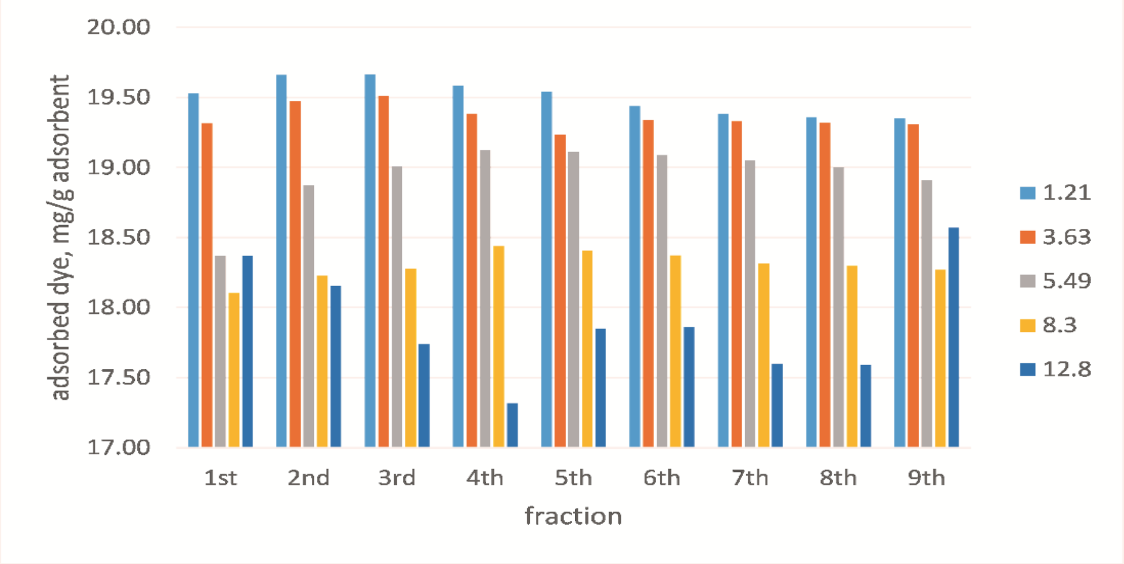

Figure 6. Adsorption of AO 7 at five different pH values on Y residue washed with acetone

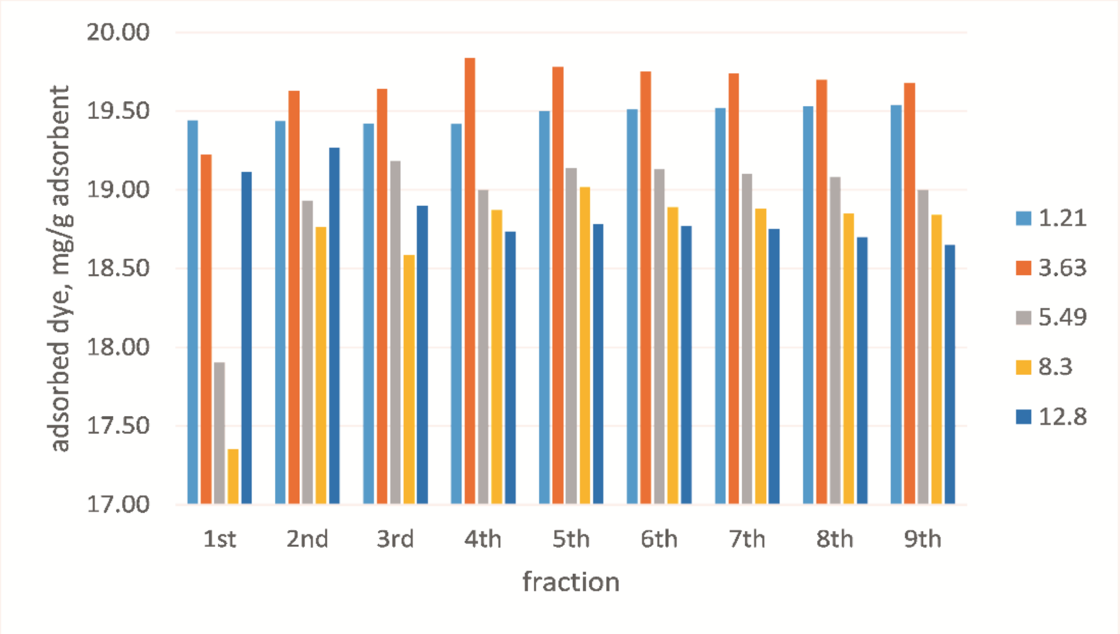

Figure 7. Adsorption of AO 7 at five different pH values on Y residue washed with 0.1 N HCl

Thereafter, a comparison of the effectiveness of AO 7 removal between the M and Y biomasses (water-washed) and rose and lavender post-distillation residues (water-washed) was performed. As a positive control was used activated carbon – a versatile and well known adsorbent. The results are presented in Figure 8.

The activated carbon, as expected, showed the best effectiveness in AO 7 decontamination. The adsorption rate and effectiveness of M and Y for AO 7 removal (from aqueous solution) was comparable with that of rose residues (at acidic pH) and slightly lower than that of lavender residues. This tendency was kept for till pH 5.49 and at higher pH M and Y residues showed better effectiveness than R and L residues. The observed results could be tentatively explained with the predominating physical character of adsorption of the dye onto the biomasses and the time and pH necessary in the begining for increasing the effectiveness. Similar observations were reported by Hambarliyska *et al.* [15] investigating the removal of β-naphthol orange with lemon balm and yarrow residues in static conditions and by Marovska *et al.* [12] investigating the adsorption of β-naphthol orange using rose and lavender biomasses in static conditions.

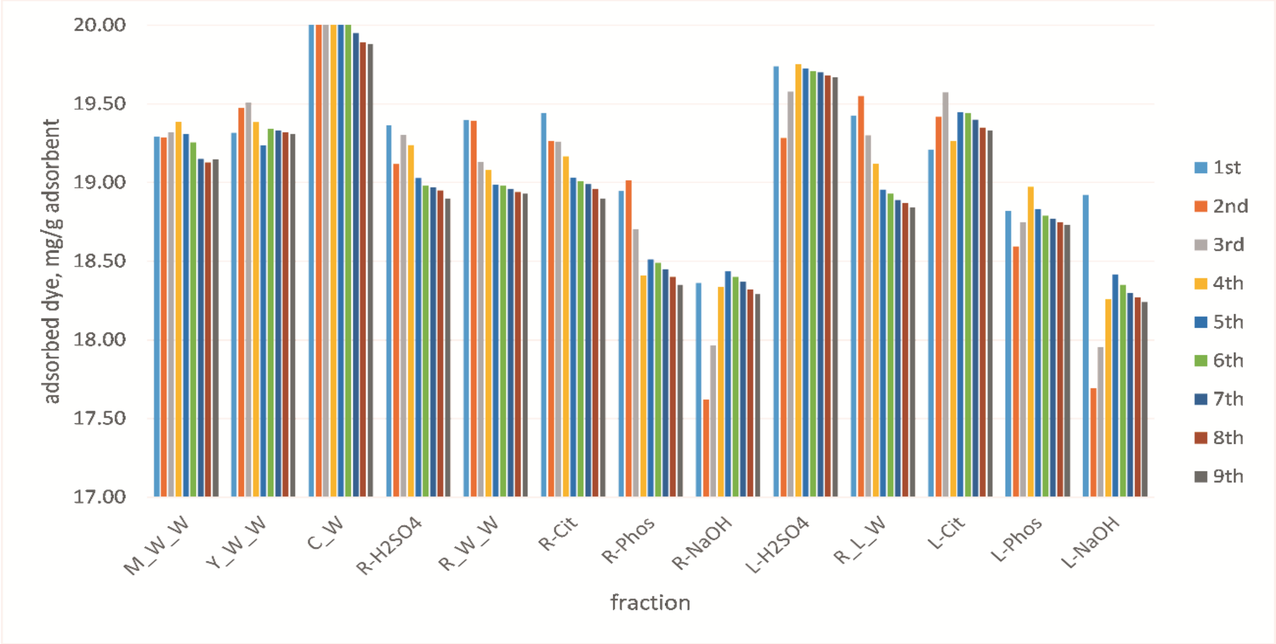

Figure 8. Comparison of AO 7 removal by M and Y residues (water-washed – M_W_W and Y_W_W) with C_W – activated carbon, rose (R) and lavender (L) residues (solutions at five different pH: 1. pH 1.21 – 0.1 M H_2SO_4; 2. pH 3.63 – aqueous dye solution; 3. pH 5.49 – 50 mM citrate buffer; 4. pH 8.30 – 50 mM phosphate buffer; 5. pH 12.80 – 0.1 N NaOH)

CONCLUSIONS

The present study investigated the removal of AO 7 (an azo dye) in dynamic conditions with two industrially generated biomasses – *M. officinalis* and *A. millefolium*. Residues pretreated with 0.1 N HCl demonstrated the highest adsorption capacity for AO 7 removal but the process was slower at the beginning of the adsorption. An adsorption/ desorption equilibrium was observed after collection of the 5th and 6th fraction. The pH is among the most important factors affecting the adsorption effectiveness and for M and Y residues higher efficiency was observed below pH 5.5. The optimal conditions for AO 7 removal could be summarized as follows: pH below 5, initial pretreatment of the plant materials with dilute acids, and contact time at least 30 min. Comparison of the M and Y residues (water-washed) with activated carbon and biomasses of rose and lavender (also generated by the essential oil industry) suggested that lemon balm and yarrow biomasses have significant potential to be used as biodegradable and renewable adsorbents for azo dyes from their aqueous solutions.

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Investigation of water absorption index, water solubility index and color changes of extrudates from corn semolina enriched with watermelon seed flour

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A full factorial 2^3 experimental design was employed to investigate the effect of watermelon seed flour content (3 % and 10 %), temperature of the matrix (160 °C and 180 °C) and moisture content (14 % and 18 %) on the water absorption index (*WAI*), water solubility index (*WSI*) and color characteristics. Extrusion was performed using a single-screw Brabender 20 DN extruder. The *WAI* ranged from 5.54 g/g to 7.26 g/g, *WSI* varied between 11.68 % and 17.03 % and the color difference (ΔE) ranged from 8.22 to 14.55. Statistical analysis indicated that the *WAI* increases with rising of moisture content, while the *WSI* decreases. Furthermore, increasing the watermelon seed flour content from 3 % to 10 % resulted in a decrease in *WAI* and an increase in *WSI.*

Keywords: extrusion, corn semolina, watermelon seed flour, color, water absorption index, water solubility index

Abbreviations: *WAI* – water absorption index; *WSI* – water solubility index; WSF – watermelon seed flour

INTRODUCTION

In recent years, there has been growing interest in the consumption of functional food products, primarily driven by increased consumer awareness of the role of diet in maintaining and enhancing health [1, 2]. The market demands the development of novel enriched food products with diverse shapes, compositions, flavors, textures, and competitive prices that meet consumers' nutritional requirements and provide valuable bioactive compounds beneficial to health [3].

Currently, there is an emerging focus on the valorization of food products and nutrient-rich waste streams. Such approaches contribute to the production of novel food products with improved qualities [4]. Watermelon seeds, often considered waste, can be utilized to develop extruded products with significant nutrient content [4, 5]. Watermelon seeds are an excellent source of fats, proteins, minerals including magnesium, potassium, phosphorus, iron, zinc, sodium, calcium, and copper, as well as vitamins (A and B groups) and various phytochemical compounds [6].

Extrusion is a modern, specialized technological process employed in the manufacture of diverse products such as grain-based snacks, meat substitutes, pasta, confectionery, baby food, and pet food, which are distinguished by superior quality and composition compared to those produced using traditional methods [1, 7]. This process enables the production of food products with controlled composition, distinctive shapes, and enhanced flavor.

The water absorption index and water solubility index serve as key indicators to assess the functional properties of extrudates and predict their behavior during storage. These indices are influenced by two primary groups of factors: those related to the raw material properties and those associated with extrusion process conditions [8]. Achieving food products with desired characteristics necessitates a thorough understanding of the extrusion process [7].

The present study aimed to investigate the water absorption index, water solubility index, and color changes of extrudates produced from corn semolina enriched with watermelon seed flour.

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a b

Fig. 1. Watermelon seeds (a) and watermelon seed flour (b)

EXPERIMENTAL

Materials

Watermelon seed flour (WSF). Watermelon seeds (*Citrullus vulgaris*) were collected from fresh ripe fruits purchased at the provincial market in Plovdiv, Bulgaria. The seeds were ground into a fine powder using an electric blender (Nutribullet Model OC22300058, 600 W, China) (Fig. 1). The resultant powder was then passed through a sieve with 1 mm mesh size to obtain flour with an average particle diameter of 0.68 mm, which was subsequently packaged for further use. The average chemical composition of the WSF was 8 % moisture, 25.4 % fat, and 3 % ash.

Corn semolina. The experiment utilized 'Familex' corn semolina, with a moisture content of 13 %, sourced from the local market in Plovdiv, Bulgaria. The WSF and corn semolina were mixed in the specified proportions, and water was added to achieve the desired moisture content (Table 1).

Extrusion

A single-screw laboratory extruder, Brabender 20 DN (Duisburg, Germany) [9], was employed for extrusion under varying process parameters (Table 1). The extrusion was conducted under fixed conditions, including nozzle diameter of 3 mm, screw compression ratio of 3:1, extruder screw speed of 200 rpm, feeding screw speed of 30 rpm, and temperatures set at 140 °C and 150 °C for the first and second extruder zones, respectively.

Statistical analysis

A full factorial design (N = 2^3), was applied during the processing. The independent variables were watermelon seed flour content (X_1), temperature of the matrix (X_2), and moisture content (X_3). The experimental design is presented in Table 1. The variation levels for each factor were selected based on literature data and preliminary studies [10, 11]. Each experimental condition was replicated three times.

Table 1. Design with natural and coded values of factors

№	Natural values			Coded values		
	Watermelon seed flour content, %	Temp. of the matrix, ºC	Moisture content, %	X_1	X_2	X_3
1	3	160	14	-1	-1	-1
2	3	180	14	-1	+1	-1
3	10	160	14	+1	-1	-1
4	3	160	18	-1	-1	+1
5	3	180	18	-1	+1	+1
6	10	160	18	+1	-1	+1
7	10	180	14	+1	+1	-1
8	10	180	18	+1	+1	+1

A linear regression model including interactions between factors, was employed to describe the relationships using coded variables:

$$y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} X_i X_j \qquad (1)$$

where b_o - free coefficient;

b_i - coefficient of linear effect;

b_{ij} - coefficient of interaction.

The critical value of Fisher's criterion (F_c) was used to evaluate the adequacy of the models. All statistical analyses were performed using the Statgraphics XVII Centurion trial version software.

WAI and WSI analysis

The water absorption index (*WAI*) and water solubility index (*WSI*) were determined following the AACC Method 56-20 [12]. The extrudate was ground, and a 0.2 g sample was mixed with 5 cm³ of distilled water. The mixture was suspended in water at 30 °C for 30 min with gentle agitation. Subsequently, it was centrifuged using a CH 90-2A (China) centrifuge at 3000 rpm for 20 min. The supernatant was decanted into a pre-weighed evaporating dish and dried at 105 °C until a constant weight was obtained. After cooling, the dried sample was weighed. Threefold repetition was used to obtain all experimental results.

The *WAI* was calculated as follows:

$$WAI = \frac{m_g}{m_s}, \text{ g/g} \qquad (2)$$

The *WSI* was defined as:

$$WSI = \frac{m_{ds}}{m_s} . 100, \% \qquad (3)$$

where m_g – weight of sediment, g;

m_s – weight mass of sample, g;

m_{ds} – weight of dry solids after evaporation of the supernatant, g.

Color analysis

The extrudates were ground using a laboratory grinder to a particle size of 200 µm, after which their color was measured using an NS800 spectrophotometer (3nh, China) based on the CIE L*a*b color system. In this system, L represents lightness (L = 0 corresponds to black and L = 100 to white), $+a$ indicates red, $-a$ green, $+b$ yellow, and $-b$ blue. The color difference (ΔE) was calculated using the following formula:

$$\Delta \mathrm{E} = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \qquad (4)$$

where L, a and b - values of the extruded samples; L_o, a_o and b_o - values of the non-extruded mixtures (Table 2).

Threefold repetition was used to obtain all experimental results.

Table 2. Color parameters of non-extruded mixtures

WSF content (%)	L_0	a_0	b_0
3	79.12	5.96	33.85
10	77.02	5.90	33.86

RESULTS AND DISCUSSION

Water absorption and solubility index are some of the important indicators of the quality and behavior of extrudates during processing and storage. Table 3 presents the mean values and standard deviations of the water absorption index (*WAI*) and water solubility index (*WSI*) as influenced by the three factors studied. The results indicate that *WAI* ranges from 5.54 g/g to 7.26 g/g. The maximum *WSI* value of 17.03 % is obtained at watermelon seed flour content 10 %, temperature of the matrix 160 ºC, moisture content 18 % and the lowest value of 11.68 % at watermelon seed flour content 3 %, temperature of the matrix 180 ºC, moisture content 18 %. As can be seen from the results, the increase in WSF content from 3 % to 10 % leads to a higher value of *WSI,* which may be due to the fact that the higher concentration of watermelon seeds enhances the solubility of the matrix in water and provides more soluble compounds or increases the surface area for water interaction. Seth *et al.* [13] examined the influence of feed composition (10 % – 40 %), moisture content (12 % - 24 %) and extrusion temperature (100 °C – 140 °C) on extrudate characteristics of yam-corn-rice based snack food and reported that water absorption index increased significantly with the increase in all examined variables. Eftekhariyazdi *et al.* [14] investigated the combined effect of the pumpkin-flour ratio (25 % – 75 %), feed moisture content (14 % – 22 %) and barrel screw speed (120 rpm – 180 rpm) on the physical attributes of extrudates and found strong positive effect of moisture content on the *WAI*. The increase in the pumpkin-flour ratio from 25 % to 75 % increases the values of *WSI*.

Table 3. Results for *WAI* and *WSI*

№	*WAI*, g/g	*WSI*, %
1	5.54 ± 0.08	13.10 ± 0.08
2	6.59 ± 0.22	12.78 ± 0.10
3	6.00 ± 0.02	16.84 ± 0.20
4	7.26 ± 0.23	11.98 ± 0.13
5	6.50 ± 0.13	11.68 ± 0.27
6	6.57 ± 0.19	17.03 ± 0.10
7	6.01 ± 0.05	15.26 ± 0.09
8	6.73 ± 0.17	15.09 ± 0.14

The Pareto chart (Fig. 2A) illustrates that, with respect to *WAI*, the factors moisture content (X_3), WSF content (X_1), as well as the interactions between factors (X_2X_3) and ($X_1X_2X_3$), are statistically significant. The greatest effect is attributed to the moisture content (X_3), followed by the interactions ($X_1X_2X_3$) and (X_2X_3), and subsequently by watermelon seed flour content (X_1). Regarding *WSI* (Fig. 2B), the results indicate that the single factors (X_1), (X_2), and (X_3), together with the interactions (X_1X_2) and (X_1X_3), are significant. The most pronounced positive effect on *WSI* is exerted by the watermelon seed flour content (X_1), followed by the negative effects of temperature of the matrix (X_2) and moisture content (X_3).

The adequate linear mathematical models shown below, comprising statistically significant coefficients, were developed at a significance level of 0.05:

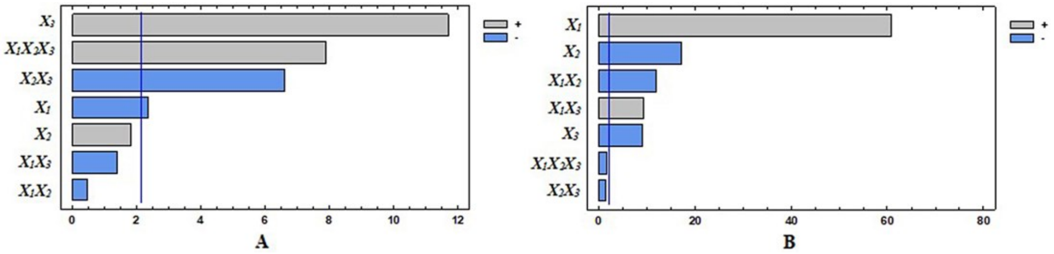

Fig. 2. Pareto charts for *WAI* (A) and *WSI* (B)

$$WAI = 6.399 - 0.073X_1 + 0.3654X_3 - 0.2063X_2X_3 + 0.2463X_1X_2X_3 \quad (5)$$

$$R^2 = 92.81\ \% \quad F = 0.24 < F_c = 3.2$$

$$WSI = 14.22 + 1.8358X_1 - 0.5167X_2 - 0.2742X_3 - 0.3625X_1X_2 + 0.28X_1X_3 \quad (6)$$

$$R^2 = 99.57\ \% \quad F = 0.39 < F_c = 3.6$$

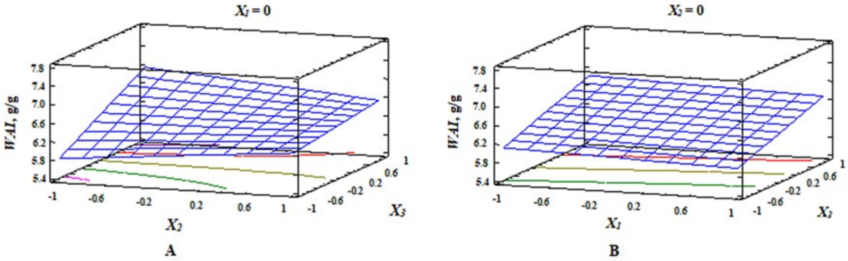

Fig. 3. Response surface of *WAI*, depending on: **A.** temperature of the matrix (X_2) and moisture content (X_3); **B.** watermelon seed flour content (X_1) and moisture content (X_3)

The adequacy test of the obtained models for *WAI* and *WSI* in coded form shows that the calculated value of the Fisher criterion is smaller than its critical value. The determination coefficient value for both parameters is above 92.81 %.

The combined effect of matrix temperature (X_2) and moisture content (X_3) is illustrated in Fig. 3A. These two parameters are the principal variables significantly affecting the *WAI*, as they are directly associated with starch gelatinization. The highest *WAI* value is attained at a lower level of temperature of the matrix and a higher level of moisture content, whereas the lowest value is observed when both factors are at lower levels (Fig. 3A). *WAI* increases 1.50 times when the moisture content rises from 14 % to 18 %, under otherwise identical conditions.

This indicates that higher moisture content enhances water absorption capacity, which may be attributed to the increased ability of extrudates to absorb water – potentially influencing the texture and overall quality of the final product. This trend is relevant for optimizing formulation and processing parameters to achieve the desired water absorption characteristics in food products. Comparable results were observed in our previous study involving the extrusion of corn semolina and cocoa bean shells [15]. It is evident that most of the variation in *WAI* is primarily governed by moisture content, rather than matrix temperature.

The influence of *WSF* content (X_1) and moisture content (X_3) on the *WAI*, at constant temperature of the matrix (X_2), is presented in Fig. 3B. An increase in watermelon seed flour content results in a slight reduction in *WAI* at both the lower and upper levels of moisture content. In contrast, an increase in moisture content (X_3) induces a more pronounced effect on *WAI* when the *WSF* content (X_1) is at its lower level.

The effect of *WSF* content (X_1) and matrix temperature (X_2) on *WSI*, at a constant moisture content (X_3), is illustrated in Fig. 4A. The data clearly indicate that the factor (X_1) exerts a dominant influence compared to factor (X_2), probably due to the presence of components in the watermelon seed flour – such as soluble proteins and low molecular weight polysaccharides – which contribute to the increase in *WSI*. The highest *WSI* value is observed at a higher level of (X_1) and a lower level of (X_2). This may be attributed to enhanced starch gelatinization, dextrinization, and molecular degradation under elevated thermal conditions. Such structural modifications improve the solubility of starch and associated compounds, thereby resulting in increased *WSI* values.

The combined influence of matrix temperature (X_2) and moisture content (X_3) on *WSI* at a constant level of *WSF* content (X_1), is presented in Fig. 4B. The response surface appears nearly flat, suggesting that neither temperature of the matrix nor moisture content exerts a significant effect on *WSI* within the investigated range. This observation is corroborated by the standardized Pareto chart (Fig. 2B) and the coefficients obtained in eqn. 6.

The impact of *WSF* content (X_1) and moisture content (X_3) on the *WSI*, at a fixed temperature of the matrix (X_2), is illustrated in Fig. 4C. An increase in watermelon seed flour content results in a corresponding rise in *WSI*, likely attributable to the presence of components such as soluble proteins, simple sugars, and low-molecular-weight starch fragments, which enhance the solubility of the extrudates. Within the studied range, moisture content exerts minimal or no significant effect on *WSI*. The highest *WSI* value is recorded at the highest level of (X_1) and the lowest level of (X_3), whereas the lowest value occurs at the lowest level of (X_1) and the highest level of (X_3).

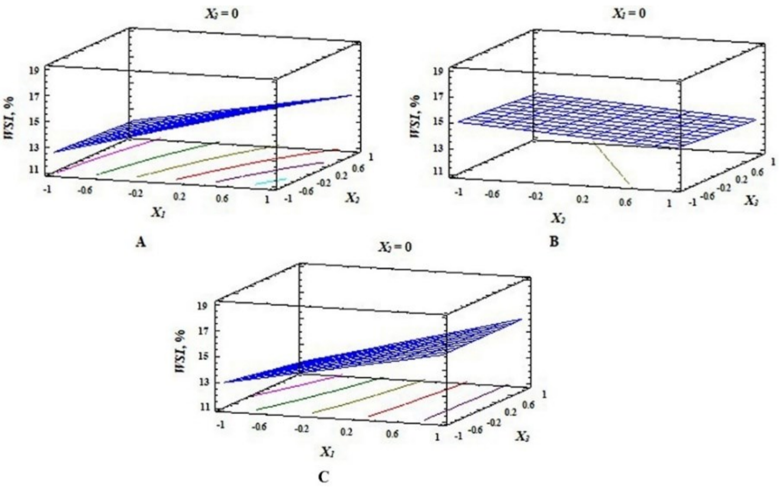

Fig. 4. Response surface of *WSI*, depending on: **A.** watermelon seed flour content (X_1) and temperature of the matrix (X_2); **B.** temperature of the matrix (X_2) and moisture content (X_3); **C.** watermelon seed flour content (X_1) and moisture content (X_3)

Table 4. Color changes of extrudates

№	*L*	*a*	*b*	Δ*E*
1	65.16 ± 0.21	6.19 16 ± 0.10	29.77 ± 0.20	14.55 ± 0.16
2	76.00 ± 0.20	5.09 ± 0.12	23.91 ± 0.18	10.46 ± 0.18
3	71.38 ± 0.18	16.91 ± 0.16	39.56 ± 0.21	9.09 ± 0.20
4	78.94 ± 0.16	3.87 ± 0.14	23.41 ± 0.24	10.65 ± 0.14
5	79.79 ± 0.21	4.55 ± 0.08	25.79 ± 0.22	8.22 ± 0.22
6	66.73 ± 0.20	10.60 ± 0.10	30.55 ± 0.20	11.78 ± 0.12
7	69.03 ± 0.16	6.41 ± 0.14	23.90 ± 0.26	12.78 ± 0.10
8	65.45 ± 0.21	8.07 6 ± 0.12	25.88 ± 0.20	14.22 ± 0.18

Lightness (*L*) values range from 65.16 to 79.79, redness (*a*) from 3.87 to 16.91, yellowness (*b*) between 23.41 and 30.55, and total color difference (*ΔE*) from 8.22 to 14.55, as shown in Table 4.

The extrudates displayed a darker appearance, supported by the significantly lower (*L*) values compared to those of the initial, non-extruded mixtures (L_0). This reduction in lightness indicates that the extrusion process affects the surface color of the material, most likely due to thermal, non-enzymatic browning reactions, such as the Maillard reaction [16]. The lowest (*L*) value was observed when all processing parameters were at their minimum levels, whereas the highest was recorded at 3 % watermelon seed flour content, 180 °C matrix temperature and 18 % moisture content.

The derived model for (*ΔE*) is presented in eqn. 7.

$$\Delta E = 11.4663 + 0.4996X_1 - 0.2479X_3 + 1.582X_1X_2 + 1.287X_1X_3 - 0.3638X_1X_2X_3 \qquad (7)$$

where $R^2 = 99.13\ \%$ $\quad F = 0.16 < F_c = 3.6$.

The individual effects of WSF content (X_1), matrix temperature (X_2), moisture content (X_3), as well as the interactions between factors, on the total color difference (ΔE) are presented in Fig. 5. It is evident that all investigated factors are significant, except for X_2 and the interaction between (X_2) and (X_3). The greatest positive effect among the single factors is exerted by (X_1), followed by a negative influence of (X_3).

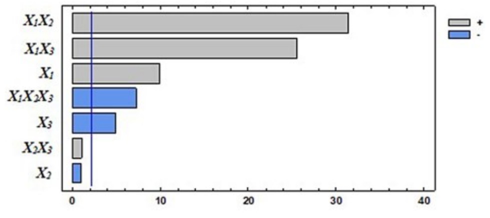

Fig. 5. Pareto chart for (Δ*E*)

The combined effect of watermelon seed flour content (X_1) and matrix temperature (X_2) on the total color difference (Δ*E*) is illustrated in Fig. 6A. It is evident that the highest (Δ*E*) values(7)are obtained at elevated levels of both factors. An increase in WSF content leads to a corresponding rise in (Δ*E*), indicating more pronounced color changes, likely due to intensified Maillard reactions or pigment transformations resulting from thermal and compositional variations.

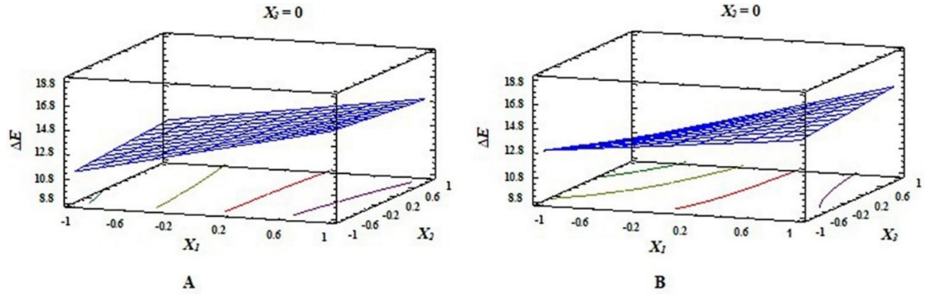

Fig. 6. Response surface of Δ*E*, depending on: **A.** watermelon seed flour content (X_1) and matrix temperature (X_2); **B.** watermelon seed flour content (X_1) and moisture content (X_3)

To better understand the visual impact of formulation and processing variables, the effect of WSF and moisture content on the total color difference was examined. Figure 6B illustrates the influence of WSF content (X_1) and moisture content (X_3) on the total color difference (Δ*E*).

The response surface reveals a clear positive correlation between both independent variables and (Δ*E*) values. As the levels of (X_1) and (X_3) increase, (Δ*E*) also steadily rises, indicating enhanced changes in product color. This combination contributes to more pronounced alterations in visual appearance, likely due to increased pigment diffusion or component interactions during processing. Such changes are critical for ensuring product consistency and consumer acceptance in food formulations.

CONCLUSION

Appropriate mathematical regression models were developed to investigate the effects of WSF content (3 % and 10 %), matrix temperature (160 °C and 180 °C), and moisture content (14 % and 18 %) on *WAI*, *WSI*, and the color changes of extrudates produced from corn semolina enriched with watermelon seed flour. The *WAI* values ranged from 5.54 g/g to 7.26 g/g, while *WSI* values varied from 11.68 % to 17.03 %. Moisture content exhibited the most pronounced positive effect on *WAI*, whereas watermelon seed flour content had the strongest positive influence on *WSI*. The total color difference (Δ*E*) values ranged from 8.22 to 14.55. Among the single factors, WSF content had the greatest positive impact on (Δ*E*), followed by a negative effect associated with moisture content.

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Phenolic profile and antioxidant activities of different vegetative parts from *Colchicum autumnale* L. and *Colchicum diampolis* Delip. et Česchm grown in Bulgaria

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The genus *Colchicum* L. is well known for its medicinal uses. The therapeutic potential of these plants was mainly associated with the presence of tropolone alkaloids, with colchicine being the best studied bioactive substance. The accompanying bioactive compounds are very poorly studied in the Bulgarian populations of the genus *Colchicum*. Thus, the purpose of the present study was to investigate the phenolic profile and antioxidant activity of different vegetative parts (flowers, leaves, corms, and seeds) of two wild species grown in Bulgaria, namely C*olchicum autumnale* L. and the endemic species C*olchicum diampolis* Delip. et Česchm. Gas chromatography-mass spectrometry (GC-MS) analyses of phenolic compounds revealed the presence of 11 phenolic acids (salicylic acid, 4-hydroxybenzoic acid, protocatechuic acid, α-resorcylic acid, γ-resorcylic acid, gentisic acid, (*E*)-p-coumaric acid, (*E*)-ferulic acid, vanillic acid, (*E*)-caffeic acid and syringic acid) with the domination of p-coumaric acid. The antioxidant potential was determined through four of the most used spectrophotometric methods – 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC). The highest antioxidant activity was detected in obtained extracts from flowers and leaves of all populations.

Keywords: *Colchicum autumnale* L., *Colchicum diampolis* Delip. et Česchm, phenolic acids, antioxidant activity

INTRODUCTION

In the past few years, there is a growing demand for phytochemicals with antioxidant activity. Antioxidants have the ability to neutralize free radicals and supply protection against damage owing to these free radicals [1]. Free radicals are generated by various metabolic processes and the uncontrolled or increased formation of free radicals in the body may lead to oxidative stress [2]. Oxidative stress has been known to contribute to various diseases such as cancer, atherosclerosis, diabetes, neurological disorders, and hypertension [3]. The most effective antioxidant compounds seem to be phenolic acids and flavonoids of many plant raw materials, especially in fruits, flowers, seeds and herbs [4].

The genus *Colchicum* (*Colchicaceae*) comprises approximately 90 geophytic species [5]. Its distribution extends from North America, Asia, North Africa, Europe, and Eurasia [6]. Generally, in Bulgarian flora, 10 species are documented [7], with five protected under the Biodiversity Act. These include both spring-flowering (*C. diampolis*, *C. doerfleri* Halascy, *C. davidovii* Stef.) and autumn-flowering species (*C. bivonae*, *C. autumnale*, *C. turcicum* Janka, *C. haynaldii* Heuff.). *Colchicum* species (*Colchicaceae* family) are one of the significant plants with medicinal properties associated with the presence of tropolone alkaloids,

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colchicinoids, mainly colchicine [8]. Ancient Greek physicians used the plant as a therapeutic agent for gout. In India and Africa, various preparations of colchicum are still used traditionally to treat gastroenterological, musculoskeletal, and cutaneous diseases [9]. Colchicine and its natural analogues are used clinically for the treatment of several disorders such as Familial Mediterranean Fever, amyloidosis, Behcet's disease, cirrhosis, psoriasis, and many other dermatological diseases. Pharmacological studies have shown that the *Colchicum* species possess antioxidant, antibacterial, acetylcholinesterase, anti-inflammatory, and antiarthritic properties [9].

Concerning *Colchicum* species from the Bulgarian flora, studies were focused mainly on the botanical characteristic and distribution of the species [10, 11]. Data about detailed phytochemical analyses and biological activity of the genus is insufficient. Recently, Dincheva *et al.* [12] described comprehensive GC-MS metabolite profiling of *C. autumnale* L. (Ribaritza), *C. bivonae* Guss. (Slivnitsa), and *C. diampolis* Delip. et Ceschm. (Iskra) and revealed the presence of 66 metabolites, including free amino, organic, phenolic, and fatty acids, sugars, and alkaloids. Phenolic acids possessed valuable biological and pharmacological properties (strong antioxidant, anti-inflammatory, anticancer, antimicrobial, antiallergic, antiviral, antithrombotic, hepatoprotective activity and many more) [13] that are of great importance for *Colchicum* species. In this context, the presented study aimed at finding and comparing the phenolic profile and antioxidant activity of different vegetative parts (flowers, leaves, corms, and seeds) of two wild grown in Bulgaria species of the genus *Colchicum,* namely C. *autumnale L.* and the endemic species *C. diampolis* Delip. et Ceschm.

MATERIALS AND METHODS

Plant material

The plant materials of *C. autumnale L.* and *C. diampolis* Delip. et Ceschm. (namely flowers, leaves, corms, and seeds), were collected from their natural habitats. Samples of *C. autumnale L.* were collected from Gela village, Smolyan municipality, Rhodope Mountains (41°39'53.9"N, 24°33'48.2"E) and from Iskrets village, municipality Svoge, Western Stara Planina (42°59'02.9"N, 23°17'27.6"E). Plants of *C. diampolis* were collected from Karnobat Municipality, Eastern Stara Planina (42°39'00"N, 26°58'05"E). The samples were collected under an official permit № 965/27.01.2023 from the Ministry of Environment and Water of Bulgaria. The samples were identified by Dr. I. Semerdjieva (Agricultural University, Plovdiv, Bulgaria/Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences). Voucher specimens of target species were deposited at the herbarium at the Agricultural University (SOA), Plovdiv, Bulgaria. The herbarium specimen numbers were as follows: *C. autumnale* – 063587 and 063588; *C. diampolis* - 062422. Approximately twenty plants were collected from the same population. The collected plant materials were thoroughly cleaned by rinsing with tap water and then with distilled water to remove soil and other contaminants. The plant parts, including flowers, leaves, seeds, and corms, were carefully separated and then lyophilized in a laboratory freeze dryer Alpha 1-2 LSC basic (Martin Christ GmbH, Osterodeam Harz, Germany). The dried plant materials were ground into a fine powder using a sample disruption/homogenizer system QIAGEN Tissue Lyser II (Retsch GmbH, Haan, Germany). Powdered materials were used for extract preparation.

Extraction methods

Dried ground plant parts were extracted with 40% ethanol in an ultrasonic bath SIEL UST 5.7-150 (Gabrovo, Bulgaria) at 35 kHz frequency and 240 W power, at 40℃ for 30 min. The residue of the plant material was removed through filter paper filtration, and the obtained water-ethanol extracts were used for analysis of total phenols, total flavonoids content and antioxidant activity.

The extraction procedure for GC-MS analysis of phenolic acids was described previously by Dincheva *et al.* [12].

Analysis of total polyphenol and total flavonoid content

The total polyphenol content was measured using Folin–Ciocalteu's assay, described by Vrancheva *et al.* [14]. Analysis of total flavonoids was determined spectrophotometrically following the method described by Vrancheva *et al.* [14].

Determination of antioxidant activity

- *DPPH scavenging assay.* The ability of extracts to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by the method described by Kivrak *et al.* [15] and Andonova *et al.* [16].
- *ABTS*$^{•+}$ *scavenging assay.* The radical scavenging activity of the extracts against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

(ABTS) was estimated according to Thaipong *et al.* [17] and Andonova *et al.* [16].

- *Ferric reducing antioxidant power (FRAP) assay.* The FRAP assay was carried out according to the procedure of Benzie and Strain [18] and Andonova *et al.* [16].
- *Cupric reducing antioxidant capacity (CUPRAC) assay.* The CUPRAC assay was carried out according to the procedure of Apak *et al.* [19] and Andonova *et al.* [16]. The antioxidant activity determined by DPPH, ABTS, FRAP, and CUPRAC assays was expressed as mmol Trolox equivalents (TE) per g dry weight (dw) by using a calibration curve built in the range of 0.05–0.5 mmol Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Fluka), dissolved in methanol (Merck).
- *GC-MS analysis of phenolic acids.* The analysis was performed using a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with a 5975C mass-selective detector (Agilent Technologies, Santa Clara, CA, USA) according to method described by Dincheva *et al.* [12]. To calculate retention indices (RI), a mixture of aliphatic hydrocarbons (C_{10}–C_{40}) from Sigma was injected under the same temperature program. Compounds were identified by comparing the RIs and spectral data with references from a custom reference library, the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/analysisinput.aspx, accessed 6 June 2024) [20] and the NIST'08 database (National Institute of Standards and Technology, Gaithersburg, MD, USA) [21]. The results were presented in mg/g dw.

Statistical analysis

Three independent extracts from each plant part were prepared and each extract was analyzed in triplicate for total phenolic and flavonoid contents, individual phenolic acids, and antioxidant activity. The presented values are means with standard deviations (± SD) calculated by Microsoft Office Excel® 2010.

RESULTS AND DISCUSSION

The total polyphenolic content varied between 6.17 ± 0.57 and 269.01 ± 3.44 mg GAE/g dw in the different plant parts, as the highest values were established in the extracts of flowers and leaves of all tested species (Table 1). Besides, flowers and leaves of both populations of *C. autumnale* contained higher amounts of total polyphenols than that of *C. diampolis.* In terms of total flavonoids, flowers and leaves were also determined to be richer than corms and seeds extracts (in the range of 0.18 ± 0.00 and 3.31 ± 0.18 mg QE/g dw). The established total flavonoid content in flowers and leaves of *C. autumnale* from Gela was about ten times lower than that in *C. autumnale* from Iskrets and *C. diampolis* population.

In accordance with our data, Suica-Bunghez *et al.* [22] found that the flower extract of *C. autumnale* contained a higher amount of total polyphenols and flavonoids than root (bulb) extract. Davoodi *et al.* [23] reported significantly lower total phenol (0.56 g gallic acid/100 g corm) and total flavonoid content (0.37 g quercetin/100 g corm) in 80 % water-methanol corms extract of *C. autumnale* than our data. It is interesting to note that Senizza *et al.* [24] found that different root extracts of *Colchicum triphyllum* contained higher total phenolic acids than flower and leaf extracts. The differences observed in total phenolic and total flavonoid content could be explained with different solvents and extraction methods used, interspecies differences, climatic and geographical differences, as well.

To obtain detailed information about the phenolic profile, GC-MS analysis was carried out. Eleven phenolic acids were identified (salicylic acid, 4-hydroxybenzoic acid, protocatechuic acid, α-resorcylic acid, γ-resorcylic acid, gentisic acid, (*E*)-p-coumaric acid, (*E*)-ferulic acid, vanillic acid, (*E*)-caffeic acid, and syringic acid) with the domination of p-coumaric acid (Table 2). Salicylic and 4-hydroxybenzoic acid were detected only in the leaf extracts of both populations of *C. autumnale* and in the leaf and seed extracts of *C. diampolis*. Protocatechuic acid and α-resorcylic acid were in the highest concentration in the leaves of all samples, as flowers contained the highest amount of γ-resorcylic acid. Ferulic acid was not detected in the corms of all samples and seeds of *C. autumnale* (Iskrets). Vanillic and caffeic acid were found only in flowers of *C. diampolis* and flowers, corms, and seeds of two *C. autumnale* populations. Flowers were the only plant parts with the presence of syringic acid.

The same phenolic acids were identified in the plant parts of other populations of *C. autumnale* (from Ribaritsa village) and *C. diampolis* (from Iskra village) grown in Bulgaria, but in different quantities [12].

The relatively high amounts of total polyphenols, total flavonoids and individual phenolic acids in the analyzed plant species is a prerequisite for their antioxidant activity. In order to investigate the antioxidant potential, four methods differing in conditions and mechanism of action were applied (DPPH, ABTS, FRAP and

CUPRAC, respectively). The highest ability to quench DPPH radical was detected in the extract of leaves of *C. autumnale* from Isktets (582.65 ± 0.47 mM TE/g dw), followed by the extract of flowers of *C. diampolis* (566.61 ± 0.17 mM TE/g dw), Table 3. Flower and leaf extract of all samples tested also showed the highest antioxidant potential according to ABTS method. Corm and seed extracts possessed the lowest antioxidant activity defined by DPPH and ABTS methods. Flower and leaf extracts also had the highest ability to reduce Fe^{3+} (FRAP) and Cu^{2+} (CUPRAC) in comparison with corm and seed extracts. The highest antioxidant activity of flowers and leaves determined by all methods could be explained with the highest quantity of total polyphenols and individual phenolic acids found in their extracts. Similarly to our finding Suica-Bunghez *et al.* [22] reported that flower extract of *C. autumnale* had a higher ability to scavenge DPPH radical than root extract (flowers – 52.81%, roots – 34.60 % inhibition of DPPH). Rocchetti *et al.* [9] also reported that leaf and flower extracts of *Colchicum szovitsii* possessed higher antioxidant activity (defined by DPPH, ABTS, FRAP and CUPRAC methods) than root extracts.

Table 1. Total polyphenol content (TPC) and total flavonoid content (TFC) of different vegetative parts from *C. autumnale* and *C. diampolis*.

	C. diampolis				*C. autumnale* (Gela)				*C. autumnale* (Iskrets)			
	*F	L	C	S	F	L	C	S	F	L	C	S
TPC, mg GAE/g dw	157.49 ± 3.96	96.73 ± 4.73	16.61 ± 0.90	24.46 ± 0.17	192.56 ±1.52	269.01 ± 3.44	6.17 ± 0.57	27.54 ± 0.45	174.99 ± 4.71	204.05 ± 5.79	19.80 ± 0.62	28.29 ± 1.25
TFC, mg QE/g dw	2.79 ± 0.02	3.31 ± 0,02	0.23 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.71 ± 0.01	0.23 ± 0.01	0.20 ± 0.01	2.60 ± 0.02	2.73 ± 0.02	0.18 ± 0.01	0.27 ± 0.01

*F – flowers; L – leaves; C – corms; S – seeds.

Table 2. GC-MS analysis of phenolic profile of different vegetative parts from *C. autumnale* and *C. diampolis*. The results are presented in mg/g dw.

		C. diampolis				*C. autumnal* (Gela)				*C. autumnale* (Iskrets)			
Phenolic acid	RI	*F	L	C	S	F	L	C	S	F	L	C	S
Salicylic acid	1505	**nd	1.07 ± 0.02	nd	0.89 ± 0.02	nd	0.93 ± 0.02	nd	nd	nd	0.82 ± 0.02	nd	nd
4-Hydroxy benzoic acid	1620	nd	0.29 ± 0.01	nd	0.38 ± 0.01	nd	1.27 ± 0.02	nd	nd	nd	1.10 ± 0.02	nd	nd
Proto catechuic acid	1820	0.44± 0.01	1.31 ± 0.03	0.46 ± 0.01	0.14 ± 0.01	0.38 ± 0.01	1.14 ± 0.03	0.64 ± 0.01	0.24 ± 0.01	0.34 ± 0.01	1.01 ± 0.02	0.93 ± 0.02	0.31 ± 0.01
α-Resorcylic acid	1762	0.34± 0.01	0.61 ± 0.01	0.26 ± 0.01	0.50 ± 0.01	0.29 ± 0.01	0.53 ± 0.01	0.13 ± 0.01	0.20 ± 0.01	0.26 ± 0.01	0.47 ± 0.01	0.43 ± 0.01	0.25 ± 0.01
γ-Resorcylic acid	1765	0.40± 0.01	0.14 ± 0.01	nd	0.12 ± 0.01	0.35 ± 0.01	0.12 ± 0.01	0.15 ± 0.01	0.09 ± 0.01	0.31 ± 0.01	0.11 ± 0.01	0.10 ± 0.00	0.12 ± 0.01
Gentisic acid	1779	0.31± 0.01	0.50 ± 0.01	0.04 ± 0.00	0.43 ± 0.01	0.27 ± 0.01	0.24 ± 0.01	0.74 ± 0.02	0.26 ± 0.01	0.24 ± 0.01	0.39 ± 0.01	0.36 ± 0.01	0.33 ± 0.01
(*E*)-p-Coumaric acid	1942	0.73± 0.02	0.95 ± 0.02	0.56 ± 0.01	0.08 ± 0.00	0.63 ± 0.01	0.48 ± 0.01	0.86 ± 0.02	0.40 ± 0.01	0.56 ± 0.01	0.73 ± 0.02	0.41 ± 0.01	0.50 ± 0.02
(*E*)-Ferulic acid	2099	0.16± 0.01	0.07 ± 0.00	nd	0.06 ± 0.00	0.14 ± 0.01	0.06 ± 0.00	nd	0.12± 0.01	0.13 ± 0.01	0.05 ± 0.00	nd	nd
Vanillic acid	1773	0.29± 0.01	nd	nd	nd	0.25 ± 0.01	nd	0.40 ± 0.01	0.15± 0.01	0.22 ± 0.01	nd	0.25 ± 0.01	0.19 ± 0.01
(*E*)-Caffeic acid	2136	0.70± 0.01	nd	nd	nd	0.91 ± 0.02	nd	0.90 ± 0.02	0.57± 0.01	0.81 ± 0.02	nd	0.28 ± 0.01	0.71 ± 0.02
Syringic acid	1838	0.27± 0.01	nd	nd	nd	0.24 ± 0.01	nd	nd	nd	0.21 ± 0.01	nd	nd	nd

*F – flowers; L – leaves; C – corms; S – seeds; ** nd - not detected

Table 3. Antioxidant activity of different vegetative parts from *C. autumnale* and *C. diampolis*.

	Colchicum diampolis				*Colchicum autumnale* (Gela)				*Colchicum autumnale* (Iskrets)			
	*F	L	C	S	F	L	C	S	F	L	C	S
DPPH, mM TE/g dw	566.61 ± 0.17	45.69 ± 0.91	2.33 ± 0.01	6.72 ± 1.12	144.57 ± 2.97	110.44 ± 4.98	0.87± 0.21	18.31± 0.88	53.34 ± 0.04	582.65 ± 0.47	6.58 ± 0.17	16.96 ± 0.54
ABTS, mM TE/g dw	192.30± 0.67	118.50 ± 1.94	25.23 ± 0.21	31.89± 1.89	236.89± 0.84	243.56 ± 5.05	10.77± 0.13	32.58 ± 0.88	178.43 ± 3.68	235.89 ± 1.56	24.05± 0.14	31.34 ± 0.12
FRAP, mM TE/g dw	143.34± 1.40	92.14 ± 0.87	14.90 ± 0.11	23.00± 0.17	230.67± 1.89	219.58 ± 2.47	42.74± 1.06	25.83 ± 0.39	129.98 ± 1.33	181.99 ± 1.22	14.13 ± 0.13	24.52 ± 0.40
CUPRAC, mM TE/g dw	266.54± 6.88	189.29 ± 4.08	31.80 ± 0.54	57.69± 0.76	349.49± 10.98	446.66 ± 14.42	88.60± 3.94	58.00± 1.31	203.55 ± 9.62	247.67 ± 7.08	25.99 ± 0.24	76.50 ± 1.69

*F – flowers; L – leaves; C – corms; S – seeds.

CONCLUSION

To the best of our knowledge, this is the first report of detailed analyses on antioxidant activity of wild grown in Bulgaria populations of *C. autumnale* and *C. diampolis*. Flower and leaf extracts of all *Colchicum* populations showed the highest ability to quench DPPH and ABTS radical and the highest capability to reduce Fe^{3+} (FRAP) and Cu^{2+} (CUPRAC), as well. The highest antioxidant potential of flower and leaf samples could be explained with the highest quantity of total polyphenols and individual phenolic acids determined in their extracts. The present study showed that all plant parts of investigated populations of *Colchicum* species are a valuable source of phenolic acids, and that the observed antioxidant activity could provide the basis for their further inclusion in different systems for better health benefits.

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Biological activity of the *Rosa alba* L. absolute against gentamicin-induced nephrotoxicity

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This study evaluated the nephroprotective effect of *Rosa alba* L. absolute (*R. alba*) against gentamicin (GM)-induced nephropathy in Balb/c mice (n = 24). The biological impact of *R. alba* absolute was evaluated using electron paramagnetic resonance (EPR) spectroscopy. The *in vivo* EPR assay measured the levels of nitric oxide (NO•), reactive oxygen species (ROS) products, and pro-oxidant malondialdehyde (MDA) in tissue homogenates from the kidneys of the experimental animals. Balb/c mice were divided into three groups (n = 6): Group 1: control group, on a common diet; Group 2: GM intoxicated (administered 200 mg kg^{-1} day^{-1}, 7 days); Group 3: Combination of GM (administered 200 mg kg^{-1} day^{-1}, 7 days) + *R. alba* absolute, administered per os (PO) (80 mg kg^{-1} day^{-1} of body weight). The antioxidant protective effects of white oil-bearing rose were confirmed by the modulation of ROS, NO•, and MDA. The *R. alba* absolute influence on antioxidant enzymes (glutathione (GSH), superoxide dismutase (SOD), catalase (CAT)) was monitored. The levels of antioxidant enzymes significantly decreased in the GM group, compared to Groups 1 and 2 ($p < 0.05$). In the treated groups, the application of *R. alba* absolute improved the studied parameters ($p < 0.05$). The results show that *R. alba* extract reduces the oxidative toxic effect of GM, and exhibits a potential nephroprotective effect against GM-induced renal injury.

Keywords: *Rosa alba* absolute, oxidative stress, gentamicin nephrotoxicity.

INTRODUCTION

Gentamicin (GM) is an aminoglycoside antibiotic with a pronounced antibiotic activity against Gram-negative bacteria. Clinically, the antibiotic is used in urinary tract infections [1], but its 7-day use is accompanied by increased toxicity and impaired metabolism, which impairs renal function [2]. The accumulation of gentamicin in the body is associated with its buildup within the Golgi apparatus, as well as in the endosomal and lysosomal compartments of proximal tubular cells in the kidneys. This intracellular accumulation is typically accompanied by distinct inflammatory responses in the affected renal tissues [1, 2]. Various hypotheses suggest that cytoplasmic accumulation of GM leads to the induction of oxidative stress (OS), followed by a decrease in mitochondrial activity, inflammation and fibrosis [3]. Prolonged therapy exacerbates OS by increasing the levels of short-lived reactive oxygen species (ROS/superoxide ion ($•O_2^-$), hydrogen peroxide (H_2O_2)), nitrogen species (RNS/nitric oxide (•NO), peroxynitrite ($ONOO^-$)), and oxidized lipids [3, 4]. Redox signaling ROS and RNS are directly involved in metabolic regulation and adaptation to xenobiotic stress [5], and their intracellular imbalance is modulated by enzymatic and non-enzymatic systems (glutathione (GSH), uric acid, vitamin C), by natural ferrostatins (vitamin E), and by plant polyphenols [6-8]. GM therapy has been shown to reduce enzymatic efficiency, impair lipid peroxidation [9], and induce renal genotoxicity through increased OS. The potential of plant compounds containing polyphenols, flavonoids, rutin, quercetin, etc, leads to the mitigation of inflammatory processes in the body and modification of ROS and RNS production, especially in acute kidney injury. The white oil-bearing rose is a shrubby plant from the *Rosaceae* family, with significant antioxidant properties *in vitro*, not showing mutagenic effects

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[10]. Its aromatic products (essential oil, wastewater, or absolute) are characterized by active pharmacological applications due to phytocomponents such as: geraniol, nerol, citronellol, quercetin, isoquercetin, eugenol, methyleugenol, tannins, nonadecane, geranial acetate, squalene, kaempferol, quercetin, rutin, etc. [11]. In traditional medicine, *R. alba* L. is used to treat palpitations, headaches, colds, leprosy, stomatitis, bile, burning sensation, ophthalmology, bronchitis, rheumatism, diabetes, inflammation, microbial infection, uterine infection, stomach problems [12], etc. Nootropic effect, antimicrobial, and antidiabetic activity of *R. alba* L. have also been proven [13]. Previous research was focused on the redox-modulating capacity and antineoplastic activity of the *R. alba* L. wastewaters showed that *R. alba* L. exhibits the properties of a good heavy metal scavenger; it has a relatively stable anti-cytotoxic effect, and acts as a detoxifying agent under conditions of oxidative damage [10, 11]. Georgieva *et al.*, [11] commented that *R. alba* L. demonstrated a high total polyphenol content (7.6 mg/mL) compared to quercetin. In addition, *R. alba* L. exhibits dose-dependent antioxidant activity in terms of scavenging DPPH• radicals, superoxide ion ($•O_2^{-}$) radicals, and $ABST^{+}$ concentrations, i.e., *R. alba* L. is a detoxifying agent under OS conditions [11]. In addition to wastewaters, *R. alba* extracts also registered antimutagenic effects and high antioxidant activity. The *R. alba* L. essential oil, when tested at concentrations of 250, 500, and 1000 µg/mL, showed weak cytotoxic and genotoxic effects on *H. vulgare* plant cells [14].

In this study, we investigated for the first time whether *R. alba* absolute exhibits protective therapeutic effects on GM-induced nephropathy in mouse models. This product is likely to prevent gentamicin-induced renal disease and inhibit oxidative damage by regulating the levels of ROS products, nitric oxide (NO•), and lipid peroxidation, and by restoring antioxidant enzymes (glutathione (GSH), superoxide dismutase (SOD), catalase (CAT)) in kidney tissue homogenates and blood serum of Balb/c mice.

EXPERIMENTAL

Plant material

R. alba absolute was provided by the Institute of Rose and Essential Oil Crops - Kazanlak. Briefly: fresh rose blossom from *R. alba* L. (500 g of whole blossoms - without external moisture) is subjected to a three-fold extraction with n-hexane, by maceration under static conditions in a scheme of battery-connected extractors. The duration of the extractions is 5 min - 30 min - 15 min, respectively. This model allows for complete extraction of aromatic substances with minimal solvent consumption. The obtained extracts are combined, filtered and evaporated under vacuum at 40 °C until the hexane is completely removed to obtain a specific - yellow-orange, waxy and paraffin-like semi-solid substance. The product thus obtained is dissolved in 96 % ethanol, with continuous shaking and gentle heating. The resulting fraction is cooled at room conditions, then stored for 24 h at sub-zero temperatures (-20 °C). It is filtered cold (laboratory system with funnel, suction flask and water pump) to remove the waxes. This manipulation is performed three times, until the waxes are completely exhausted. The filtrates are combined, dehydrated with Na_2SO_4 and subjected to vacuum evaporation to remove the ethanol. The final product obtained is the so-called absolute – a yellow-orange viscous liquid, with a typical smell of rose.

The authenticity of the rose species was confirmed by Trakia University, Stara Zagora, Bulgaria, and the voucher specimens were deposited at the IBER-BAS herbarium with the following numbers: *R. alba* - SOM 178484.

Gas chromatography analysis

The chemical composition of the absolute was determined by gas chromatography. GC system (Agilent 7820A) coupled with a flame ionization detector was used. The capillary column EconoCap™ EC™-5 (30 m × 0.32 mm × 0.25 µm film of 5 % phenyl, 95 % methylpolysiloxane) was used. The oven temperature program and detector and injector temperatures were set as described in BDS 17381 - 96 "Concret of roses. Rose absolute". Hydrogen was used at a flow rate of 1.1 mL min^{-1} as carrier gas. The calculation of the relative percentages was performed by GC-FID peak area without a correction factor. The identification of constituents was performed by comparing the retention indices and matching with co-injections of authentic compounds.

Animals

Balb/c mice (n=24; 35 – 37.8 g; 9 weeks old, Neurobiology Institute, Slivnitsa, Bulgaria), used in this study, were kept in the animal care facility by the Bioethics Committee, TrU, Stara Zagora, Bulgaria. The following protocol was followed throughout the experiment: six animals in a polycarbonate cage, temperature 21 °C, relative humidity 50 %, dark/light cycle photoperiod from 7 to 19 h, unhindered access to the usual diet and fluids for the maintenance of laboratory animals, filtered water (pH = 5.5; *ad libitum*). All procedures were in accordance with the requirements of the Animal Ethics Committee, with a license (317/6000-0333/09.12.2021) following Directive 2010/63/EU on the animals' protection used for experimental and other scientific work.

Experimental procedure

The mouse model of GM-induced nephrotoxicity was developed by daily intraperitoneal injection of 200 mg $kg^{-1}day^{-1}$ for 7 consecutive days [15, 16]. GM induction causes oxidative stress disorders and generates a cascade of redox-homeostatic imbalances directly affecting the renal tubules.

R. alba absolute has shown pharmacological properties at doses ranging from 250 to 1000 µg mL^{-1} [11]. Its pharmacological effects may be different depending on the concentrations of flavonoids and polyphenolic components. Based on preliminary *in vitro* studies [11, 14] of antioxidant activity and cytoprotective effect, 80 mg kg^{-1} *R. alba* absolute was used in the present study. *R. alba* absolute was mixed in isotonic NaCl solution (0.9 %) and homogenized for 15 min before injection.

Animals were randomly divided into four groups (n = 6), according to (1) controls - basal diet (19.6 % protein, 4.03 % fat, 6.89 % fiber, 10.71 % moisture; 8.97 % ash), injected intraperitoneally with 1 mL of isotonic NaCl solution (0.9 %); (2) *R. alba* absolute administered orally (PO) only (80 mg kg^{-1} day^{-1} body weight); (3) GM only - injected IP (200 mg kg^{-1} day^{-1} body weight) to induce acute nephrotoxicity; (4) GM + *R. alba* absolute therapy - GM was injected IP (200 mg kg^{-1} body weight), and *R. alba* absolute (80 mg kg^{-1} day^{-1} body weight) was administered 2 h after GM injection (Fig. 1).

The *R. alba* administration at a dose of 80 mg kg^{-1} day^{-1} (body weight) improved the vitality of the animals, similar to the control group. The physiological state and behavior of the animals were monitored daily. Anxiety, weakness, muscle twitching, vomiting, abdominal pain, shortness of breath, blood in urine and feces, and a percentage mortality (0 %) were not recorded.

Twenty-four hours after the last dose, mice were weighed and anesthetized by IP injection (Nembutal, 50 mg kg^{-1}). Fresh kidney tissues were chilled, homogenized, and analyzed. The mice's kidneys were weighed, and the right kidney was placed in ice-cold 0.05 M PBS (pH= 7.5; 4°C), homogenized individually, and analyzed.

Lipid peroxidation and endogenous antioxidant activity

Lipid peroxidation in kidney tissue was assessed by the method of Plaser *et al.* [17], against equivalent concentrations of malondialdehyde (MDA) in µmol/mg protein (TERMO Sci., RS232C, USA).

Renal catalase (CAT) activity was assessed by the Aebi method [18], at an absorbance of 240 nm. The activity of cellular nephritic superoxide dismutase (SOD) was analyzed by Sun *et al.* [19], at 420 nm absorption. Glutathione (GSH) levels in kidney homogenates were assessed using the method of Akerboom and Sies, 1981 [20]. Reduced GSH was assessed by continuous DTNB reduction, expressed as nanomoles of GSH per ml of protein. A blank without blood/tissue homogenate was prepared similarly and absorbance was recorded at 412 nm.

Oxidative stress analysis in renal tissue

ROS production: A total of 100 µL of homogenized kidney tissue was mixed with 900 µL (50 mM) of N-*tert*-butyl-alpha-phenylnitrone (PBN) dissolved in DMSO. The mixture was centrifuged at 4000 × *g*, 10 min at 4 °C, by [21].

The nitric oxide (•NO) generation relative to the spin–adduct formed between the spintrap carboxy 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl (CPTIO.K) and •NO in kidney tissue were based on established EPR methods [22, 23]. Briefly, 50 µM CPTIO.K was dissolved in a mixture of 50 mM Tris (pH = 7.5), and DMSO (9:1) and was centrifuged at 4000× *g* for 10 min at 4 °C. Then, 100 µL kidney samples were mixed in 100 µL of CPTIO.K, and spin–adducts were recorded.

The superoxide ($•O_2^-$) concentration in kidney tissue was determined relative to the spin–adduct formed using the spin-trap CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine), based on methods [24, 25]. For this purpose, 30 µL of kidney tissue was activated in 30 µL of CMH (1:1) on an ice bath, and after 5 min incubation, it was ready for use.

Spectral EPR analyses were performed with fivefold measurement of EPR spectra, with the following characteristics: 3503–3515 G center field; 6.42–20.00 mW microwave power; 5–10 G modulation per sample, and the results were presented in arbitrary units (a.u.). Spectral processing was performed using Bruker WIN-EPR accessed 2019 and *Simfonia*, software version 1.2.

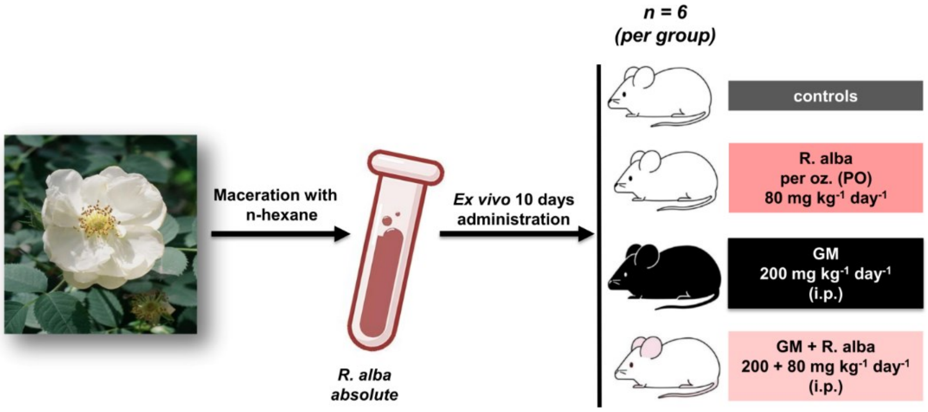

Fig. 1. Experimental protocol design of *R. alba* absolute *in vivo* acute administration.

Statistical analysis

Statistical analysis was performed with Statistica 8, StaSoft, Inc. (Madrid, Spain), and the results were expressed as means ± SE. All data were expressed as means ± SE and obtained by one-way ANOVA, and in the LSD post hoc test, $p > 0.05$ was considered statistically significant. LSD post hoc tests were used to define which groups were different from each other.

RESULTS AND DISCUSSION

In addition to the economically important essential rose oil from the Bulgarian varieties *Rosa alba* L., *Rosa damascena* Mill., *Rosa gallica* L., and *Rosa centifolia* L., other residual products (concrete, absolute, rose water) are generated during production, which have not yet been fully investigated, and interest in studying their use, especially in cellular and animal models [10-13, 14] is growing.

R. alba absolute chemical compositions

R. alba concrete is primarily used for the preparation of *R. alba* absolute. Chromatographic analysis of the present *R. alba* absolute revealed various acyclic, related monoterpene alcohols, while sesquiterpene hydrocarbons and sesquiterpene oxygenates were present in small concentrations (Table 1).

As shown in Table 1, the highest monoterpene alcohols included aryl-alcohols (phenylethyl alcohol, 16.2 %), geraniol (9.89 %), and citronellol/nerol (6.17 %). The relatively absent content of the phenylpropanoid eugenol (0.02 %) practically confirms the low toxicity and lack of carcinogenicity (Opinion of the Scientific Committee on Food on methyl eugenol, 2001); Rusanov *et al.* [26] of *R. alba* absolute. Geraniol exhibits a robust inhibitory effect on CCL_4-induced toxicity and normalizes the architecture of renal corpuscles, with their glomeruli and proximal and distal renal tubules [27].

Table 1. Characteristics of *R. alba* absolute chemical compounds in GC-FID analysis.

Rosa type	No	Compounds	Relative percent, (%)
	1	Ethanol (C_2H_6O)	0.86
R. alba absolute	2	Benzyl alcohol (C_7H_8O)	1.58
	3	Phenylethyl-alcohol (C_8H_{10})	16.2
	4	Citronellol/Nerol ($C_{10}H_{20}$)	6.17
	5	Geraniol ($C_{10}H_{18}O$)	9.89
	6	Eugenol ($C_{12}H_{20}O_2$)	0.02
	7	Methyleugenol ($C_{11}H_{14}O_2$)	0.01
	8	trans-β-Caryophyllene ($C_{15}H_{24}$)	1.31
	9	Heptadecane ($C_{17}H_{36}$)	0.19
	10	Nonadecene ($C_{19}H_{38}$)	3.00
	11	Nonadecane ($C_{19}H_{40}$)	6.69

*Results are shown as mean ± RSD (n=2)

In addition, the antioxidant and anti-inflammatory properties of citronellol/nerol reduce the oxidative damage to the kidneys caused by GM-induced nephrotoxicity [28]. GC-FID analysis also revealed a component predominance of trans-β-caryophyllene/caryophyllene. The natural bicyclic sesquiterpene has a unique structure involving a cyclobutane ring and a trans-double bond in a 9-membered ring. Scandiffio *et a*l. [29] commented on the modulatory and pharmacological effects of the sesquiterpene hydrocarbon (E)-β-caryophyllene (BCP) in organs such as liver and brain [30, 31]. In addition, BCP exerts therapeutic effects as an antioxidant agent and anti-inflammatory protector [32] in the kidneys [28, 33].

R. alba absolute ameliorate lipid peroxidation against GM-induced acute inflammation

Megalin and tubulin endocytosis promote GM deposition in proximal renal tubules. This alters lysosomal aggregation, phospholipid metabolism, and mitochondrial toxicity [3, 13]. Consequently, OS promotes the ROS/RNS accumulation and additional acute oxidative stress [30] damages. At the mitochondrial level, ROS/RNS modulation occurs through metabolic factors, proinflammatory deactivators, as well as endogenous and exogenous antioxidant deactivation of organelles [6, 7]. The biologically active components, monoterpene alcohols, sesquiterpene hydrocarbons, and sesquiterpene oxygen compounds, contribute to nephron protection due to antioxidant and anti-inflammatory mobilization [27, 28, 32, 33].

Based on these facts, we aimed to investigate the protective effect of *R. alba* absolute extract against acute kidney injury induced by GM therapy. Furthermore, we investigated the antioxidant regulatory mechanism of *R. alba* absolute by maintaining endo-exogenous deactivation, redox homeostatic imbalance and lipid peroxidation.

The study of the effects after 7-days of GM administration on renal enzyme activities and the lipid peroxidation degree is presented in Figure 1. Renal SOD (1.296 ± 0.37 *vs.* 4.78 ± 0.89 U/gHb, $p < 0.005$; Figure 2A), CAT (1.66 ± 0.17 *vs.* 3.85 ± 0.05 U/gPr, $p < 0.05$; Figure 2B), and GSH (228.40 ± 33.4 *vs.* 364.5 ± 17.39 µmol/mL, $p < 0.004$, Figure 2C) decreased statistically significantly compared to controls, and indicating acute oxidative impairment of renal functions. However, GM significantly increased MDA concentration in kidneys (5.99 ± 0.87 vs. 3.044 ± 0.287 µmol/mL, $p < 0.05$; Figure 2D) compared with controls.

GM-treated mice showed almost twofold decreased SOD, CAT enzyme activity and partial depletion of GSH activity. This fact confirms that GM stimulates the mitochondrial respiratory chain to accumulate H_2O_2, i.e. •O_2^-, $^1O_2$, HO•, OH^-, NO•, and peroxy (R/ $ONOO^-$) radicals in the kidney tissue. GM induces acute renal inflammation and oxidative damage accumulation in the renal tubules and glomeruli. These OS damages are probably a consequence of the inflammatory cells activation and physiologically impaired redox modulation, which increases lipid peroxidation [7]. The reported twofold increase in MDA concentrations after 7 days of GM accumulation is a sign of activated nephrotoxicity. The presented results are in accordance with previous studies [3, 7-9]. Conversely, combined administration of *R. alba* absolute at a dose of 80 mg kg^{-1} showed statistically significant toxicity inhibition and modulation of GM-induced renal disorders. These processes occur simultaneously with a statistically significant increase in endo-exogenous enzymatic protection.

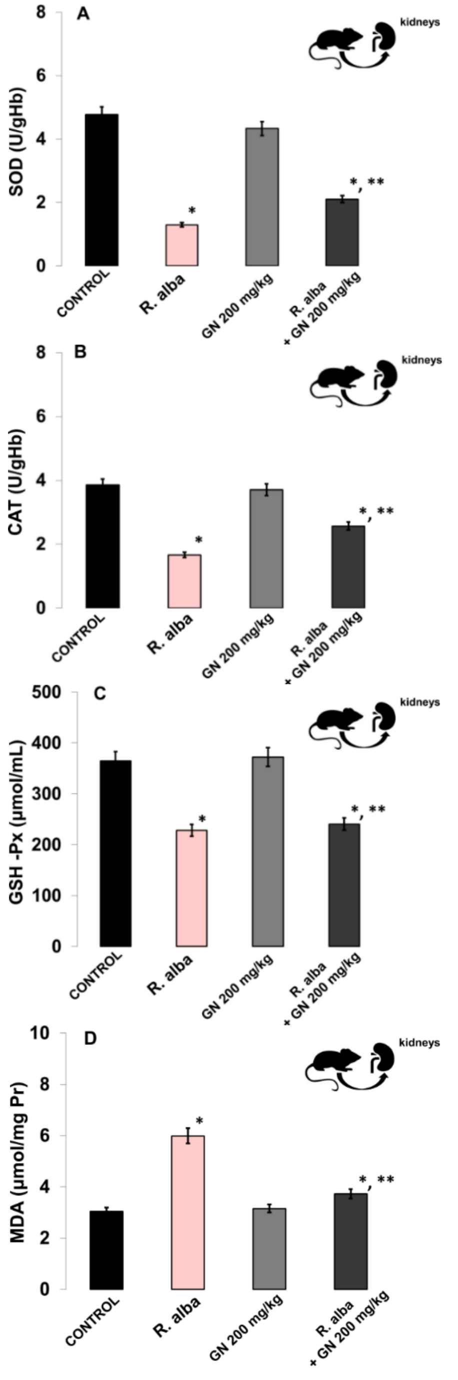

Figure 2. *R. alba* absolute effects on GM-induced nephritic enzyme activities and lipid peroxidation in tested groups: controls, *R. alba* absolute treated mice, GM-treated mice and *R. alba* absolute + GM-treated mice: (A) superoxide dismutase (SOD, U/gPr), (B) catalase (CAT, U/gPr), (C) reduced glutathione (GSH; µmol/ml), (D) malondialdehyde concentration (MDA, µmol/mgPr). The results are presented as mean ± SD (n=6). One-way ANOVA with multiple comparisons using Student's t-test was used to determine significant differences to (*) $p < 0.05$ *vs.* controls, (**) $p < 0.005$ *vs.* GM-treated mice.

R. alba absolute at a dose of 80 mg kg^{-1} is probably able to deactivate the H_2O_2, •O_2^-, HO•, OH^- radical accumulation, i.e. reduce and deactivate the renal OS damages. Statistically significant reduced activity of SOD (2.106 ± 0.31 *vs*. 4.78 ± 0.89 U/gHb, $p < 0.005$; Figure 2A), CAT (2.573 ± 0.22 *vs*. 3.855 ± 0.05 U/gPr, $p < 0.05$; Figure 2B), GSH (243.60 ± 31.9 *vs*. 364.5 ± 17.39 µmol/mL, $p < 0.004$, Figure 2C) and MDA (3.725 ± 0.4 *vs*. 5.99 ± 0.96 µmol/mL, $p < 0.05$, Figure 2D) was recorded, compared to controls (Figures 2A-D).

Antioxidantly, *R. alba* absolute (80 mg kg^{-1}), both alone and in combination GM + *R. alba* absolute, reverses hyperpolarization and restores the endogenous/exogenous redox homeostasis. This occurs after a possible reversal of the respiratory chain role, compared to GM accumulation, and to controls. *R. alba* L. (wastewaters, extracts, etc.) has been reported to possess metal-chelating properties [11]. *R. alba* L. sequesters copper (II) to a non-redox-active form, i.e. prevents oxidative damage to cell membranes and increases cell survival. Reduced cellular oxidation is accompanied by reduced initiation of •OH radicals [34, 35] and direct destruction of membrane lipids [7, 11]. Georgieva et. al. [11] drew attention to the fact that antioxidants such as *R. alba* L., have the ability to chelate and reduce iron (III) ions, which allows for the potential maintenance of membrane-metal homeostasis. In addition, the chelation and reduction of iron (III) ions supported by *R. alba* absolute protectively control ferroptotic processes by suppressing toxic ROS/RNS. Therefore, *R. alba* absolute restores the SOD, CAT enzymatic activity, by further activating GSH levels, which leads to H_2O_2 detoxification and increased cellular reduction of lipid peroxides (L-OOH). Moreover, *R. alba* absolute contains high polyphenol levels (7.6 mg mL^{-1}*)* [10, 11] and acyclic monoterpene alcohols (geraniol (9.89 %); citronellol/nerol (6.7 %)). These agents have been shown to be effective in protecting against GM-mediated and doxorubicin-mediated renal injuries and fibrosis development, due to potent ihibition of residual lipid peroxidation [28, 36].

R. alba absolute ameliorated ROS, NO• stress levels against GM-induced acute inflammation

Cell-permeable spin probes (based on aminoxyl, nitronyl nitroxide, or hydroxylamine (CPTIO.K, CMH) radicals) allow for the accurate determination of ROS/ RNS and directly reflect differences in redox status, *in vivo* [37]. The spin probes are easily reduced to the corresponding diamagnetic forms and act as catalysts for the destruction of renal NO• to the formation of the imino-nitroxide radical, or the dimutation of renal $•O_2^-$ to H_2O_2 and molecular oxygen (O_2) [38, 39].

To confirm the protective role of *R. alba* absolute against GM-mediated oxidative renal disorders and inflammatory processes, the redox-modulated activity was investigated.

GM administration significantly increased the ROS products (2.855 ± 0.31 vs. 1.09 ± 0.08 a.u., $p < 0.005$; Figure 3) and NO• levels (56.61 ± 5.31 *vs*. 17.88 ± 1.66 a.u., $p < 0.002$; Figure 3) in kidney homogenates, compared to controls. The strong antioxidant protection of *R. alba* absolute after GM induction was reflected in the reduced toxic ROS production (1.456 ± 0.19 *vs*. 2.855 ± 0.31 au, $p < 0.05$) and in cellularly absorbed NO• concentrations (39.61 ± 3.001 *vs*. 56.83 ± 5.31 au, $p < 0.05$) in kidneys. These findings highlight the protective effects of the combined treatment of GM+ *R. alba* absolute on renal OS, compared to mice treated with GM alone. It should be noted that *R. alba* absolute (7 days) likely alleviated oxidative lesions in renal proximal tubule cells through redox dismutation of renal ROS production and NO• radicals to nontoxic molecular oxygen (O_2), compared to the GM-treated group. Furthermore, ROS/ RNS dismutation activated the enzymatic minimization of inflammatory responses.

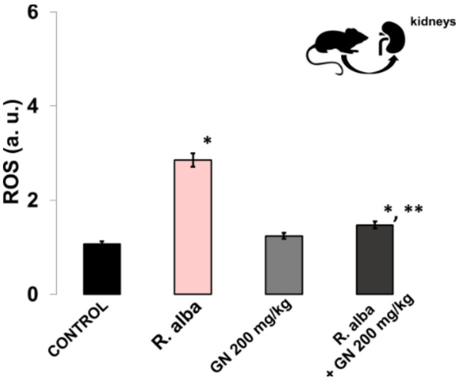

Figure 3. *R. alba* absolute effects on GM-induced nephrotoxicity and oxidative changes in ROS production. The radicals were scavenged in triplicate by EPR spectroscopy using *Win-EPR* and *Simfonia* software and expressed in arbitrary units (a.u.). The results are presented as mean ± SD (n = 6). One-way ANOVA with Student's t-test was used to determine statistically significant differences in relation to $^*p < 0.05$ *vs*. controls and $^{**}p < 0.005$ *vs*. GM-treated mice.

R. alba absolute containing phenylethyl alcohol, citronellol/nerol, geraniol, trans-β-caryophyllene prevents the transformation of $•O_2^-$ into toxic $ONOO^-$ and •OH radicals. Simultaneously, the active antioxidant components restore the NO bioavailability as a signaling molecule, regulating renal blood flow. Protection with *R. alba* absolute for 7-days showed significant catalysis of ROS production and RNS elevation by reducing oxidative stress damage, followed by activation of the mitochondrial respiratory chain [40, 41]. Also, antioxiantly *R. alba* absolute corrects GM accumulation by directly reducing collagen deposition, suppressing vasoconstriction, and antioxidant-mediated renal fibrosis [41, 42]. Verma *et al.,* [42] reported that *R. alba* L. (oil; rose water etc.) effectively controlled lipid profile abnormalities and preserved normal renal architecture and renal function.

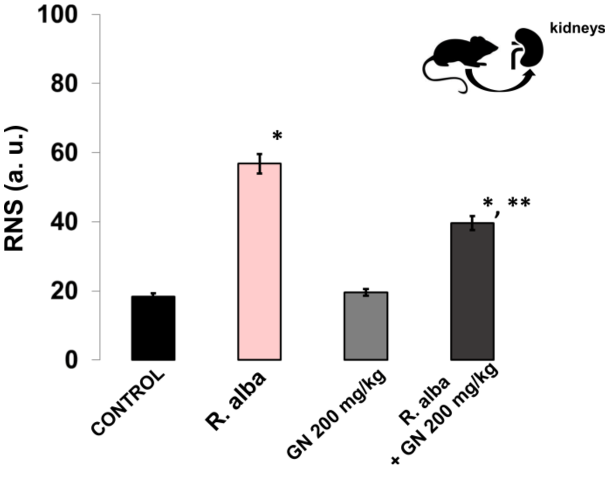

Figure 4. The *R. alba* absolute effects on GM-induced nephrotoxicity and oxidative changes in nitric radicals (NO•). The radicals were scavenged in triplicate by EPR spectroscopy using Win-EPR and Sim-Fonia software and expressed in arbitrary units (a.u.). The results are presented as mean ± SD (n = 6). One-way ANOVA with Student's t-test was used to determine statistically significant differences in relation to $^*p < 0.05$ *vs.* controls and $^{**}p < 0.005$ *vs.* GM-treated mice.

In line with this, Ilieva *et al.* [10] demonstrated that Bulgarian oil-bearing roses *R. alba* L., *Rosa damascena* Mill., *R. centifolia* L., and *R. gallica* L. exhibit significant antioxidant activity, ROS/RNS-protecting effects, and toxicological safety against non-tumorigenic human embryonic kidney cells (HEK-293) and mouse fibroblasts (CCL-1). *R. alba* absolute, through its antioxidant activities, deactivates free radicals in the presence of reduced ferric and copper ions. These processes, in turn, catalyze the overproduction of toxic •OH radicals and inhibit ferroptosis [10, 11, 36, 43]. Furthermore, the active citronellol/nerol, geraniol, and trans-β-caryophyllene in *R. alba* absolute likely promote renal blood flow, increasing glomerular filtration and remodulating the NF-kB pathway and Th2 immune response [36, 43, 44].

***Study limitations**: Due to ethical considerations, the number of animals studied per group was six, which is sufficient to reveal the most significant differences, but may be insufficient to establish additional correlations between some parameters. In the present study, in vivo GM activity was not investigated by R. alba absolute co-administration, which would also reveal additional information about the antimicrobial properties of the combination therapy. Further studies are needed to profile the R. alba absolute toxicity, including dose-response analyses, histopathological validation, and identification of possible doses with a beneficial effect on renal intoxication in longer-term use.*

CONCLUSIONS

The present study highlights the antioxidant activity and anti-inflammatory potential of *R. alba* absolute (80 mg mL^{-1}) under GM-induced nephrotoxicity. The antioxidant protection is mediated by a reduction in lipid peroxidation and restoration of enzyme activities. *R. alba* absolute directly related to the redox modulation of free radicals, which facilitate mitochondrial antioxidants and anti-inflammatory properties, without compromising the efficacy of bactericidal therapy.

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Estimation of energy and emission properties of waste from medicinal and aromatic plants

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This study evaluates the feasibility of utilizing post-extraction waste from medicinal and aromatic plants - lavender, thyme, yarrow, wormwood, sandy everlasting, oregano, costmary, tarragon, sage, and hyssop - as a feedstock for bioenergy production. Key fuel properties of the residual biomass were assessed, including heat of combustion, higher heating value (HHV), ash content, volatile matter, and moisture content. Elemental analysis was performed to determine the concentrations of carbon, hydrogen, nitrogen, and sulfur. The results demonstrate that these residues possess high potential as solid biofuels, characterized by favorable composition: high carbon (45.4–49.8%) and hydrogen (3.9–8.2%) contents, low ash (6.21–18.42%), nitrogen (1.78–3.16%), chlorine (0.022–0.586%), and sulfur (0.033–0.294%) contents, and high HHV (16.89–19.23 MJ/kg). Compared to coal, combustion of this biomass could significantly reduce emissions, with estimated reductions of up to 32% for CO and 31%CO_2, 40% for NO_x, 99% for SO_2, and 67% for particulate matter, depending on the specific biomass type.

Keywords: medicinal and aromatic plant waste, biofuel, elemental analysis, emission factors

INTRODUCTION

In recent years, alternative energy sources characterized by low emissions, including greenhouse gases and dust particles, have been increasingly sought after [1, 2]. Waste plays a crucial role in the European Commission's strategy for energy security and greenhouse gas (GHG) emission reduction. As a renewable energy source, the use of waste biomass has been expanding worldwide, primarily for household heating as an alternative to fossil fuels [3, 4]. The demand for plants with high productivity, low nutrient requirements, and valuable biological composition for various bioeconomic applications is of utmost importance. Perennial plants, in particular, offer advantages by reducing production costs and energy inputs compared to annual field preparation and planting. Additionally, such a biomass production system can enhance ecosystem services and support soil conservation, aligning with the concept of "low energy consumption and high output" regarding energy investments and additional costs. Essential oil crops are primarily cultivated for their essential oils [5-7]. The area dedicated to essential oil production in the European Union is steadily increasing, reaching approximately 80,000 hectares [8]. In recent years, global trade in medicinal and aromatic plants has grown by 10–12% annually [9]. Consequently, waste generation in this industry has also increased, reaching up to 30 million tons annually [10]. A substantial amount of waste biomass is generated during harvesting, pre-processing, drying, collection of harvested crops, and plant feedstock processing. After essential oil extraction, solid residue remains, which can be utilized for fuel energy production through pyrolysis, gasification, or hydrothermal carbonization processes [11]. Despite containing valuable substances, these residues are often discarded through stockpiling, landfilling, or open-field burning, leading to resource waste and significant environmental pollution [10, 12]. Despite the strong economic potential of utilizing plant biomass waste, limited research has focused on the calorific value of these residues [8, 13-14].

This study aims to evaluate the energy potential of residues obtained after steam distillation of essential oils from lavender, thyme, yarrow, wormwood, sandy everlasting, oregano, costmary, tarragon, sage, and hyssop. Additionally, it examines the emission factors of toxic exhaust components and explores the feasibility of using these residues for energy production

EXPERIMENTAL

Aromatic plants, including lavender, thyme, yarrow, wormwood, sandy everlasting, oregano, costmary, tarragon, sage, and hyssop, were collected from southern Bulgaria at the flowering stage. After

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essential oil extraction *via* steam distillation, the plant residues were dried in an oven at 105°C and ground using a laboratory grinder. The physicochemical characterization of the plant residues was conducted based on calorific value, ultimate analysis, and proximate analysis.

The heating value was determined following the ISO standard (BDS EN ISO 18125) [15] using an IKA C6000 oxygen bomb calorimeter (IKA Werke GmbH, Germany).

The samples were analyzed according to standard methods: moisture content [16], ash content [17], volatile matter [18].

Total carbon, hydrogen, nitrogen, and sulfur contents were determined by dry combustion using a Vario Macro CHNS analyzer (Elementar GmbH, Germany) [19].

Using the results from the ultimate analysis, emission factors for CO, CO_2, NO_x, SO_2, and dust emissions were estimated through equations (1) – (7) [20]:

CO emission factor

$$\mathrm{CO} = \frac{28}{12} x\ \mathrm{Ec}\ x\ (C\_CO/C) \qquad (1)$$

where: CO – carbon monoxide emission factor (kg/kg); 28/12 – molar mass ratio of carbon monoxide to carbon; Ec – emission factor of chemically pure coal (kg/kg); C_CO/C – proportion of carbon emitted as CO (for biomass: 0.06).

Emission factor of chemically pure coal

$$Ec = c.uc \qquad (2)$$

where: c – carbon content in biomass (kg/kg); uc – proportion of carbon oxidized during combustion (for biomass: 0.88).

CO_2 emission factor

$$\mathrm{CO_2} = \frac{44}{12} x\ (Ec - \frac{12}{28}\ x\ CO - \frac{12}{16}\ x\mathrm{ECH_4} - \frac{26.4}{31.4}\ x\ ENMVOC) \qquad (3)$$

where: CO_2 – carbon dioxide emission factor (kg/kg); 44/12 – molar mass ratio of carbon dioxide to pure coal; 12/28 – molar mass ratio of carbon to carbon monoxide; 12/16 – molar mass ratio of carbon to methane; ECH_4 – methane emission factor; ENMVOC – non-methane VOC emission index (for biomass: 0.009).

Methane emission factor

$$\mathrm{ECH_4} = \frac{16}{12} x\mathrm{Ec} \times (\mathrm{C\_CH4/C}) \qquad (4)$$

where: ECH_4 – methane emission factor (kg/kg);16/12 – molar mass ratio of methane to carbon; C_CH_4/C – proportion of carbon emitted as CH_4 (for biomass: 0.005).

NOx emission factor

$$NOx = \frac{46}{14} \times Ec \times N/C \times (N\_NOx/N) \qquad (5)$$

where: NO_x – emission factor for nitrogen oxides (kg/kg); 46/14 – molar mass ratio of nitrogen dioxide to nitrogen (NO in air oxidizes to NO_2); N/C – nitrogen to carbon ratio in biomass; N_NOx/N – proportion of nitrogen released as NO_x (for biomass: 0.122).

Emission factor of SO_2

$$\mathrm{SO_2} = \frac{2S}{100} \qquad (6)$$

where: SO_2 – sulfur dioxide emission factor (kg/kg); 2 – molar mass ratio of SO_2 to sulfur; S – sulfur content in the fuel (%).

Dust emissions

$$Edust = 1{,}5 \times A \times \frac{100 - \eta 0}{100 - k} \qquad (7)$$

where: Edust – dust emission factor (kg/Mg); 1.5 – coefficient denoting 15% of ash released as volatile dust; A – ash content in the fuel (%); η_0 – dust removal efficiency (for biomass: 20%); k – combustible component content in the dust (for biomass: 5%).

RESULTS AND DISCUSSION

Proximate and ultimate analysis

The proximate and ultimate analysis results for essential oil plant waste are presented in Tables 1 and 2. According to CEN/TS 14961 [21], biomass for solid biofuels is categorized into three main groups: woody biomass, herbaceous biomass, and fruit biomass. Essential oil plant waste falls under the herbaceous biomass group - specifically, agricultural and garden herbs. The key parameters of biomass waste were compared against the requirements of ISO 17225-6 standard [22] for herbaceous biomass and data from other studies.

Heating value and energy potential: The lower heating value (LHV) is a key parameter for assessing the suitability of biomass as a biofuel, as it reflects the usable energy released during combustion, excluding the latent heat of vaporization of water. According to ISO 17225-6 [22], the minimum acceptable LHV for solid biofuels is 14.5 MJ/kg. The LHV of the tested plant wastes ranged from 15.39 MJ/kg (costmary) to 17.86 MJ/kg (wormwood), indicating that all samples meet or exceed the standard requirement and possess good energy potential.

Table 1. Proximate analysis and heating values of plant waste

Plant waste	M,%	Ash, %	FC, %	VM, %	LHV, MJ/kg	HHV, MJ/kg
Lavender	8.42± 0.08	7.28± 0.03	11.50± 0.10	80.08± 0.21	17.41± 0.01	19.23± 0.02
Wormwood	6.26± 0.04	6.21± 0..03	12.72± 0.11	81.02± 0.10	17.86± 0.04	19.21± 0.05
Sandy everlasting	6.12± 0.06	8.44± 0.15	15.08± 0.15	78.80± 0.40	17.05± 0.01	18.31± 0.01
Oregano	7.48± 0.24	9.00± 0.05	17.82± 0.18	74.70± 0.30	16.76± 0.01	18.30± 0.04
Costmary	7.80± 0.40	18.42± 0.12	17.50± 0.18	74.70± 0.10	15.39± 0.03	16.89± 0.01
Tarragon	7.53± 0.03	12.88± 0.05	17.94± 0.20	74.53± 0.08	16.13± 0.01	17.63± 0.01
Sage	8.23± 0.10	14.57± 0.09	17.03± 0.15	74.74± 0.15	16.42± 0.01	18.10± 0.01
Hyssop	6.86± 0.08	7.20± 0.04	13.34± 0.10	79.80± 0.50	17.31± 0.05	18.75± 0.06
ISO 17225-6	≤12 -15	≤ 6-10	-	-	≥14.5	-
CEN/TS 14961-1 Harvest (July – Oct)	-	6.5 (2.5-10)	-	-	16.6	-

M - moisture, FC - fixed carbon, VM - volatile matter, LHV - lower heating value, HHV- higher heating value

Table 2. Ultimate analysis of tested wastes

Waste	N, %	S, %	Cl, %	C, %	H, %	O, %
Lavender	1.78± 0.01	0.033± 0.005	0.022± 0.001	49.3± 0.2	5.4± 0.2	36.20± 0.10
Wormwood	2.98± 0.12	0.096± 0.012	0.172± 0.008	49.3± 0.4	8.2± 0.5	33.21± 0.10
Sandy everlasting	1.58± 0.02	0.120± 0.002	0.443± 0.002	48.4± 0.3	4.8± 0.7	36.66± 0.12
Oregano	1.67± 0.03	0.062± 0.001	0.115± 0.004	48.6± 0.1	3.9± 0.7	36.70± 0.11
Costmary	2.57± 0.12	0.294± 0.005	0.254± 0.001	45.4± 0.4	5.2± 0.7	28.12± 0.10
Tarragon	3.16± 0.01	0.181± 0.003	0.586± 0.002	46.7± 0.3	8.1± 0.7	28.98± 0.10
Sage	2.46± 0.01	0.152± 0.002	0.043± 0.002	48.3± 0.5	5.6± 0.7	28.92± 0.10
Hyssop	1.80± 0.03	0.278± 0.001	0.054± 0.002	49.8± 0.4	5.8± 0.7	35.12± 0.11
ISO 17225-6	1.5-2	0.2- 0.3	0.1-0.3	-	-	-
CEN/TS 14961-1 Harvest (July – Oct)	1.3	0.1 (0.1-0.2)	0.5 (0.2-0.6)	46	5.7	40

The higher heating value (HHV), which represents the total energy content of the biomass including the energy from the condensation of water vapor, ranged from 16.89 MJ/kg (costmary) to 19.21 MJ/kg (wormwood) and 19.23 MJ/kg (lavender). Lavender and wormwood wastes demonstrated the highest HHVs (19.21–19.23 MJ/kg), making them excellent candidates for biofuel applications. In contrast, the lower HHV of costmary suggests reduced energy efficiency. These findings are consistent with previously reported values for lavender (19.2 MJ/kg) [14] and thyme waste (17.8 MJ/kg) [14], mint waste (15.90–16.64 MJ/kg) [8], miscanthus (17.99 MJ/kg), wood (18.35 MJ/kg), and rapeseed straw (15.97 MJ/kg) [23].

Moisture content and ash content: Moisture content significantly influences combustion efficiency, as high moisture reduces the net energy output by requiring additional energy to evaporate water during combustion. The moisture content of the tested biomass ranged from 6.12% (sandy everlasting waste) to 8.23% (sage waste), well within the ISO 17225-6 standard limits of 12–15% [22]. These low moisture levels contribute positively to combustion performance.

Ash content is another critical factor in determining biomass fuel quality. Excessive ash can lower combustion efficiency, increase particulate emissions, and cause operational issues such as slagging and fouling in combustion systems. In this study, ash content varied from 6.21% (wormwood) to 18.42% (costmary). The ash content in wormwood, sandy everlasting, oregano, and hyssop wastes falls within the acceptable ISO 17225-6 range (≤6–10%) [22]. Moisture and ash values for lavender, wormwood, sandy everlasting, oregano, and hyssop meet standard criteria, supporting their suitability as solid biofuels. These results align with earlier findings for lavender waste (6.7–7.8%) [13] and mint waste (7.23–10.29%) [8].

Fixed carbon and volatile matter: Fixed carbon content is a desirable property in biomass fuels, as it significantly influences combustion stability and duration of burning. Although ISO 17225-6 [22] does not define standard limits for fixed carbon, values in this study ranged from 11.5% (lavender waste) to 17.82% (oregano waste) and 17.94% (tarragon waste). The higher fixed carbon content in oregano and tarragon suggests superior combustion stability. Similar fixed carbon values have been reported for mint waste (9.40–15.77%) [8] and lavender waste (15.3%) [13].

Volatile matter is another critical factor in biomass combustion. A higher volatile matter content typically results in easier ignition and more intense combustion. In the current study, volatile matter ranged from 74.53% (tarragon waste) to 81.02% (wormwood waste), aligning with values reported for mint waste (64.99–70.36%) [8], Miscanthus (72.5%), and rapeseed straw (73.5%) [23]. The balance between fixed carbon and volatile matter suggests that the tested plant wastes possess stable combustion characteristics, making them suitable for energy applications.

Table 2 presents the ultimate analysis results of the tested plant wastes, including carbon (C), hydrogen (H), oxygen (O), nitrogen (N), and sulfur (S) content. These values are compared to ISO 17225-6 requirements [22] and to results from related studies.

Carbon and hydrogen are key energy-contributing elements in biomass. During combustion, these elements undergo exothermic oxidation reactions, producing CO_2 and H_2O, thereby determining the gross calorific value of the fuel [24]. The total carbon content in the tested samples ranged from 45.4% to 49.8%, with hyssop waste having the highest concentration. These values are consistent with previous findings for mint waste (44.82–47.05%) [8], lavender (45.4–48.1%) [13], willow biomass (50.84%) [25], and wood (49.80%) [26].

Hydrogen content ranged from 3.9% (oregano waste) to 8.2% (wormwood waste). This range is comparable to that reported for mint waste (5.54–5.76%) [8], lavender waste (5.8–6.77%) [13], willow biomass (5.86%) [25], and wood (6.30%) [26]. These values also closely match the hydrogen content found in cereal and forage straws, such as wheat, barley, flax, and timothy (6.1–6.4%).

Oxygen content, on the other hand, tends to negatively influence the energy value of biomass. In the tested samples, oxygen content ranged from 28.12% (costmary waste) to 36.70% (oregano waste), which is in line with values observed in mint waste (29.14–36.09%) [8] and lavender waste (37.8%) [13].

The elemental composition of biomass is crucial for estimating heating values, combustion air requirements, and composition of flue gases [27]. It also plays a key role in assessing the environmental impact of biomass combustion. Nitrogen and sulfur, in particular, are undesirable in biomass fuels due to their role in generating harmful emissions. During combustion, nitrogen contributes to nitrogen oxides (NO_x) formation, while sulfur leads to sulfur oxides (SO_x) emissions, which are associated with particulate pollution, acid rain, and equipment corrosion [24].

Nitrogen content in the tested biomass ranged from 1.78% (lavender waste) to 3.16% (tarragon waste). Only the wastes of sandy everlasting, oregano, lavender, and hyssop had nitrogen levels below the 1.5–2.0% threshold set by ISO 17225-6 [22], making them more suitable for combustion from an environmental standpoint. For comparison, nitrogen content in mint waste has been reported in the range of 0.23–0.70% [8], and in lavender at approximately 1.3% [13].

Sulfur content in biomass is generally low but still influences emission profiles. The tested samples exhibited sulfur levels ranging from 0.033% (lavender waste) to 0.294% (costmary waste), remaining within the acceptable limits (0.2–0.3%). These values are consistent with reported sulfur contents in lavender waste (0.1%) [13] and mint waste (0.0–0.19%) [8]. A low sulfur content is advantageous, as it minimizes SO_2 emissions and reduces environmental risks.

Chlorine content in the tested plant wastes ranged from 0.022% in lavender waste to 0.586% in tarragon waste, remaining within the acceptable limits for solid biofuels. A lower chlorine concentration is advantageous, as it decreases the risk of corrosion in combustion systems and limits the formation of harmful emissions, such as hydrogen chloride and dioxins.

Emission factors

Determining emission factors is essential for estimating pollutant levels released during fuel combustion. These factors depend on the physicochemical characteristics of the biomass used. Table 3 presents the emission factors for carbon monoxide (CO), carbon dioxide (CO_2), nitrogen oxides (NO_x), sulfur dioxide (SO_2), and particulate matter (dust) for the investigated plant wastes, with coal data provided for comparison.

Significant differences were observed in CO emissions among the plant wastes. The highest CO emission rate was recorded for lavender and wormwood waste (60.74 kg/Mg), while sage waste had the lowest (55.44 kg/Mg), reflecting a variation of up to 5%.

NO_x emissions ranged from 2.44 kg/Mg for tarragon waste to 11.15 kg/Mg for hyssop waste. Compared to hard coal, only tarragon and sage waste exhibited lower NO_x emission levels.

The CO_2 emission factor was highest for lavender and wormwood waste (1487.07 kg/Mg) and lowest for sage waste (1357.34 kg/Mg).

SO_2 emissions ranged from 0.067 kg/Mg for lavender waste to 0.588 kg/Mg for costmary waste. Dust emissions were highest for costmary waste (23.27 kg/Mg) and lowest for tarragon waste (7.83 kg/Mg).

The emission factors observed are comparable to those reported for other plant-based materials such as mint waste [8], *Eucalyptus globulus* [28], and larch needles [29]. Overall, the studied plant wastes exhibit emission profiles similar to those of various biomass sources, with particularly low SO_2 emissions. CO and CO_2 emission levels are close to those reported for mint [8], larch needles [29], and hazelnut husk and leaves [29–30]. In terms of NO_x, the studied wastes show significantly lower emissions compared to straw pellets, sunflower stalks, corn stalks, and wood pellets [31], as well as *Eucalyptus globulus* [28].

Wastes from hyssop and costmary showed elevated dust emissions, comparable to those from mint (9.14–13.0%) [32], tree leaves (10.80%) [29] and hazelnut waste (10.95%) [30]. High particulate emissions are attributed to the elevated ash content in these materials. Therefore, technological measures such as installing additional dust filters should be considered to mitigate particulate emissions.

Table 3. Emission factors (kg/Mg) for analyzed wastes and coal

Waste	CO	CO_2	NO_x	SO_2	Edust
Lavender	60.74± 0.30	1487.07± 7.33	6.29± 0.10	0.067± 0.005	9.20± 0.10
Wormwood	60.74± 0.30	1487.07± 7.33	10.51± 0.18	0.192± 0.015	7.84± 0.10
Sandy everlasting	59.63± 0.29	1459.92± 7.08	5.57± 0.09	0.240± 0.015	10.66± 0.15
Oregano	59.88± 0.28	1465.95± 7.08	5.89± 0.09	0.124± 0.010	11.37± 0.15
Costmary	55.93± 0.27	1369.41± 6.55	9.07± 0.19	0.588± 0.050	23.27± 0.30
Tarragon	57.29± 0.28	1402.60± 6.78	2.44± 0.06	0.073± 0.008	7.83± 0.10
Sage	55.44± 0.27	1357.34± 6.50	3.28± 0.06	0.284± 0.040	11.79± 0.10
Hyssop	57.53± 0.28	1408.63± 6.78	11.15± 0.25	0.362± 0.040	16.27± 0.15
Hard coal	82.01± 0.64	1969.00± 15.15	4.09± 0.08	5.200± 0.100	23.57± 0.50

The emission assessment method used in this study provides an effective means of evaluating the environmental impact of plant waste for energy production without the need for advanced analytical equipment. This approach offers a practical tool for rapid estimation of biofuel emissivity, an aspect often overlooked in biomass suitability assessments.

Differences in emission factors among the tested wastes may influence their selection for energy use, especially when environmental performance and combustion efficiency are priorities. Among the tested materials, tarragon waste demonstrated the lowest overall emissions, while lavender and wormwood waste exhibited the highest CO and CO_2 emissions. Therefore, utilizing tarragon waste for energy purposes may offer advantages in reducing greenhouse gas (GHG) emissions.

Environmental impact of essential oil crop waste combustion

The use of waste from essential oil crops as biofuel results in substantial emission reductions compared to coal combustion [20]. Depending on the type of waste, emission levels were reduced by: 25.9–32.4% for carbon monoxide (CO); 24.4–31.1% for carbon dioxide (CO_2);19.8–40.2% for nitrogen oxides (NO_x); 88.7–98.7% for sulfur dioxide (SO_2); and 66.8% for particulate matter (dust).

These reductions confirm the significant environmental benefits of utilizing such biomass wastes as alternative fuels.

CONCLUSIONS

The study revealed significant differences in the calorific values and emission characteristics of the tested plant wastes. Lavender (17.41 MJ/kg; 19.23 MJ/kg), wormwood (17.86 MJ/kg; 19.21 MJ/kg), hyssop (17.31 MJ/kg; 18.75 MJ/kg), sandy everlasting (17.05 MJ/kg; 18.31 MJ/kg), oregano (16.76 MJ/kg; 18.30 MJ/kg), and sage waste (16.42 MJ/kg; 18.10 MJ/kg) exhibited relatively high lower and higher heating values, indicating good potential as biofuels. In contrast, costmary (15.39 MJ/kg; 16.89 MJ/kg) and tarragon (16.13 MJ/kg; 17.63 MJ/kg) wastes showed lower energy content.

The chemical composition of the plant materials significantly influences both energy potential and emission factors. For instance, costmary waste had the highest ash content (18.42%), which adversely affected its calorific value.

Emission factors varied depending on the waste type. Wormwood and lavender waste produced the highest emissions of CO (60.74 kg/Mg) and CO_2 (1487.07 kg/Mg), while hyssop waste showed the highest NO_x emissions (11.15 kg/Mg). Costmary waste generated the most particulate emissions (23.27 kg/Mg), likely due to its high ash content.

When selecting appropriate biomass for energy production, it is essential to consider both energy efficiency and environmental impact. If maximizing energy output is the primary objective, lavender, wormwood, hyssop, sandy everlasting, and oregano waste are suitable candidates. However, if minimizing environmental impact is prioritized, tarragon waste is the preferable choice due to its low emissions profile.

Ultimately, the selection of biomass for energy use should be guided by specific local contexts, including regulatory frameworks, environmental standards, and energy demands.

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