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Research Article

Analytical Investigation of Active Compound Contents of *Panax ginseng* C.A. Meyer and *Ginkgo biloba* L. Supplements Fortified with Apitherapy Products

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Abstract: Extracts of therapeutic plants of nature, such as *Ginkgo biloba* L. and *Panax ginseng* C.A. Meyer (*P.ginseng*) are highly requested. Recently admixtures of these extracts with apitherapy products are also available. In this study, as research materials, *P.ginseng*, and *Ginkgo biloba* L. supplements in the form of tablets, paste, and liquid extracts prepared as a mixture with bee products were compared in terms of their ginsenosides, phenolics, terpene lactones, and antioxidant capacities. Within this study, it was aimed to clarify the active ingredients *P.ginseng* extracts, *Ginkgo biloba* L. extracts, and their mixtures with bee products by developing advanced and novel analytical methods. Additionally, it was purposed to unveil the contribution of bee products to phytotherapy supplements of *P.ginseng* and *Ginkgo biloba* L. on the basis of secondary metabolites. Ultrasound-assisted hydroalcoholic extraction was applied at sample preparation and supernatants were analyzed at UPLC-MS/MS and UV-Visible spectrophotometer. The antioxidant activities were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. As a result, total ginsenoside contents of the products per serving were ranged between 9.7 mg to 150.2 mg. Terpene lactones of the ginkgo products were ranged between 0.1 mg to 9.5 mg per serving. The antioxidant activities of all products were determined between 13% and 92%. Quercetin, rutin hydrate, CAPE, kaempferol, galangin, chrysin, gallic acid, pinocembrin, and isorhamnetin were found as the predominant phenolics. Due to their higher antioxidant capacities, superior phenolic concentrations, and diversities, it was enlightened that apitherapy products can augment the phytotherapy efficacies of *P.ginseng* and *Ginkgo biloba* L. supplements.

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1. INTRODUCTION

With the development of modern medicines and revisions to the methods of treatment, phytotherapy which has been thrown into the background for a while has become popular again due to the difficulties in the treatment of chronic diseases and increased side effects from some modern therapies. Plant extracts of therapeutic nature, such as *Panax ginseng* C.A. Meyer and *Ginkgo biloba* L., dating back to ancient times, and mixtures containing these extracts are being

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requested by the consumers. In recent years, the mixture forms of these extracts produced from such plants with alternative apitherapy products are available at pharmacies and herbalists and these preparations attract great attention.

Ginseng is a slow-growing, perennial, deciduous herb that belongs to the Araliaceae family and the *Panax* genus. The most known and important cultivated species are *Panax ginseng* C.A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (American ginseng). *P. ginseng* has been the main source of ginseng root used at traditional medicines and it is classified into three types depending on drying conditions (Popovich *et al.*, 2012). *Panax quinquefolius* L. is native to North America. It is 'cool' and is traditionally used for decreasing the 'internal heat' as opposed to *P. ginseng* which has heating effects (Gui & Ryu, 2014; Popovich *et al.*, 2012). Carbohydrates such as polysaccharides, starch and cellulose are the major components of *P.ginseng* (60–70%) (Lee *et al.*, 2015). *P.ginseng* also contains antioxidative compounds, polyacetylenes, and gomisins, which protect the liver and peptides that behave likewise to insulin (Qi *et al*., 2011; Shin *et al.*, 2015). In traditional Chinese medicine, ginseng is commonly mentioned as an "adaptogen," a substance that can aid an organism in getting over various types of stress and restore homeostasis. Immunomodulating and bio-modulating action, beneficial effects within the hematopoietic, cardiovascular, endocrine, immune, and central nervous systems are among the most important features of ginseng in clinical trials and laboratory (Kim, 2018; Kim *et al.*, 2017; Kim *et al.*, 2018). Different ginsenosides are responsible for these health-promoting effects, therefore it is important to determine the composition of various ginsenosides in commercial ginseng products. Ginsenosides are the major secondary metabolites found in roots more than other parts of the ginseng plant, which are a class of triterpene saponins (Kim *et al.*, 2017). In both *P. ginseng* and *Panax quinquefolius* L., ginsenosides Re, Ro, Rg1, Rb1, Rb2, Rc and Rd are present in different amounts, while ginsenoside Rf can only be identified in *P. ginseng* (Dou *et al.*, 1998) and its products are commercially marketed as dietary supplements with the forms of dried root powders, liquid extracts, teas and formulated into tablets, capsules, etc. The recommended dose of *P. ginseng* standardized extract containing 1.5 to 7 % ginsenosides is 100-300 mg per day or 0.5 to 2 g per day of the root extract (Court *et al.*, 1996). Through modern analytical technology, at least 289 ginsenosides were reported from 11 species of genus *Panax*. The most general subtype of ginsenosides (126 reported compounds) has C-17 side chains. In addition, 66 20(S)-/ 20(R)-protopanaxadiol, 50 20(S)- or 20(R)-protopanaxatriol, 19 oleanolic acid, 15 ocotillo, and 13 miscellaneous saponin compounds have been reported (Kim, 2018; Yang *et al.*, 2014).

In literature, Soxhlet extraction, ultrasound-assisted extraction, alkaline hydrolysis, refluxing methods have been typically used to extract ginsenosides from roots of *P. ginseng* with hydroalcoholic solvents as the typical organic modifiers (Hong *et al.*, 2009; Shin *et al*., 2001a, 2001b). High-performance liquid chromatography (HPLC) has been generally used to determine the ginsenosides with refractive index (RI), ultraviolet (UV) detections and mass spectrometers (MS) (Chan *et al.*, 2000; Li *et al.*, 2005; Xia *et al.*, 2018; Yu *et al.*, 2021).

The other widely consumed herbal nutritional product is *Ginkgo biloba* L. which is one of the oldest species (family Ginkgoaceae). It is native to China and also grows throughout Japan, Korea, Europe, and the United States. *Ginkgo* trees reach a height of 20 – 35 m and can live for as long as 1000 years (Jacobs & Browner, 2000). The medicinal properties of *Ginkgo biloba* L. can be traced back to 5.000 years in China. The healer Chen Noung described the medicinal properties of the *Ginkgo biloba* L. in the first known pharmacopeia (Chang & Chang, 1997). Medicinal extracts are made from dried leaves and have been used for various purposes (DeFeudis, 1998). Uses of *Ginkgo biloba* L. extracts include the treatment and/or prevention of age-related physical and mental impairments, Alzheimer's, cardiovascular and bronchial disease (Diamond *et al.*, 2000; Le Bars *et al.*, 2002; Pietri *et al.*, 1997). Such pharmacological

activities are attributed to the synergistic activity of the flavonoids and the terpene trilactones (Smith & Luo, 2004; Stiker *et al*., 2000; van Beek, 2002). The flavonoids are composed of a large group of polyphenols and contain flavonol glycosides, flavones, flavan-3-ols, acylated flavonol glycosides and proanthocyanidins. Among these molecules, the flavonol glycosides are the most abundant group. Furthermore, numerous flavonol glycosides are sometimes found as their aglycones such as kaempferol, quercetin and isorhamnetin that are usually present in the leaves with small amounts (van Beek, 2002). The ginkgo terpene trilactones: bilobalide (sesquiterpene) and ginkgolides (diterpene); ginkgolide B, ginkgolide A, ginkgolide J and ginkgolide C are unique to *Ginkgo biloba* L. and they show broad spectrum of pharmacological activities (de Jager *et al*., 2006). Commercial *Ginkgo biloba* L. products are usually commercialized as mixtures, based on the content of terpene lactones and flavonol glycosides (van Beek & Montoro, 2009). A standardized leaf extract contains 22–27% of flavone glycosides and 5–7% of terpene lactones. Ginkgolide A, C, B with 2.8–3.4% and bilobalide with 2.6–3.2% along with less than 5 ppm ginkgolic acid are expected to be observed at standardized leaf extract (Kiefer, 2004). The most considered standardized leaf extract in studies is EGb761 (Mahadevan & Park, 2008). The standard dose of EGb761 is 120 mg (~1.7 mg/kg) once or twice daily for clinical puposed usage; hence, a standard dose will contain ~3–4 mg ginkgolides A, B, and C, 3–4 mg bilobalide, and 29 mg flavonoids (Nash & Shah, 2015). Quality control of *Ginkgo biloba* L. extracts and leaves can be routinely carried out by reversed-phase HPLC (RP-HPLC) with evaporative light scattering detection (ELSD) (Dubber & Kanfer, 2006; Kaur *et al*., 2009), refractive index (RI) (Wang & Ju, 2000), diode array detector (DAD) (Tang *et al*., 2010) and mass spectrometry detection (Wang et al., 2017). As an orthogonal technique gas chromatography (GC) system equipped with a flame ionization detector (FID) or mass spectrometry (MS) detection (Czigle *et al*., 2019) is also used. At sample preparation steps; cold and hot extraction, pressurized solvent extraction and ultrasound-assisted extraction is typically applied to achieve the simultaneous analysis of terpene trilactones and flavonol aglycones (Kaur *et al.*, 2009; Liu *et al.*, 2015).

Apitherapy has been gaining great attention nowadays due to its superior health effects. Bee products like pollen, royal jelly, honey, and especially propolis are rich in flavonoids, phenolic acids, minerals, and diverse anti-inflammatory and immune booster substances. The antioxidant capacities, phenolic acids, and flavonoid contents of *P. ginseng* and *Ginkgo biloba* L. extracts mixed with bee products are expected to be increased significantly. This research aimed to determine and compare the bio-active phytochemical contents, antioxidant capacities, and phenolic profiles of *P. ginseng* and *Ginkgo biloba* L. extract products as well as their preparations in mixture with bee products applying developed methodologies. In addition to many studies that investigated the health benefits and pharmacological properties of bee products, our research addedly focused on the investigation of active substance diversities at commercially available supplements and tried to find answers to how apitherapy products may change the phenolic and antioxidant properties of *P. ginseng* and *Ginkgo biloba* L. extracts that have been used in traditional medicine for years. Moreover, a high-resolution phenolic profile determination method with a comprehensive compound library and using mass-based detection was targeted to be developed. Thanks to new methods in terms of ginsenoside, terpene lactone, and phenolic monitoring, we envisage that this research will contribute a lot to the literature for advanced phytotherapy research.

2. MATERIAL and METHODS

2.1. Sample Collection

Products containing *P. ginseng* extract (n=12) and samples containing *Ginkgo biloba* L. extract (n=9) were purchased from the pharmacies and herbalists. Two of the samples that contain *Ginkgo biloba* L. extracts and seven of the samples containing *P. ginseng* extract were in form

of mixtures with alternative bee products. Information of the samples regarding their serving sizes and their forms are given in Table 1.

Table 1. Forms and serving sizes of *P. ginseng & Ginkgo biloba* L. samples.

Product	Product Type/Form	Serving size	Product	Product Type/Form	Serving size
A1[a]	Paste	7 g	B1	Tablet	1 tablet / 50 mg
A2[a]	Paste	7 g	B2[c]	Paste	7 g
A3[a]	Paste	7 g	B3	Liquid Extract	7 g
A4[a]	Tablet	1 tablet / 780 mg	B4	Tablet	1 tablet / 722 mg
A5[b]	Liquid Extract	7 g	B5	Capsule	1 tablet / 654 mg
A6	Tablet	1 tablet / 722 mg	B6[a]	Paste	7 g
A7	Tablet	1 tablet / 1770 mg	B7	Capsule	1 capsule / 395 mg
A8[a]	Paste	7 g	B8	Tablet	1 tablet / 870 mg
A9	Capsule	1 capsule / 670 mg	B9	Capsule	1 capsule / 450 mg
A10	Capsule	1 capsule / 1560 mg			
A11	Paste	7 g			
A12[a]	Liquid Extract	25 g			

A1-12: *Panax ginseng* C.A. Meyer products; B1-9: *Ginkgo biloba* L. products; [a] Contains more than a single bee product; [b] Contains royal jelly (RJ); [c] Contains honey

2.2. Reagents and Chemicals

The analytical standard mixture of *Ginkgo biloba* L. terpene lactones, containing bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J (0.1 mg/ml of each component in acetonitrile) was purchased from Cerilliant Corporation® (Round Rock, Texas, USA) and a standard mixture of ginsenosides that contain Rg_2, Rb_1, Rb_2, Rc, Re, Rd, Rg_1 and Rf (0.1 mg/ml of each component in methanol) were also obtained from Cerilliant Corporation® (Round Rock, Texas, USA). Individual standards of ginsenoside Ra_1, Ra_3, and Rb_3 were supplied from Fleton Natural Products Co., Ltd® (Chengdu, China). A second working stock standard mixture for additional ginsenosides (Ra1, Ra3, and Rb3) was prepared by dissolving them in methanol at 0.1 mg/ml concentrations. The analytical standards of each phenolic substances (Cinnamyl aldehyde, 3,4-dimethoxy benzaldehyde, phloroglucinol, 4-hydroxybenzoic acid, transcinnamic acid, protocatechuic acid, p-coumaric acid, 2-OH-coumaric acid, m-coumaric acid, phenyllactic acid, vanillic acid, homogentisic acid, gallic acid, shikimic acid, caffeic acid, quinic acid, ferulic acid, syringic acid, 3,4-dimethoxy cinnamic acid, methylsyringate, resveratrol, chrysin, pinocembrin, apigenin, genistein, galangin, naringenin, pinobanksin, caffeic acid phenethyl ester (CAPE), ellagic acid, kaempferol, luteolin, epicatechin, catechin, quercetin, hesperetin, taxifolin, epigallocatechin, isorhamnetin, chlorogenic acid, rutin, myricetin, and rosmarinic acid) were purchased from Sigma-Aldrich® (Munich, Germany). Stock standards of each phenolic substances were prepared by dissolving them in ethanol individually at 10 mg/ml concentration. For calibration purposes, a phenolic stock standard mixture at 0.01 mg/ml was prepared by adding 200 µl from each phenolic stock standard in a falcon tube and diluting it to an appropriate volume. Afterward, the phenolic stock standard mixture was diluted for the constitution of the 6 linear calibration points (0.05 µg/ml, 0.1 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1.0 µg/ml, and 2.0 µg/ml) using deionized water, thereby external standard calibration plots with the range from 0.05 µg /ml to 2.0 µg/ml concentrations were obtained for each phenolic compound. DPPH (2,2-diphenyl-1-picrylhydrazyl) was also purchased from Sigma-Aldrich®. 19.7 mg DPPH was dissolved in 25 ml ethanol and used as reagent at free radical scavenging activity assays. Acetonitrile (ACN), acetic acid, ethanol (EtOH, LC Grade) was obtained from

Merck® (Darmstadt, Germany). Methanol (MeOH) was obtained from JT. Baker® (Deventer, Holland). Ultrapure water was taken from a Milli-Q® Plus system in our laboratory (Millipore®, Billerica, MA, USA).

2.3. Terpene Lactone Analysis

100 mg of *Ginkgo biloba* L. product was weighed in a 50 ml falcon tube and 20 ml of 90% MeOH solution was added. The sample was extracted using an ultrasonic bath (Sonorex® RK 154 BH, Bandelin Electronic® GmbH & Co.KG, Berlin, Germany) at 50°C for 15 min. It was then centrifugated at 3000 rpm for 10 min. (Hettich®, Rotina 35R Type, Tuttlingen, Germany) and the supernatant was transferred to a new falcon tube. This extraction step was repeated twice more. Transferred supernatants were combined (~50 ml) vortexed-mixed and completed to a volume of 50 ml with deionized water. Prepared samples were transferred to glass vial by diluting 1:10 (w/v) with deionized water. Calibration standards at 0.5, 1.0, 2.0, and 4.0 µg/ml were also prepared by diluting the standard mixture solution of terpene lactones using deionized water. All standards and samples were kept at 4°C until analysis. The chromatographic analysis was performed on Waters® ACQUITY UPLC (Waters®, Milford, MA, USA) system equipped with a binary solvent delivery system and autosampler. The chromatographic separation was achieved on Waters® ACQUITY UPLC HSS (High Strength Silica) T3 column (2.1 mm x 75 mm, 1.8 µm). The mobile phase consists of solution A (water containing 0.005% acetic acid) and solution B (methanol containing 0.005% acetic acid). The gradient elution was optimized as follows: 10% B (0–0.5 min.), 10–70% B (0.5–7 min.), 70–10% B (7-7.01 min.), 10% B (7.01-8 min.). The flow rate was 0.35 ml/min. The column and autosampler temperatures were maintained at 40°C and 4°C, respectively. The injection volume was 10 µL. The detection was done via Waters® Xevo TQ tandem quadrupole mass spectrometer (Micromass® MS Technologies, Manchester, UK) with electrospray ionization (ESI) interface in multiple reaction monitoring (MRM) and positive ionization mode. The needle capillary voltage was set at 3 kV. The flow rate and temperature of desolvation gas were 850 L/h and 450 °C, respectively. The flow rate of cone gas was 50 L/h and source temperature operated at 150°C. These conditions were optimized by analyzing the reference compounds and comparing the signal-to-noise ratio (S/N) for each analyte. All ESI and mass spectrometer conditions were optimized individually for each target compound and listed in Table 2. Dwell times were automatically set by the software. Data acquisitions and quantifications were performed using Waters® Mass-Lynx software with the Target Lynx program.

Table 2. MRM transitions and mass spectrometry parameters for terpene lactone analysis.

Analyte / Ionization Mode (+/-)	Precursor Ion (m/z)	Product Ion (m/z)	Dwell Time (sec.)	Cone Voltage (V)	Collision Energy (eV)
Bilobalide / +	325.1	163.0	0.005	30	20
Ginkgolide A / +	407.1	350.9	0.005	50	15
Ginkgolide B / +	423.1	367.1	0.005	22	16
Ginkgolide C / +	438.5	382.9	0.005	30	16
Ginkgolide J / +	469.0	423.0	0.005	30	15

2.4. Ginsenoside Analysis

500 mg of *P. ginseng* product was weighed and 15 ml of 90% MeOH was added. Ultrasound-assisted extraction was performed at 50°C for 15 min. The sample was centrifugated at 3000 rpm for 10 min. After the transfer of supernatant to a new falcon tube, the extraction process

was repeated two more times and all supernatants were combined (~45 ml) after each extraction step. Extracts were vortexed-mixed and completed to volume of 50 ml. Final prepared samples were transferred to glass vial by diluting 1:10 (w/v) with ultra-pure water. Calibration standards at 0.5, 1.0, 2.0, and 4.0 µg/ml concentrations were prepared by appropriate dilution of the standard mix. solutions of ginsenosides. Waters® ACQUITY UPLC system equipped with a binary solvent delivery system and autosampler was used to perform the separation. A chromatographic resolution was crucial and this was achieved using a Waters® Acquity BEH (Bridge Ethylene Hybrid) C18 column (2.1 mm x 50 mm, 1.7 µm). The liquid chromatography phases consisted of water as mobile phase A and acetonitrile (ACN) as mobile phase B without any modifiers. The gradient elution was optimized as follows: 15% B (0–1 min.), 15–35% B (1–9 min.), 35–75% B (9–12 min.), 75–15% B (12–12.01 min.), 15% B (12.01-14 min.). The flow rate was 0.4 ml/min. The column oven set to 40°C and autosampler temperature was maintained at 4°C. The injection volume was 10 µl. The high accuracy mass spectrometric data were recorded on Waters® Xevo TQ tandem quadrupole mass spectrometer with ESI source in MRM mode. The optimized MS parameters were as follows: the capillary voltage: 3 kV, flow rate, and temperature of desolvation gas: 800 L/h and 450 °C respectively, flow rate of cone gas: 50 L/h, and source temperature: 150°C. Ionization states and MS parameters were optimized individually for each target compound and they were listed in Table 3. Dwell times were automatically set by the Mass-Lynx software. Data acquisition was controlled by the Target Lynx software from Waters®.

Table 3. MRM transitions and mass spectrometry parameters for ginsenoside analysis.

Analyte / Ionization Mode (+/-)	Precursor Ion (m/z)	Product Ion (m/z)	Dwell Time (sec.)	Cone Voltage (V)	Collision Energy (eV)
Ra_1 / -	1209.0	945.0	0.005	80	40
Ra_1 / -	1209.0	1077.0	0.005	80	40
Rg_2 / -	783.0	391.0	0.005	85	40
Rg_2 / -	783.0	475.0	0.005	85	40
Rg_2 / -	783.0	637.0	0.005	85	40
Ra_3 / +	1263.0	437.0	0.005	80	50
Ra_3 / +	1263.0	497.0	0.005	80	50
Ra_3 / +	1263.0	789.0	0.005	80	50
Rb_1 / +	1131.0	305.0	0.005	85	55
Rb_1 / +	1131.0	365.0	0.005	85	55
Rb_1 / +	1131.0	789.4	0.005	85	55
Rb_1 / +	1131.0	772.1	0.005	85	55
$Rb_2 + Rb_3$ / +	1101.7	789.4	0.005	90	55
$Rb_2 + Rb_3$ / +	1101.8	335.0	0.005	85	55
Rc / +	1101.0	335.0	0.005	80	50
Rc / +	1101.7	789.4	0.005	85	50
Re / +	969.6	203.2	0.005	85	55
Re / +	969.6	349.4	0.005	85	55
Rd / +	969.0	365.0	0.005	85	50
Rd / +	969.6	789.4	0.005	85	50
Rg_1 / +	823.5	203.2	0.005	85	50
Rg_1 / +	823.5	643.3	0.005	85	50
Rf / +	823.0	365.0	0.005	80	50
Rf / +	823.0	424.0	0.005	80	50

2.5. Phenolic Profiling

1.0 g of all products was weighed and 30 ml 70% ethanol (EtOH) solution was added for extraction. Samples were extracted by shaking overnight using a rotary orbital shaker (Thermo Fisher ScientificTM, Inc.MaxQ 4000 Benchtop Orbital Shaker, Waltham, MA, USA). It was centrifugated at 3000 rpm for 5 min. and the supernatant was separated. Extraction was repeated and the total volume of supernatants was completed to 100 ml with EtOH. Samples were diluted to appropriate volume depending on the phenolic concentration of the sample to be able to quantify between the calibration range with deionized water and filtered to glass vials using a 0.45 µm PVDF filter (Interlab®, Istanbul, Turkey) prior to chromatographic analysis. Calibration standards at 0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 µg/ml were prepared from stock standard mixture by applying serial dilutions. The chromatographic system was Waters® ACQUITY UPLC, which consisted of a binary solvent delivery system and autosampler. The separation was performed on Waters® CORTECS T3 column (1,6 µm 2,1 x 150 mm) using a gradient elution of (A) water containing 0.01% acetic acid and (B) 80:20 ACN:MeOH containing 0.01 acetic acid at a flow rate of 0.25 ml/min. Gradient elution was applied as follows: 2% B (0–1.30 min.), 2–55% B (1.30–35 min.), 55–95% B (35–37 min.), 95–2% B (37–37.01 min.), 2% B (37.01-40 min.). The column and autosampler tray temperatures were 30°C and 10°C, respectively. The effluent from the LC outlet was directed into the ionization electrospray source of Waters® Xevo TQ tandem quadrupole mass spectrometer after 1.0 min. delay using the embedded valve on the device. The ion source and desolvation temperature were held at 150 °C and 450 °C, respectively. The flow rate of desolvation and cone gas were optimized at 850 L/h and 50 L/h respectively. The needle capillary voltage was determined at 2 kV for ideal responses. MRM mode was employed for analysis. Peak areas for all components were automatically integrated using Mass-Lynx software with the Target Lynx program (Waters®). All ESI and MS parameters were optimized individually for each target compound and listed in Table 4.

Table 4. MRM transitions and mass spectrometry parameters for phenolic compound analysis.

Analyte	Precursor Ion (m/z)	Product Ions (m/z)	Cone Voltage (V)	Collision Energy (eV)	Ionization Mode (+/-)
Cinnamyl Aldehyde	133.2	55.0 / 77.2 / 105.2	20	15 / 20 / 15	+
3,4 Dimethoxy Benzaldehyde	167.0	124.0 / 139.2	20	15 / 15	+
Phloroglucinol	124.9	56.9 / 82.8	25	15 / 15	-
4-Hydroxybenzoic Acid	137.0	92.9	20	15	-
Transcinnamic Acid	147.0	77.0 / 102.8	25	20 / 20	-
Protocatechunic Acid	152.9	108.8	25	15	-
p-Coumaric Acid	163.0	93.0 /119.0 / 147.0	25	20 / 20 / 20	-
2-OH Coumaric Acid	163.0	93.0 / 119.0 / 147.0	25	20 / 20 / 20	-
m-Coumaric Acid	163.0	93.0 /119.0 / 147.0	25	20/20/20	-
Phenyllactic Acid	165.1	102.8 / 118.9 / 146.9	25	15 / 15 / 10	-
Vanilic Acid	166.9	90.8 / 108.1 / 123.2 / 152.2	25	20 / 20 /10 /25	-
Homogentisic Acid	167.0	122.9 / 123.1	20	20 / 20	-
Gallic Acid	169.0	124.9	25	20	-
Shikimic Acid	173.0	73.0 / 93.0 / 111.0	25	20 / 20 / 20	-
Caffeic Acid	179.0	135.0	25	20	-
Quinic Acid	191.1	59.0 / 84.8 / 92.8 / 126.8	35	20 / 20 / 20/ 20	-
Ferrulic Acid	193.0	134.0 / 149.0 / 178.0	25	20 / 20 / 20	-

Table 4. *Continues*.

Syringic Acid	197.0	123.0 / 167.0 / 182.0	25	20 / 20 / 20	-
3,4 Dimethoxycinnamic Acid	206.7	102.7	25	20	-
Methylsyringate	211.2	181.0 / 196.0	25	20 / 20	-
Quercetin	301.0	150.8 / 178.9	35	20 / 20	-
Ellagic Acid	301.0	185.2 / 229.0/ 257.0/ 284.2	30	30 / 25 /25 / 30	-
Hesperetin	301.3	135.8/ 150.8/ 164.1/ 241.7	25	20 / 20 / 20/ 20	-
Taxifolin	303.0	125.0	25	20	-
Epigallocatechin	305.2	124.8/ 164.8/ 166.9/ 219.0	25	20 / 20 / 20/ 20	-
Isorhamnetin	315.0	300.0	25	20	-
Myricetin	317.0	137.2 / 151.2 / 179.2	35	25 / 25 / 20	-
Chlorgenic Acid	353.3	179.0 / 191.0	25	20 / 20	-
Rosmarinic Acid	359.0	161.0 / 197.0	25	20 / 20	-
Rutin	609.1	300.0 / 301.0	25	20 / 20	-
Resveratrol	227.0	143.0 / 185.0	30	20 / 20	-
Chrysin	253.0	151 / 209 / 225	25	20 / 20 / 20	-
Pinocembrin	255.0	151.0 / 171.0 / 213.0	25	20 / 20 / 20	-
Apigenin	269.0	117.3 / 149.0 / 151.0	40	30 / 25 / 25	-
Genistein	269.0	133.2/ 159.2/ 224.2 /240.0	40	30 / 20 / 25/ 20	-
Galangin	269.0	197.0 / 213.0 / 227.0	25	20 / 20 / 20	-
Naringenin	271.0	145.0 / 151.0	25	20 / 20	-
Pinobanksin	271.2	153.0 / 225.0 / 253.0	25	20 / 20 / 20	-
CAPE	283.0	135.0 / 161.0 / 179.0	25	20 / 20 / 20	-
Kaempferol	285.0	93.0 / 151.0 / 257.0	25	20 / 20 / 20	-
Luteolin	285.0	133.0 / 241.0 / 267.0	25	20 / 20 / 20	-
Epicatechin	289.1	108.8 / 203.0 / 245.0	25	20 / 20 / 20	-
Catechin	289.1	108.8 / 203.0 / 245.0	25	20 / 20 / 20	-

2.6. Free Radical Scavenging Activity (Antioxidant Capacity Assay)

The percentage of antioxidant activity (AA%) of each sample was assessed by DPPH free radical scavenging assay. Hydroalcoholic extracts prepared for phenolic profiling analysis were used as representative samples and reacted with the reagent of DPPH radical. For this, 2.3 ml of sample extract was mixed with 0.3 ml of DPPH solution (0.1 mmol/L) and it was incubated in the dark for 15 min. The changes in color (from deep violet to light yellow) were observed and the absorbance of sample solutions was read at 517 nm against ethanol/DPPH solution as a reagent blank using a UV-VIS spectrophotometer (Thermo Fisher Scientific® Inc., Electron Evolution 300 UV-VIS Spectrophotometer, Waltham, MA, USA).

3. RESULTS and DISCUSSION

3.1. Ginsenosides

12 commercial samples were gathered and analyzed using the developed Ultra Performance Liquid Chromatography Multiple Reaction Monitoring Mass Spectrometry (UPLC/MRM-MS) method for ginsenoside contents. Thanks to our high-resolution chromatographic separation method, as shown in Figure 1, ginsenoside molecules were resolved and analyzed in high specificity and in short analysis time. Only the ginsenoside Rb2 and ginsenoside Rb3 components were co-eluted and their concentrations are reported as total amount.

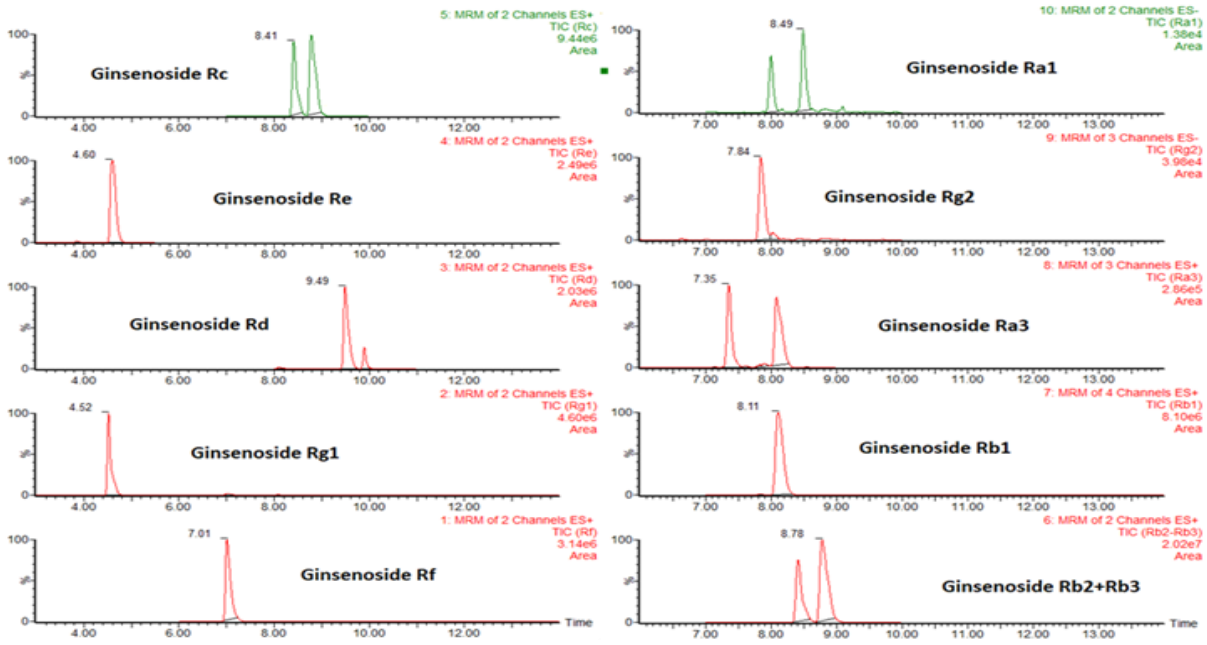
The majority of these samples are in mixture with different bee products; A1: pollen and honey, A2: pollen, honey, propolis, and royal jelly, A3: royal jelly and propolis, A4: pollen, royal jelly, and propolis, A5: royal jelly A8: propolis and honey A12: propolis, royal jelly, and honey. All quantified results were converted to their corresponding percentage for each ginsenoside component in the *P. ginseng* sample. The analysis results are given in Table 5.

Table 5. Analysis results of the ginsenoside contents.

Sample	Label claim	Serving size	Ginsenoside percentage of the product (w/w %) (mg/100mg)	Total Ginsenoside content at per serving (mg)	Recommended daily serving amount	Quantified daily intake amounts of Total Ginsenosides (mg)
A1*	-	7 g	0.16	11.19	7 g x 3	33.57
A2*	-	7 g	0.00	0.00	7 g x 3	0.00
A3*	80 mg of Ginseng Powder Extract + 20 mg of Ginsenosides at per serving	7 g	1.25	87.47	7 g x 1	87.47
A4*	235 mg of *Panax Ginseng* Extract at per tablet	1 tablet (780 mg)	2.12	16.51	2 tablet	33.02
A5*	-	7 g	0.14	9.71	7 g x 3	29.13
A6	26,96 mg of Ginsenosides + 49 mg of Ginseng Granul Extract + 490,3 mg of Ginseng Powder Extract	1 tablet (722 mg)	11.18	80.70	1 tablet	80.70
A7	50 mg of Korean Ginseng Extract at per serving	1 tablet (1770 mg)	0.96	16.91	1 tablet	16.91
A8*	12 mg of Ginsenosides per serving	7 g	0.17	12.05	7 g x 3	36.15
A9	500 mg of Korean Ginseng Powder Extract: 28,8 mg of Total Ginsenosides	1 capsule (670 mg)	5.19	29.04	1 tablet	29.04
A10	40 mg of *Panax Ginseng* Root extract	1 capsule (1560 mg)	1.90	13.30	1 tablet	13.30
A11	-	7 g	0.33	23.28	7 g x 1	23.28
A12*	%5 Red Ginseng extract at per serving	25 g	0,60	150.22	25 ml x 1	150.22

***** Samples are in form of a mixture with apitherapy products.

Figure 1. The chromatograms of ginsenosides standard mixture at 0.5 µg/ml concentration.



Total ginsenoside percentage values in the products were ranged between 0.14% and 11.80%. Label fraud has been detected at A2 coded sample, since the ginsenoside content could not be quantified as a result of the analysis. Approximate percentages of total ginsenoside contents were similar at investigated samples except for A6 and A12 coded samples which have considerably higher quantities for daily intake. In addition to the total ginsenoside content, the compound diversity of the product and amount of each separate ginsenoside molecule is also a very important point of evaluation regarding the raw material quality. Considering that the therapeutic effects of each ginsenoside compound may vary, the diversity of ginsenoside types in *P. ginseng* extracts is a matter to be considered. In the samples coded A9 and A10, the percentage distribution of the compounds can be seen as homogeneous, while Ginsenoside Rd was detected as the predominant substance over the other components in 6 of the analyzed samples (A1, A3, A4, A7, A8, A10). Ginsenoside Re, Ginsenoside Rb2, and Rb3 were other high abundance ginsenoside species at A1, A3 and A4 coded samples alongside a high level of Ginsenoside Rd. While the highest content of ginsenoside in A5 coded sample was determined as Rg2, ginsenoside Ra1 with Rg2 in sample coded A6 stands out as the most dominant species. In the sample coded A8, in addition to the ginsenoside Rd, Rb2, Rb3 components, unlike other samples, high levels of ginsenoside Rb1 and ginsenoside Rc were detected. In the A11 coded sample, ginsenoside Rg1 and ginsenoside Re components were in high amount, while in the A12 coded sample, ginsenoside Rc and ginsenoside Rb1 molecules were detected at high levels. As can be seen from the results, the ginsenoside types in the samples differ greatly due to factors such as extraction technique and ginseng types and/or origins. Results regarding the ginsenoside compound diversity are given in Table 6. Whether the products include apitherapy additives or not, they were observed that *P. ginseng* contents were appropriate for recommended daily consumption, and except for one sample, all products mostly meet the values stated on the labels. Daily intake amounts of the products were ranged between 13.3 mg/day and 150.2 mg/day. The recommended daily intake amounts were at the highest levels in two samples containing *P. ginseng* which are mixed with bee products (A3: 87.47 mg and A12: 150.20 mg).

Table 6. Analysis results of ginsenoside diversities by percentage.

Ginsenoside compounds by percentage (w/w %) in total ginsenoside composition										
Samples	Rf	Rg1	Rd	Re	Rc	Rb2+Rb3	Rb1	Ra3	Rg2	Ra1
A1	0.3	4.8	38.6	21.2	6.8	19.6	7.1	0.3	0.0	1.3
A2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A3	2.2	9.0	37.4	17.9	2.5	12.0	6.3	3.3	9.2	0.2
A4	1.0	9.8	30.6	16.8	7.5	17.3	7.4	0.6	6.9	2.2
A5	3.0	0.0	19.3	0.0	0.0	0.0	0.0	0.0	77.8	0.0
A6	0.2	3.4	15.7	6.9	4.0	11.2	2.5	0.1	26.0	29.9
A7	0.8	8.3	41.4	17.5	9.1	18.0	4.3	0.0	0.0	0.5
A8	4.5	2.7	21.8	9.9	18.5	16.4	20.6	5.7	0.0	0.0
A9	10.8	10.5	10.6	10.9	7.6	9.2	9.6	13.4	8.7	8.7
A10	6.4	5.8	19.4	11.1	11.9	13.4	13.4	11.5	3.9	3.2
A11	1.2	21.8	15.6	41.3	12.8	3.6	3.1	0.0	0.3	0.3
A12	11.1	8.1	10.6	6.1	32.2	8.0	18.0	3.0	2.3	0.4

3.2. Terpene Lactones

Ginkgo biloba L. contains mainly two active components; the terpene lactones and the ginkgo flavon glycosides, which together have been proven to be responsible for the polyvalent activities of *Ginkgo biloba* L. containing preparations. The nine commercial samples were analyzed by the developed UPLC/MS-MS method to quantify the levels of terpene lactones in these samples. Figure 2 shows typical chromatograms for the analysis of 5 of terpene lactone standards using the UPLC/MS-MS method under optimized instrument conditions.

According to the analysis results, the B4 coded sample provided the highest free form terpene lactone content (16.3 mg) for daily intake. The samples with the codes of B6 and B7 provided a higher amount of terpene lactone than other products and followed the B4 coded sample in terms of the total quantity. Only two of the analyzed products (B2, B6) were bee product mixtures. Among these products, the B6 coded sample gave the second-highest daily intake value among the analyzed products in terms of terpene lactone values. The results in Table 7 are shown that daily intake amounts of terpene lactones (mg) were ranged between 16.30 mg and 0.10 mg. While the ginkgolide B and C are found in all samples, the ginkgolide A was not quantified at only B2 coded sample. Ginkgolide J and the bilobalide have not been observed in B1, B2 and B3 coded samples. Ginkgolide J was also not quantified in the sample of B6. Analysis results of the investigation for the diversity of terpene lactones in the products are given in Table 8 The homogeneous distribution could only be detected in only a few samples, similar to the results acquired at *P. ginseng* analyzes. Samples coded B4, B5, B7, B8, B9 became prominent compared to other samples due to the reason that they contain all analyzed terpene lactone parameters and contain four terpene lactone species except ginkgolide J at high and similar percentages.

Figure 2. The chromatograms of terpene lactones standard solution analysis at 0.5 µg/ml concentration.

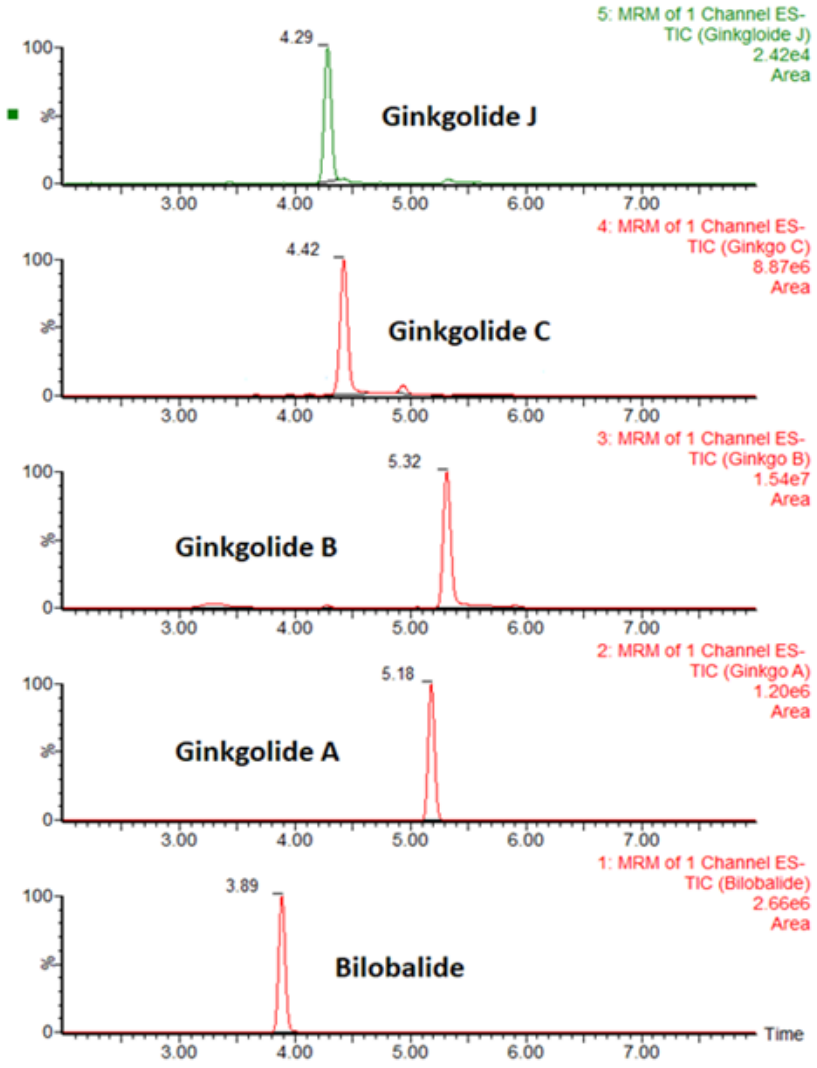


Table 7. Terpene lactone content analysis results of the *Ginkgo biloba* L. containing samples.

Samples	Label claim	Serving size	Total Terpene Lactone content of the product (w/w %) (mg/100 mg)	Terpene Lactone content at per serving (mg)	Recommended daily serving amount	Quantified daily intake amounts of Terpene Lactones (mg)
B1	90 mg of ginkgo leaf extract	1 tablet (150 mg)	0.07	0.10	1 tablet	0.10
B2*	-	7 g	0.02	1.05	7 g x 3	3.15
B3	-	7 g	0.03	2.10	7 g x 3	6.30
B4	7.05 mg of terpene lactone + 117,60 mg ginkgo leaf extract	1 tablet (722 mg)	1.13	8.16	2 tablet	16.30
B5	375 mg of *Ginkgo biloba* L. leaf extract	1 tablet (510 mg)	0.27	1.35	2 tablet	2.70
B6*	3.95 mg of terpene lactone	7 g	0.06	3.85	7 g x 3	11.55
B7	5 mg of terpene lactone + 90 mg standardized ginkgo extract leaf	1 tablet (316 mg)	1.74	5.48	2 tablet	10.96
B8	7.2 mg of terpene lactone + 120 mg of ginkgo extract	1 tablet (870 mg)	1.10	9.57	1 tablet	9.57
B9	120 mg of extract (7.2 mg of terpene lactone)	1 tablet (350 mg)	1.92	6.70	1 tablet	6.70

* Samples are in form of mixtures with apitherapy products.

Table 8. Analysis results of terpene lactone diversities.

Terpene lactones by percentage (w/w %)					
Samples	Bilobalide	Ginkgolide A	Ginkgolide B	Ginkgolide C	Ginkgolide J
B1	0.0	15.4	46.2	38.5	0.0
B2	0.0	0.0	66.7	33.3	0.0
B3	0.0	16.7	50.0	33.3	0.0
B4	21.7	20.8	17.3	31.4	8.8
B5	24.5	11.3	26.4	28.3	9.4
B6	27.3	18.2	27.3	27.3	0.0
B7	34.9	22.2	16.1	19.9	6.9
B8	36.8	18.2	14.1	24.1	6.8
B9	36.6	20.4	14.4	20.9	7.8

3.3. Phenolic profiles and antioxidant capacities

Previous analysis results have been shown that the forms of *P. ginseng* and *Ginkgo biloba* L. extracts mixed with apitherapy products have equivalent concentrations of the active ingredient (ginsenosides and terpene lactones) compared to non-mixed products on the market. The concentration of total and individual phenolic contents is given in Table 9. It can be observed that the content of the 43 components varies greatly among the different samples. Quercetin, rutin hydrate, CAPE, kaempferol, galangin, chrysin, gallic acid, pinocembrin, and isorhamnetin were monitored as predominant phenolic substances as a result of the phenolic profiling analysis. As shown in Figure 3, all these 43 components are analyzed in about 35 min. using novel UPLC/MS-MS method. Figure 4 shows the comparison of the phenolic contents of the bee product supplemented *P. ginseng* samples (A1, A2, A3, A4, A5, A8, A11, and A12) and pure *P. ginseng* extracts (A6, A7, A9, and A10).

Figure 3. The chromatograms of phenolic profiling analysis at concentration of 0.5 µg/ml standard solution.

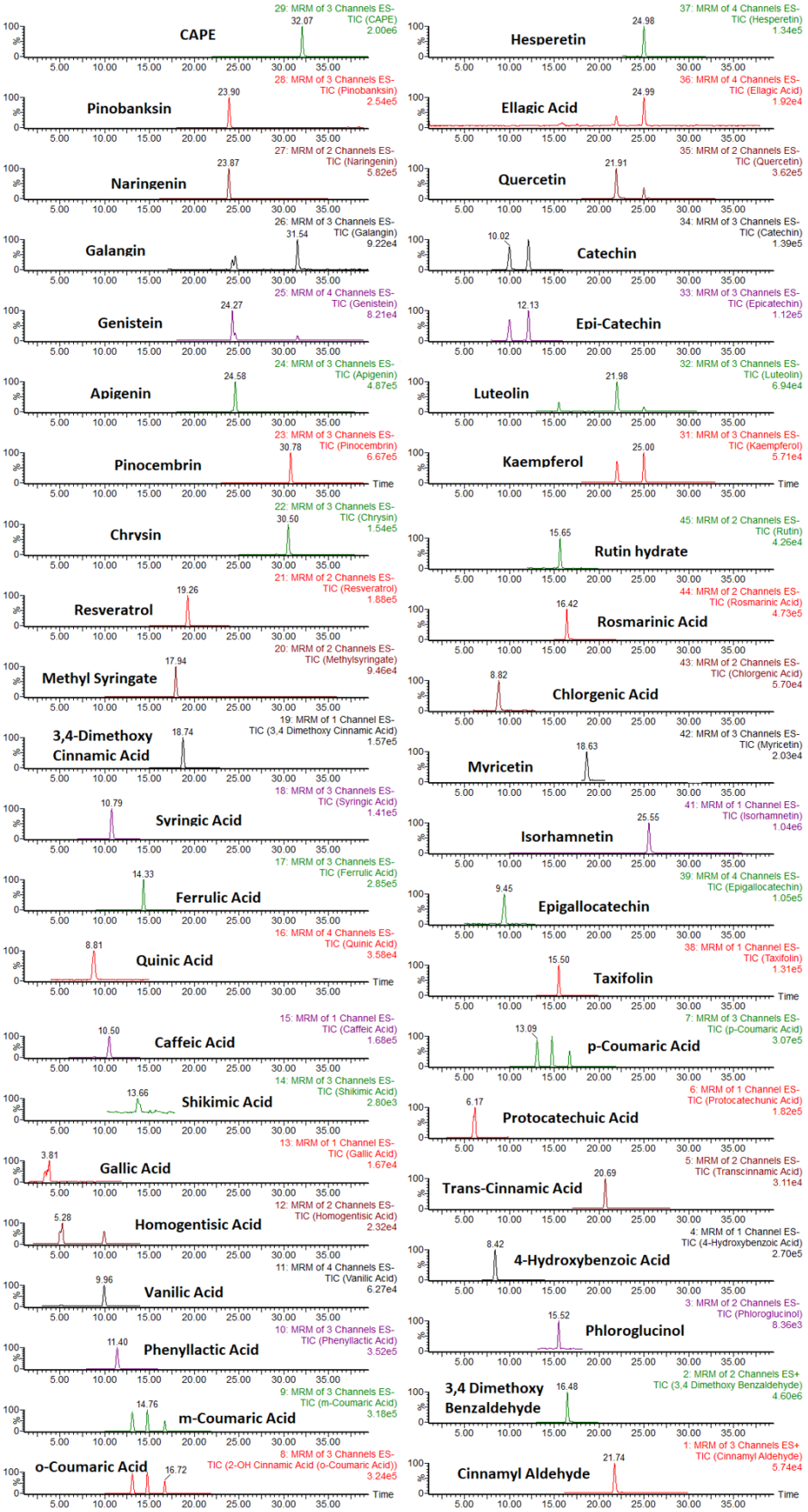
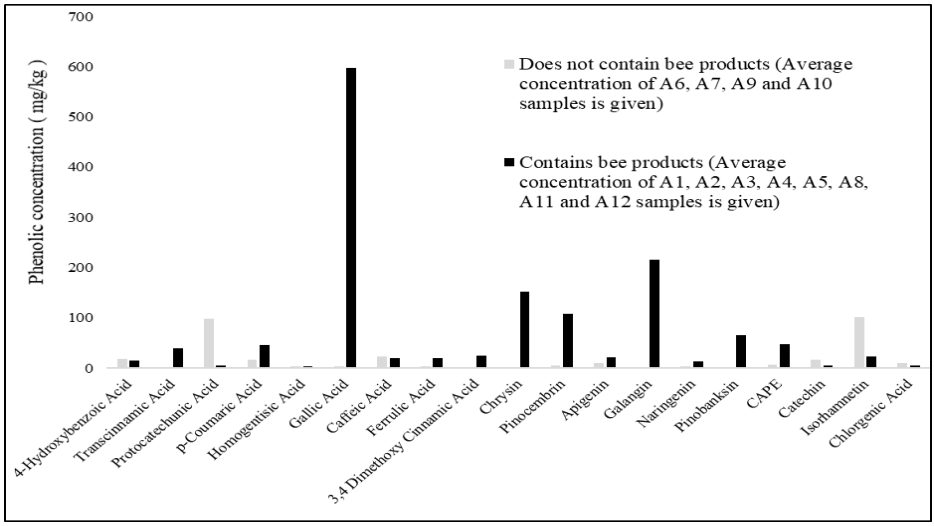


Figure 4. Comparison of selected phenolic compound contents in *P.ginseng* samples with and without bee product.



The same effect can also be seen in Figure 5 representing the *Ginkgo biloba* L. products containing bee products and comparison with those of not containing.

Figure 5. Contribution of bee product supplements in *Ginkgo biloba* L. extracts to phenolic diversity.

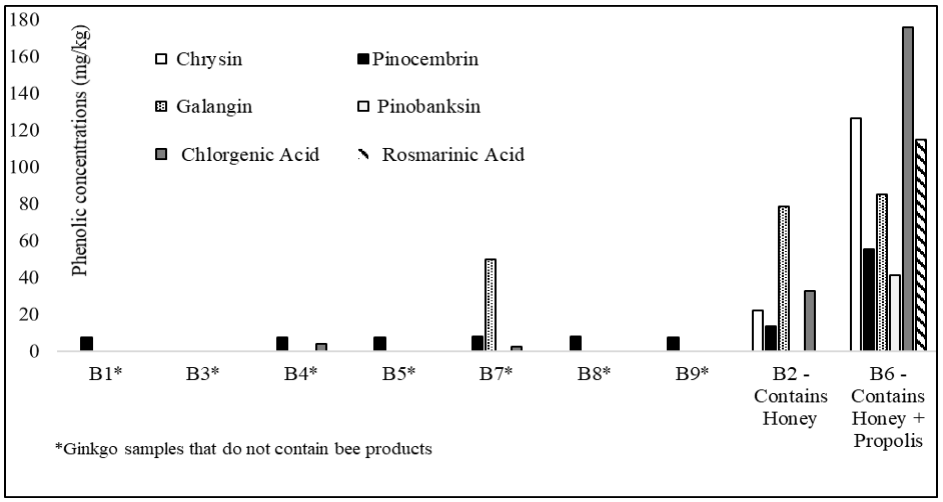


Table 9a and Table 9b also show the antioxidant activity results of the samples which were between 12% and 23%. According to these results, *Ginkgo biloba* L. and *P. ginseng* extracts supported by bee products such as pollen, propolis, and honey also provide high antioxidant capacity besides their phenolic diversities. Getting systematic knowledge about phenolic compounds and mostly flavonoids in the natural products are highly important for the phytotherapy product development strategies but also for the assessment of the therapeutic effects. Therefore, we thought, performing phenolic profiling investigations instead of total phenolic content analysis is much amenable for the samples. It was found that 4-hydroxybenzoic acid, transcinnamic acid, protocatechuic acid, p-Coumaric acid, homogentisic acid, gallic acid, caffeic acid, ferulic acid, 3,4-dimethoxy cinnamic acid, chrysin, pinocembrin, apigenin, galangin, naringenin, pinobanksin, CAPE, catechin, quercetin, isorhamnetin, chlorogenic acid, rosmarinic acid, and methylsyringate were the abundant phenolic derivates, which especially arises with the addition of bee products.

Table 9a. Total antioxidant activities and the phenolic profiles of the *P. ginseng* products.

Analyte	Sample Results (mg/kg)											
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Cinnamyl Aldehyde	0.1	-	-	1.2	-	1.0	-	-	-	-	1.7	-
3,4 Dimethoxy Benzaldehyde	-	-	-	-	-	-	-	-	-	71.9	-	-
Phloroglucinol	-	-	-	-	-	-	-	-	-	-	-	-
4-Hydroxybenzoic Acid	1.5	11.6	14.1	69.4	-	70.9	-	2.7	-	-	16.1	5.3
Transcinnamic Acid	13.6	28.4	89.6	161.3	0.9	0.2	-	6.7	2.1	-	8.0	5.2
Protocatechunic Acid	2.5	13.7	-	20.2	-	392.0	-	4.6	1.5	-	2.8	0.4
p-Coumaric Acid	-	19.8	342.1	2.9	-	68.8	-	-	-	-	-	6.5
2-OH Coumaric Acid	-	-	-	-	-	-	-	-	-	-	-	-
m-Coumaric Acid	-	-	-	-	-	-	-	-	-	-	-	-
Phenyllactic Acid	-	2.0	-	-	-	18.8	-	0.5	-	-	-	0.9
Vanilic Acid	-	-	-	-	-	91.8	-	-	-	-	-	-
Homogentisic Acid	-	4.1	1.9	7.3	0.7	-	0.8	3.1	7.4	3.5	0.7	2.3
Gallic Acid	207.1	3869.1	-	491.7	-	-	16.9	-	-	-	-	209.8
Shikimic Acid	-	-	-	-	-	-	-	-	-	-	-	-
Caffeic Acid	2.2	-	109.7	13.9	-	89.4	-	-	-	-	1.0	37.2
Quinic Acid	-	-	-	-	-	-	-	-	-	-	-	-
Ferrulic Acid	0.6	29.1	117.8	2.1	-	14.9	-	-	-	-	-	4.3
Syringic Acid	0.2	9.3	-	0.3	1.4	33.7	-	0.4	0.6	-	-	0.2
3,4 Dimethoxycinnamic Acid	-	-	31.9	155.1	-	-	-	6.2	-	-	-	8.3
Methylsyringate	-	-	-	-	-	-	-	5.7	-	-	-	-
Resveratrol	-	-	-	-	-	-	-	-	-	-	-	-
Chrysin	26.9	25.8	337.2	497.8	26.5	-	-	157.9	-	-	21.9	120.8
Pinocembrin	10.5	24.5	248.9	338.9	8.5	7.4	7.7	76.1	7.5	-	13.5	140.7
Pinobanksin	-	-	261.4	127.0	-	-	-	54.5	-	-	-	80.3

Table 9a. *Continues.*

CAPE	12.4	10.0	164.0	50.5	11.1	8.1	7.7	9.2	7.6	7.2	8.0	120.6
Kaempferol	-	-	-	-	-	477.7	-	-	-	40.3	-	-
Apigenin	-	-	48.8	51.7	-	40.6	-	24.3	-	-	17.7	28.9
Genistein	-	-	-	-	-	461.1	-	-	-	-	-	-
Galangin	48.0	199.2	287.1	816.2	32.4	-	-	176.2	-	-	78.3	85.8
Naringenin	-	1.5	46.6	32.6	-	12.5	-	8.1	-	-	0.1	13.0
Luteolin	-	-	-	-	-	-	-	-	-	-	-	-
Epicatechin	-	-	-	-	-	11.6	-	5.3	-	-	-	-
Catechin	-	-	-	11.6	-	68.8	-	28.8	-	-	-	-
Quercetin	27.2	24.9	25.6	52.5	-	3897.7	29.4	26.2	16.9	188.6	33.0	80.0
Ellagic Acid	-	-	-	-	-	-	-	-	-	-	-	-
Hesperetin	-	-	2.1	4.1	2.6	-	3.2	2.9	3.0	3.3	2.0	2.8
Taxifolin	-	-	-	-	-	-	-	-	-	-	-	-
Epigallocatechin	-	1.5	0.5	0.4	1.0	63.0	0.1	1.1	1.4	1.1	1.1	0.5
Isorhamnetin	17.1	15.1	33.1	46.9	12.6	368.3	12.6	19.8	12.5	15.7	17.1	18.4
Myricetin	-	-	-	-	-	-	-	-	-	-	-	-
Chlorgenic Acid	4.9	-	-	-	-	3.8	-	-	35.3	-	32.6	1.8
Rosmarinic Acid	-	-	-	-	-	-	-	-	-	-	-	-
Rutin	-	-	-	2.0	-	1609.3	-	0.4	-	-	33.4	-
Total Phenolics (mg/g)	0.4	4.3	2.2	3.0	0.1	7.8	0.1	0.6	0.1	0.3	0.3	1.0
Total Antioxidant Activity (%)	90.5	83.9	87.7	89.1	13.0	85.2	20.7	61.1	30.8	38.4	63.7	92.0

Table 9b. Total antioxidant activities and the phenolic profiles of *Ginkgo biloba* L. products.

Analyte	Sample Results (mg/kg)								
	B1	B2	B3	B4	B5	B6	B7	B8	B9
Cinnamyl Aldehyde	-	1.7	-	1.0	1.3	-	2.2	-	-
3,4 Dimethoxy Benzaldehyde	-	-	-	-	-	-	-	-	-
Phloroglucinol	-	-	-	-	-	-	-	-	-
4-Hydroxybenzoic Acid	49.2	16.1	1.3	70.9	142.8	5.9	249.8	159.3	306.5
Transcinnamic Acid	-	8.0	-	0.2	-	14.0	11.0	-	15.6
Protocatechunic Acid	35.4	2.8	2.6	392.0	158.5	21.7	901.2	94.2	914.4
p-Coumaric Acid	7.6	-	-	68.8	73.9	-	178.1	73.5	314.2
2-OH Coumaric Acid	-	-	-	-	-	-	-	-	-
m-Coumaric Acid	-	-	-	-	-	-	-	-	-
Phenyllactic Acid	-	-	-	18.8	-	1.7	-	-	-
Vanilic Acid	-	-	-	91.8	36.4	-	125.5	55.4	174.8
Homogentisic Acid	3.3	0.7	1.6	-	-	3.5	4.9	7.6	23.1
Gallic Acid	-	-	-	-	-	-	-	-	128.8
Shikimic Acid	-	-	-	-	-	-	-	-	-
Caffeic Acid	0.4	1.0	-	89.4	5.4	3.8	10.2	12.6	16.4
Quinic Acid	-	-	-	-	-	-	-	-	-
Ferrulic Acid	-	-	-	14.9	-	-	19.3	4.4	34.8
Syringic Acid	4.8	-	0.2	33.7	11.2	2.7	33.0	9.8	42.1
3,4 Dimethoxycinnamic Acid	-	-	-	-	-	-	-	-	-
Methylsyringate	-	-	-	-	-	2.4	-	-	-
Resveratrol	-	-	-	-	-	-	-	-	-
Chrysin	-	21.9	-	-	-	126.2	-	-	-
Pinocembrin	7.5	13.5	-	7.4	7.4	55.0	7.7	7.7	7.5
Pinobanksin	-	-	-	-	-	41.0	-	-	-
CAPE	9.4	8.0	8.3	8.1	7.6	9.7	7.4	7.7	7.3
Kaempferol	-	-	-	477.7	-	109.1	5009.5	640.8	5432.2
Apigenin	21.1	17.7	-	40.6	36.5	21.7	85.9	52.0	98.4

Table 9b. *Continues.*

Genistein	-	-	-	461.1	-	-	114.3	-	161.5
Galangin	-	78.3	-	-	-	84.9	49.7	-	-
Naringenin	3.2	0.1	-	12.5	21.6	10.3	40.6	23.2	36.6
Luteolin	-	-	-	-	-	-	54.8	-	44.3
Epicatechin	-	-	-	11.6	-	-	-	-	-
Catechin	-	-	-	68.8	33.6	8.3	19.3	10.9	24.6
Quercetin	19.7	33.0	-	3897.7	178.5	185.4	4483.7	913.5	5065.9
Ellagic Acid	-	-	-	-	-	-	-	-	-
Hesperetin	-	2.0	-	-	2.8	-	117.6	10.2	105.8
Taxifolin	-	-	-	-	11.5	-	2.1	4.6	14.4
Epigallocatechin	0.8	1.1	1.5	63.0	9.0	1.3	2.8	2.1	1.5
Isorhamnetin	15.6	17.1	12.5	368.3	20.0	24.0	706.8	249.7	810.3
Myricetin	-	-	-	-	-	-	-	-	-
Chlorgenic Acid	-	32.6	-	3.8	-	175.8	2.4	-	-
Rosmarinic Acid	-	-	-	-	-	114.9	-	-	-
Rutin	102.6	33.4	-	1609.3	347.5	14.6	1859.5	2004.5	1894.4
Total Phenolics (mg/g)	0.3	0.3	0.1	7.8	1.1	1.0	14.1	4.3	15.7
Total Antioxidant Activity (%)	72.5	63.7	20.3	85.2	85.3	91.1	71.2	83.5	74.1

According to results, propolis and pollen were mainly contributed to the phenolic profiles more than the royal jelly and honey supports.

Previous research based on the identification of the secondary metabolites of *P. ginseng* and *Ginkgo biloba* L. supplements includes tedious sample preparation steps, insensitive detection methods, and identifications based on passive detector acquisitions such as refractive index detection, UV detection, and so forth **(**Wang & Ju, 2000; Li *et al.*, 2005; Tang *et al.*, 2010). UPLC-ESI-MS/MS and developed methods enabled to identify and quantify the compounds of interest more accurately, specifically owing to their unique three-dimensional data (retention time, abundance, and mass to charge ratio). In addition to this, comprehensive phenolic profiling analysis of these herbal extracts was not achieved until this novel method was implemented. In this study, ultrasound-assisted solid-liquid extracts of the samples were analyzed directly in the UPLC-ESI-MS/MS system for three different phytochemical classes (terpene lactones, ginsenosides, and phenolics) without any additional purification, concentration, or derivatization. By using a single extract obtained from these samples, antioxidant and active ingredient measurements could be made simultaneously. In this way, the preliminary process has been simplified.

4. CONCLUSION

The active ingredient contents of *P. ginseng* and *Ginkgo biloba* L. supplements available in the market and the forms that supplemented with bee products were enlightened. Contents of the supplied samples were evaluated in terms of potential phytotherapy efficacy by means of research carried out using novel developed analytical methods. Within the scope of the study, phenolic substances analysis method including 43 compounds was developed using Ultra-Performance Liquid Chromatography, electrospray ionization (ESI) Tandem Mass Spectrometry (UPLC-ESI-MS/MS) system. Thus, sensitive and high-resolution profiling study was achieved. In addition, analytical methods were developed based on MS detection for the monitoring of ginkgolide, bilobalide, and ginsenoside active compounds. At our investigation, it has been determined that *P. ginseng* and *Ginkgo biloba* L. supplements available in the market predominantly contain sufficient amounts of active ingredients, as well as the analyzed amounts and compositions, were variable. The novel and practical analytical methods proved that the health benefits of the aforementioned products will increase owing to the fact that an increase in the phytochemical molecule diversity and antioxidant capacities if the content of the raw extract is supported with alternative apitherapy products.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Ismail Emir Akyildiz: Writing-Original draft preparation, Methodology, Conceptualization. **Sinem Raday:** Writing-Reviewing and Editing. **Ozge Erdem:** Resources. **Sezer Acar:** Investigation. **Ilknur Coskun:** Supervision. **Emel Damarli:** Project administration.

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Research Article

Investigation of Phenolic Composition, Antioxidant Capacity, and Antidiabetic Effect of *Ornithogalum lanceolatum* L.: An *in vitro* Study

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Abstract: Diabetes Mellitus is a global health problem that leads to macro- and microvascular diseases associated with hyperglycemia. Phytotherapy is one of the alternative ways to cope with this type of disease. The genus *Ornithogalum* is consumed as a wild edible plant and traditionally used for ailments. This study aims to investigate the phenolic composition using High-Performance Liquid Chromatography as well as antioxidant and antidiabetic effects using spectrophotometric method of *Ornithogalum lanceolatum* L. aerial parts and bulb. In order to determine the antioxidant capacity total phenolic content, total flavonoid content and DPPH and ABTS free radical scavenging activities were analyzed in *O. lanceolatum*. Moreover, in vitro inhibitory effects of the *O. lanceolatum* aerial parts and bulb on digestive enzymes were determined by evaluating the α-amylase and α-glucosidase activities. Protocatechuic acid was found to be the main compound in both plant parts. However, the amounts of the total phenolic acids and flavonoids were found higher in the aerial parts than those in bulb as well. Furthermore, *O. lanceolatum* aerial parts exhibited more radical scavenging activity than bulb. The α-amylase and α-glucosidase IC_{50} inhibition activities of aerial parts were found more efficient than those for bulb. It can be concluded that *O. lanceolatum* can enhance the antioxidant status and also can prevent nutraceutically postprandial hyperglycemia by inhibiting α-amylase and α-glucosidase enzymes. These findings reveal the importance of traditional remedies in the ethnopharmacological use of herbs.

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1. INTRODUCTION

Diabetes mellitus (DM) is a chronic and metabolic disease characterized by elevated blood glucose level that occurs when insulin cannot be produced enough or used effectively. An estimated of 422 million people suffer from DM worldwide and 1.6 million people solely died in 2016. The prevalence and incidence of DM in the world is increasing rapidly. Diabetes is a major cause of cardiovascular disease, high blood pressure, neuropathy, nephropathy, retinopathy, foot damage, and skin complications (WHO, 2016). The metabolic abnormalities of diabetes are caused by hyperglycemia such as non-enzymatic glycosylation and glucose

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auto-oxidation leading continuous production of free radicals. Increased free radical production causes the activation of some major pathways such as polyol pathway, protein kinase C, increase in formation of advanced glycation end-products, and overactivity of the hexosamine pathway. At the same time, these pathways lead to the production of free radicals again and play a role in the pathogenesis of complications (Zhang *et al*., 2020), therefore; lowering the high blood glucose is crucial in reducing the risk of diabetic complications. Various medications are already used to reduce hyperglycemia. Therapeutic herbal approaches are also traditionally used in the treatment and management of diabetes. Herbs have some bioactive compounds such as phenolic acids and flavonoids that exert a protective effect against various diseases. Besides, augmenting the antioxidant status helps both reducing free radicals and preventing the activation of the above-mentioned pathways (Ayepola *et al*., 2014).

Recently, medicinal and wild edible plants have gained attention as they offer both an important source of food and natural remedies for various ailments (Milella *et al*., 2014). They also have nutritive and dietetic value, largely owing to the presence of complex carbohydrates, mineral salts, vitamins, and polyphenolic compounds (Sekeroglu *et al*., 2006; Temiz, 2021). Turkey is one of the countries that have rich flora in terms of medicinal, aromatic, and wild edible plants. *Ornithogalum lanceolatum* known as burlumbuş, bulumbuç, burlumbuç, bulumbışık, is a wild edible plant, a member of Asparagaceae family, which shows propagation from Turkey to north Israel. It is collected in spring season and used in salads or consumed after cooking. *O. lanceolatum* is also traditionally used against arthralgia as topical painkiller in Turkey. Some *Ornithogalum* species are used especially in Turkish traditional and folk medicine against liver diseases, digestive system disorders, cough, asthma, edema, renal insufficiency, rheumatism, and diabetes (Koyuncu *et al*., 2018; Plančić *et al*., 2014; Renda *et al*., 2018). Based on ethnobotanical reports, this study was designed for scientific evaluation of *O. lanceolatum*. To the best of our knowledge, no antidiabetic investigation has been carried out until today, and very limited experimental studies have been reported in the literature on *O. lanceolatum*. This study aimed to evaluate folkloric information on the antidiabetic effect and antioxidant capacity as well as to determine phenolic composition of the extract from the aerial parts and bulb of *O. lanceolatum*.

2. MATERIAL and METHODS

2.1. Chemicals

All chemicals and reagents used in the study were HPLC and analytical grade and were procured from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Acarbose (Glucobay®, Turkey) was procured from pharmacy.

2.2. Plant Material and Extraction

Ornithogalum lanceolatum was picked up from Sertavul, Mersin, Turkey, in April 2020. The plants were immediately transported to the laboratory and foreign materials were removed. The plant was identified by a specialist. The plant specimens are being kept at the plant laboratory of Vocational School of Technical Sciences Karamanoğlu Mehmetbey University. The plants were dried outdoors and under shade. The all dried parts of the plant (aerial parts and bulb) were separately crumbled and extracted twice with 80% aqueous ethanol at 50°C for 3 h by continuous stirring (Wisd WiseStir MSH-20D). After being filtered with nylon cloths, the extract was filtered with 22 µm ptfe syringe filter. The *Ornithogalum lanceolatum* extracts (OLE) were stored in amber bottles at -20 °C for 4 weeks until further analyses.

2.3. HPLC Analysis of Plant Samples

The phenolic profiles of samples were determined as previously described (Özcan *et al*., 2018), with some modifications using the HPLC system (Agilent Technologies 1260 Infinity, USA).

Separation of phenolic compounds was performed using an Inertsil ODS-3 C18 (250 × 4.6 mm, 5 µm) column through gradient solvent system at 25°C. Elution was carried out at the flow rate of 1.0 mL/min using a binary mobile phase mixture of water/acetic acid (98:2 v/v) (A) and acetonitrile/water/acetic acid (50:49.5:0.5 v/v) (B). The gradient program was used as follows: 0 min 85% A, 30 min 80% A, 60 min 55% A. Diode array detector was used for monitoring at 254, 280, and 320 nm. The identification of each compounds was based on their retention times and spectral matching by comparison with external standards.

2.4. Determination of Total Phenolic and Total Flavonoid Content

Total phenolic content (TPC) in the OLE was determined by the modified Folin-Ciocalteu reagent method (Singleton and Rossi, 1965) using gallic acid as a standard. TPC were calculated as mg gallic acid equivalent 100 g^{-1} dry weight (mg GAE/100 gr dw). Total flavonoid content (TFC) was determined by the $AlCl_3$ method (Zhishen *et al*., 1999) using quercetin as a standard. TFC were calculated as mg quercetin equivalent 100 g^{-1} dry weight (mg QE/100 gr dw).

2.5. DPPH Radical Scavenging Activity

DPPH (2,2-Diphenyl-1-picrylhydrazyl) method was performed to evaluate the free radical scavenging activity with minor modifications as described by Pyo *et al*. (2004). Briefly, 100 µL diluted OLE or Trolox standard series and 3.90 mL methanolic solution of $DPPH^{\bullet}$ ($6x10^{-5}$ M) were mixed in a tube and vortexed. The tubes were incubated for 60 min at room temperature in the dark; thereafter, absorbance was measured against methanol at 517 nm (Shimadzu UV-3600, Kyoto, Japan). DPPH activity was expressed as IC_{50}, which was calculated graphically.

2.6. ABTS Assay

ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (7 mM) was reacted in potassium persulfate (2.45 mM) to produce the ABTS radical cation ($ABTS^{\bullet+}$) in the dark at room temperature for 12-16 h. The $ABTS^{\bullet+}$ working solution was diluted with distilled water to give an absorbance of 0.70±0.02 at 734 nm (Re *et al*., 1999). Briefly, 20 µL diluted OLE or Trolox standard series was added to 1980 µL adjusted $ABTS^{\bullet+}$ solution in tubes and vortexed. The tubes were left exactly for 6 min at room temperature in the dark, and then the absorbance was immediately measured at 734 nm. The percent inhibition was calculated graphically and expressed as IC_{50}.

2.7. Determination of α-amylase and α-glucosidase Inhibition Activity

α-amylase inhibition activity of the OLE was determined as previously described (Kim *et al*., 2005) with some modification. A total of 250 µL α-amylase (0.05 U/mL) in phosphate buffer (0.02 M, pH 6.9) was mixed with 200 µL various concentrations of the extract or acarbose and incubated at 37°C for 10 min. Thereafter, 250 µL 1% starch solution as the substrate was added and incubated at 37°C for 15 min. After the reaction was quenched with 500 µL 1% dinitrosalicylic acid, the tubes was boiled in water for 10 min. After cooling the tubes, the mixture was diluted with 5 mL distilled water. The absorbance of mixture was recorded at 540 nm. Acarbose was used as a standard. The α-amylase inhibition activity of the extract was expressed as IC_{50}, which was calculated graphically.

α-glucosidase inhibition activity was carried out with the method described by Kim *et al*. (2005) with some modification. The 60 µL of 1 U/mL α-glucosidase in phosphate buffer (0.1 M, pH 6.8) was mixed with 120 µL various concentrations of the extract or acarbose and incubated at 37°C for 10 min. Thereafter, 120 µL 4-nitrophenyl α-D-glucopyranoside (5 mM) was added as the substrate and tubes were kept at 37°C for 15 min. The reaction was quenched by adding 300 µL Na_2CO_3 (0.1 M) and the absorbance was recorded at 405 nm. α-glucosidase inhibition activity of the extract was expressed as IC_{50}, which was calculated graphically.

2.8. Statistical Analyses

All measurements were performed in triplicate. The data were expressed as mean and standard deviation ($\bar{X}$±SD). One-way ANOVA and *t*-test was performed and the results were correlated.

3. RESULTS and DISCUSSION

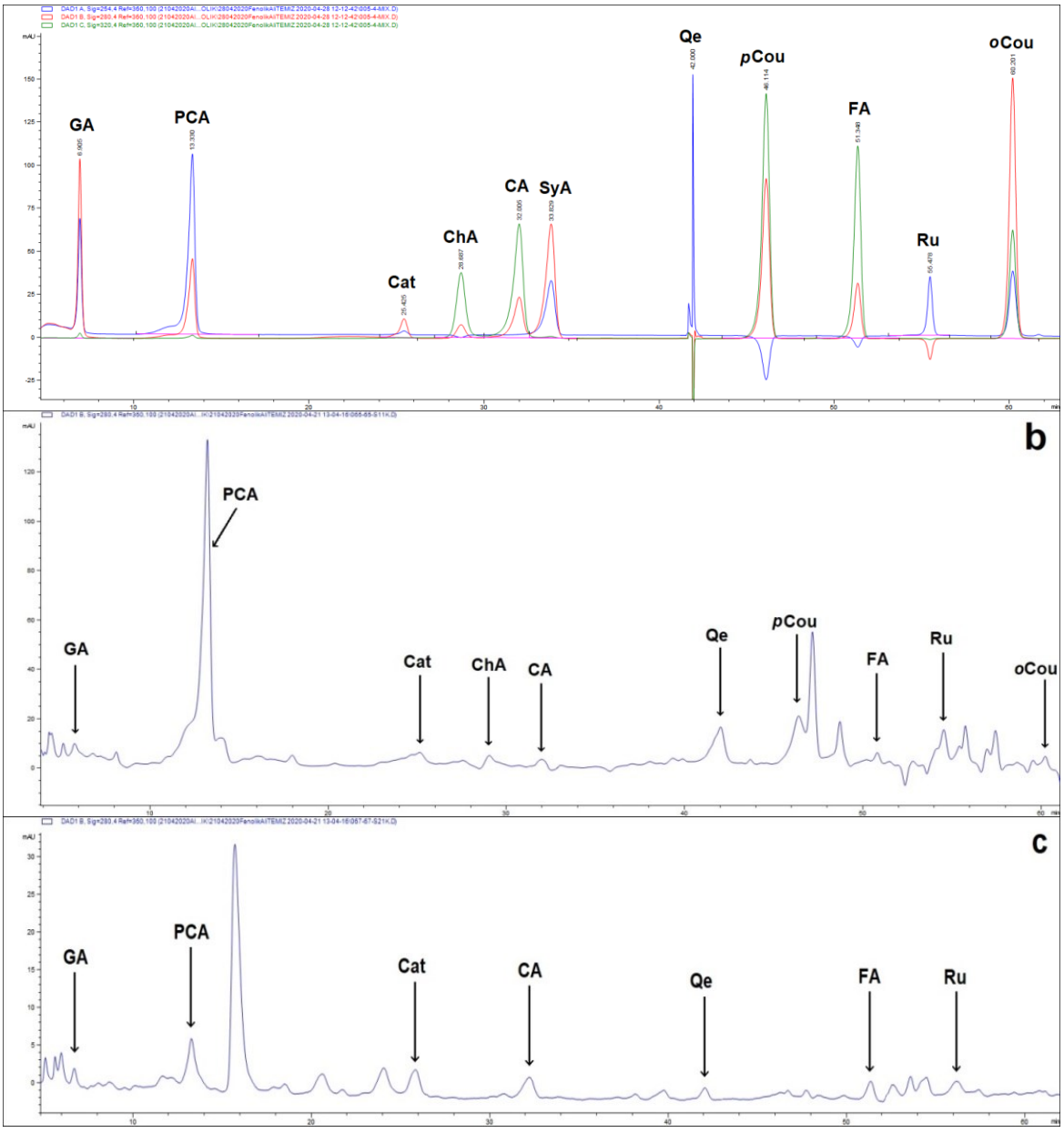
In the present study the antioxidant and antidiabetic activities as well as the phenolic composition of *Ornithogalum lanceolatum* ethanolic extract were evaluated. The amount of phenolic compounds of *O. lanceolatum* aerial parts and bulb is shown in Table 1 and HPLC chromatograms are depicted in Figure 1. Protocatechuic acid was the main compound in both plant parts, as well as rutin, quercetin, and *p*-coumaric acid were the most predominant phenolic compounds in the aerial parts. Syringic acid was not detected in both plant parts. Chlorogenic acid, *o*-coumaric acid, and *p*-coumaric acid were not also detected in bulb. On the other hand, the unidentified compound at 15.826th sec in HPLC might tentatively be a flavonoid which accumulated in bulb. Although the amount of phenolic compound in the current analysis was not fully compatible with the previous investigation on *O. lanceolatum* bulb, it has a similar phenolic composition (Özcan *et al*., 2018). The most abundant phenolic compounds in the bulb were reported to be catechin, gallic acid, catechol, and protocatechuic acid (Özcan *et al*., 2018). Biosynthesis of various phenolic compounds in plants depends on some factors, such as; species specificity, vegetation period, and growing conditions (climatic factors, water, light, altitude, and soil properties, etc.) (Chepel *et al*., 2020). Moreover, plants can produce various phenolic compounds at different growth stages and accumulate them in their bulb and/or other parts (Feduraev *et al*., 2019). In the current study, chlorogenic acid, *o*-coumaric acid, and *p*-coumaric acid that could not be detected in the bulb may be due to the plant which is in the flowering stage and which has not yet been accumulated in the bulb. Feduraev *et al*. (2019) also stated that at the fruiting stage, leaves and generative parts accumulated 3-7-fold much more of phenolic compounds in comparison to those in roots and stems.

Table 1. Amount of phenolic compounds of *Ornithogalum lanceolatum* (mg/kg dw).

	GA	PCA	Cath	ChA	CA	SyA	Que	*p*Cou	FA	Ru	*o*Cou
Aerial	88.3±0.2	1856.2±6.4	48.8±0.7	176.0±1.3	15.7±0.1	n.d	329.2±2.0	244.9±1.6	37.7±0.1	646.8±4.6	192.7±1.5
Bulb	32.8±0.1	120.8±1.0	36.4±0.7	n.d	21.5±0.1	n.d	42.0±0.1	n.d	18.2±0.1	70.3±0.8	n.d

Phenolic profile of ethanolic extracts obtained from *O. lanceolatum* aerial and bulb parts, Data shows mean ± standard deviation, dw: dry weight, GA: gallic acid, PCA: protocatechuic acid, Cath: catechin, ChA: chlorogenic acid, CA: Caffeic acid, SyA: syringic acid, Que: quercetin, *p*Cou: *p*-coumaric acid, FA: Ferulic acid, Ru: rutin, *o*Cou: *o*-coumaric acid. n.d: not determined.

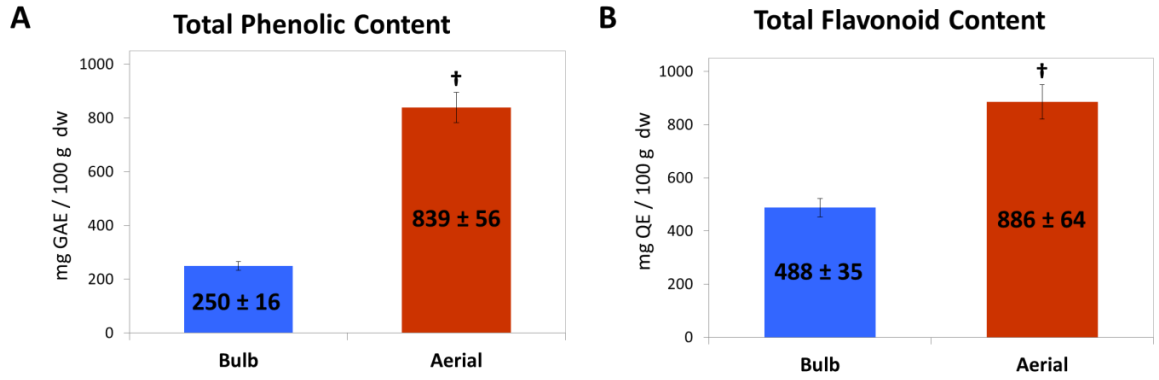
Figure 1. HPLC chromatograms of: a) the standards mixture overlay of all wavelengths (254, 280, and 320 nm), b) the obtained ethanolic extract from *O. lanceolatum* aerial parts at 280 nm wavelength, and c) the obtained ethanolic extract from *O. lanceolatum* bulb parts at 280 nm wavelength. Column: Inertsil ODS-3 C18 (250 × 4.6 mm, 5 µm); flow rate, 1.0 mL/min, temperature 25°C, gradient system. GA: gallic acid, PCA: protocatechuic acid, Cat: catechin, ChA: chlorogenic acid, CA: caffeic acid, SyA: syringic acid, Qe: quercetin, pCou: *p*-coumaric acid, FA: ferulic acid, Ru: rutin, oCou: *o*-coumaric acid.



Moreover, the phenolic composition findings were supported by TPC and TFC results (Figure 2A and B). The amounts of the total phenolic acids and flavonoids were higher in the aerial parts than those in bulb. Aerial parts and bulb had the total phenolic content with 835±79 and 249±23 mg GAE/100 g dw, respectively. The total flavonoid content of aerial parts and bulb were found to be 886±91 and 487±49 mg QE/100 g dw, respectively. It was reported that the TPC of methanolic *O. lanceolatum* and *Ornithogalum armeniacum* bulb extracts were calculated as 13.61 and 15.14 mg GAE/100 g fw, respectively (Özcan *et al*., 2018). In a previous study conducted on *O. sintenisii* aerial part and bulb were measured as 28.9±1.1 and 8.4±0.3 mg GAE/g dw for TPC, respectively. Also, in the same study, the TFC in aerial part and bulb

was determined to be 23.5±1.3 and 5.9±0.2 mg QE/g dw, respectively (Ebrahimzadeh *et al*., 2010). In another species of *Ornithogalum*, Apaydın and Yolcu (2017) reported TPC and TFC of methanol extracts of fresh *Ornithogalum umbellatum* possessed 5.821±0.008 mg GAE/g dw and 3.258 ±0.028 mg QE/g dw, respectively. *Ornithogalum orthophyllum* aerial parts and bulbs were recently determined to be 11.00±0.18 and 2.04±0.22 mg GAE/g extract for TPC (Renda *et al*., 2018). TPC and TFC results in current and previous studies showed that *Ornithogalum* species could be a promising source of antioxidants.

Figure 2. Amount of total phenolic content by Folin-Ciocalteu spectrophotometric method (A) and total flavonoid content by $AlCl_3$ spectrophotometric method (B) of ethanolic (80%) extracts from *O. lanceolatum* aerial and bulb parts. GAE: Gallic acid equivalent, QE: Quercetin equivalent, dw: dry weight, Data shows mean ± standard deviation, n=3, t-test was performed, $p<0.05$, †: Significantly different from bulb



The IC_{50} values of DPPH and ABTS of aerial part of *O. lanceolatum* were determined approximately 2-fold more efficient compared to its bulb (Table 2). Basically, a lower IC_{50} value indicates a higher inhibition activity. Herein, *O. lanceolatum* extracts exhibited DPPH and ABTS scavenging activity in proportion to their total phenolic and flavonoid content. It was reported that DPPH IC_{50} values of aerial parts and bulb of *Ornithogalum sintenisii* at flowering stage was found to be 368±15 and 669±25 µg/mL, respectively (Ebrahimzadeh *et al*., 2010). However, DPPH % inhibition of *O. umbellatum* stem and flower parts for concentration of 200 µg/mL were determined 24.92±0.012 % and 15.31±0.002 %, respectively (Aydın, 2020). In a recent study, methanol and water extracts of bulb of *Ornithogalum narbonense* were calculated as 12.60±0.30 and 4.02±0.55 mg TE/g extract, respectively for DPPH (Zengin *et al*., 2015). Besides, Zengin *et al*. (2015) reported *O. narbonense* bulb methanol and water extracts had 18.16±1.21 and 7.52±0.64 mg TE/g extract, respectively for ABTS scavenging activity. However, the ABTS IC_{50} value of polysaccharides extracted from *O. billardieri* bulb (Syn. *O. lanceolatum*) was interestingly determined as 1.51±0.1 mg/mL (Medlej *et al*., 2021). The extracts of aerial parts and bulb of *O. lanceolatum* have shown remarkable free radical scavenging ability comparing to other *Ornithogalum* species extracts.

Table 2. DPPH and ABTS IC_{50} values of *Ornithogalum lanceolatum* (dw).

	DPPH (mg TE/g)	ABTS (mg TE/g)	DPPH (mg/mL)	ABTS (mg/mL)
Aerial parts	5.09±0.02†	4.92±0.00†	12.53±0.03†	11.08±0.01†
Bulb	10.00±00	11.46±0.18	22.53±0.03	23.42±0.46

Antioxidant capacities of ethanolic (80%) extracts obtained from *O. lanceolatum* aerial and bulb parts. TE: Trolox equivalent, dw: dry weight, Data shows mean ± standard deviation, n=3, t-test was performed, $p<0.05$, †:Significantly different from bulb

α-amylase and α-glucosidase inhibition activities of *O. lanceolatum* are shown in Table 3. α-amylase inhibition IC_{50} value of aerial parts was more efficient than bulb (3.06±0.02 and 6.02±0.04 mg/mL, respectively). Similarly, α-glucosidase inhibition activity of aerial parts was higher than that for the bulb. The α-glucosidase inhibition IC_{50} values were 4.98±0.08 and 8.86±0.16 mg/mL in aerial part and bulb, respectively. Besides, α-amylase and α-glucosidase IC_{50} values for acarbose were found to be 0.38±0.02 and 0.57±0.02 mg/mL, respectively. The plant parts had higher IC_{50} inhibition values compared to acarbose, meaning that, it was less effective in digestive enzymes inhibition. Huyssteen *et al.,* (2011) stated that the aqueous extract of *O. longibracteatum* bulb showed 131.9% significant increase *in vitro* glucose utilization activity in Chang liver cells. Moreover, in a previous study, it was reported that administration of *Ornithogalum caudatum* alcohol extract to diabetic mice for 14 days declined blood glucose concentration (Cao *et al*., 2015). Phytochemicals in herbs can exert antidiabetic effects through various mechanisms. These mechanisms may be through inhibition of digestive enzymes and/or by acting like insulin-mimetic to reduce postprandial blood glucose (Temiz & Temur, 2019). Besides they might have antidiabetic effects such as reducing glucose absorption in the small intestine, stimulation of insulin secretion from islets, improving insulin sensibility, and increasing the uptake and bioavailability of glucose to the cells (Temiz and Temur, 2019; Temiz, 2021; Yang *et al*., 2015; Zhang *et al*., 2020). Backgrounds of these mechanisms have complex pathways in the cells. The antidiabetic action mechanisms of medicinal plants have not been fully clarified. Therefore, these studies may lead to the elucidation of the antidiabetic effect mechanisms with future studies.

Table 3. α-amylase and α-glucosidase inhibition IC_{50} values of *Ornithogalum lanceolatum* (dw).

	α-amylase (mg/mL)	α-glucosidase (mg/mL)
Aerial parts	3.06.±0.02†,‡	4.98±0.08†,‡
Bulb	6.02±0.04‡	8.86±0.16‡
Acarbose	0.38±0.02	0.57±0.02

Carbohydrate digestive enzyme inhibition of ethanolic (80%) extracts obtained from *O. lanceolatum* aerial and bulb parts, Acarbose: positive control, Data shows mean ± standard deviation, n=3, One-way ANOVA was performed, $p<0.05$, †: Significantly different from bulb, ‡: Significantly different from acarbose

4. CONCLUSION

The aerial parts and bulb of *O. lanceolatum* have remarkable antioxidant capacity. *O. lanceolatum* aerial parts can exert an antidiabetic effect through α-amylase and α-glucosidase inhibitory activities. The favorable antioxidant and antidiabetic effects of *O. lanceolatum* have revealed the potential efficacy of its use as a traditional medication in the maintenance of oxidant/antioxidant balance and in the management of diabetes mellitus. Investigation of traditional phytotherapeutic interventions may provide important new insights into the treatment of diabetes.

Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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Research Article

Evaluation of silibinin as an efflux pump inhibitor in *Bacillus subtilis*

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Abstract: Antibiotic resistance has become a global health problem for humankind. Improper use of antibiotics resulted in the increasing evolved bacterial resistance to them. There are different types of bacterial resistance mechanisms including efflux pumps. To overcome the efflux pump activity on the drugs, combinatorial therapy of the existing antimicrobials with natural products is a promising insight to prevent increasing multidrug resistance. In this study, the inhibitory action of a plant-derived molecule silibinin on efflux pumps of *Bacillus subtilis* was investigated. The cellular effect of silibinin was investigated using minimum inhibitory concentration and growth studies. In addition, the efflux pump action of silibinin was monitored by ethidium bromide accumulation assay on the organism. According to results, silibinin has a MIC value between 100-200 μgmL^{-1} on microplate assay and 100 μgmL^{-1} of silibinin inhibited the cell growth. Ethidium bromide accumulation assays were performed at a safe silibinin range (25 and 50 μgmL^{-1}) for eliminating the cell death, and ethidium bromide accumulation was increased with the increasing silibinin concentration. Ethidium bromide accumulation and growth results proved that silibinin has significant efflux pump inhibitor activity on *Bacillus subtilis* cells and silibinin is a promising inhibitor candidate to eliminate bacterial resistance mechanism.

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1. INTRODUCTION

Increased pressure imposed by improper and reckless use of antimicrobial agents has triggered the pace in development and transmission of bacterial resistance (Schwarz and Chaslus-Dancla, 2001). The non-susceptibility developed by bacteria to different classes of antimicrobials has led to the emergence of multidrug resistance (MDR) which is one of the most important global health threats (Magiorakos *et al.*, 2012). The basic types of resistance mechanisms are known as enzymatic inactivation of the antimicrobials, modification of the target sites, reducing the intracellular accumulation of antimicrobials by arranging influx/efflux mechanisms (Van Duijkeren *et al.*, 2018). Among these, efflux systems are considered as the major mechanisms

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that lead to MDR. These systems which are commonly comprised of transmembrane proteins enable the organisms to keep the concentrations of a wide range of different compounds at non-toxic levels by preventing their intracellular accumulation.

Efflux pumps are classified into six families based on their structure. These are (i) major facilitator superfamily (MFS), (ii) multidrug and toxic compound extrusion (MATE) family, (iii) small multidrug resistance (SMR) family, (iv) resistance-nodulation-cell division (RND) family, (v) ATP-binding cassette (ABC) superfamily, and (vi) proteobacterial antimicrobial compound efflux (PACE) superfamily. The latest one has been recently identified therefore its transport mechanism has not been clarified yet. Among the rest, only the ABC-type utilizes ATP while the other four families use proton motive force (PMF) for efflux (Du *et al.*, 2015; Lamut *et al.*, 2019).

The loss in available drug efficacies and the decrease in new antimicrobial discovery rates have increased the search on alternative strategies including combinatorial therapies. Combinatorial therapies of existing antimicrobials with natural products emerge as attractive approaches in the fight with increasing MDR. To this end, utilization of efflux pump inhibitors (EPIs) to interfere with efflux is one of the major strategies. Plants are immense sources of natural compounds, some of which are potential EPIs, so they are of utmost importance in the discovery of new antimicrobial agents. Isolation and identification of new EPIs will bring antimicrobials with lost efficacies back into the clinic. To date, many plant-based EPIs have been identified as reserpine (Gibbons *et al.*, 2003; Neyfakh *et al.*, 1991), piperine (Kumar *et al.*, 2008), roemerine (Avci *et al.*, 2019), baicalein (Chan *et al.*, 2011), 5'-methoxy-hydnocarpin (Stermitz *et al.*, 2000), and catechin gallates (Gibbons *et al.*, 2004).

Silibinin (or silybin) is the major component of silymarin extract from the seeds of *Silybum marianum* (or milk thistle, Asteraceae) and a member of flavonolignans. (Dobiasová *et al.*, 2020). It receives wide attention due to its anticancer, antioxidant, antibacterial, antifungal, anti-inflammatory, cardioprotective, neuroprotective, and hepatoprotective activities (de Oliveira *et al.*, 2015; Shen *et al.*, 2018; Wlcek *et al.*, 2013). More recently, silibinin has also been associated with an EPI property as it inhibited the efflux of norfloxacin through the NorA efflux pump protein in *Staphylococcus aureus* (Mahmood *et al.*, 2016).

In the present study, the ability of silibinin to inhibit the efflux pumps of *Bacillus subtilis* was evaluated using minimum inhibitory concentration determination, growth studies, and ethidium bromide accumulation assay. Berberine was used in growth studies since it is the substrate of many efflux pumps and its activity is weakened due to the activity of efflux pumps (Avci *et al.*, 2019). The clinical importance of *B. subtilis* is limited, it constitutes one of the model organisms for low G+C Gram-positives with a significant genomic abundance of multidrug transporters. *B. subtilis* is also the first microorganism in which the bacterial MDR phenomenon was discovered. Furthermore, it possesses a mechanism analogous to the mammalian multidrug transporter, P-glycoprotein (Lorca *et al.*, 2007; Neyfakh *et al.*, 1991)

2. MATERIAL and METHODS

2.1. Bacterial Strains and Chemicals

The efflux pump inhibitor (EPI) property of silibinin was tested in wild-type *Bacillus subtilis* 168 (DSM 402). Silibinin (CAS No. 22888-70-6) and berberine chloride hydrate (CAS No. 141433-60-5) were obtained from Sigma-Aldrich. Silibinin and berberine solutions were prepared in dimethyl sulfoxide (DMSO) (Duchefa, Netherlands).

2.2. Minimum Inhibitory Concentration (MIC) Determination for Silibinin

Minimum inhibitory concentration (MIC) of silibinin was determined via broth micro-dilution assay (Amsterdam, 1997). Two-fold serial dilutions of silibinin from 200 μgmL^{-1} to 0.097

μgmL^{-1} were prepared with Nutrient Broth (NB, Merck, Germany) in sterile 96-well U-bottom plates and a single line of the test plate was prepared with serial dilutions of the solvent DMSO as control. Each well was inoculated with 10^5 $CFUmL^{-1}$ cells. After a 24-hour incubation at 37°C, 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, Germany) dye reduction test was utilized to determine the wells with no visible cell growth. TTC solution at a final concentration of 0.5% (w/v) was added into each well. Plates were incubated at 37ºC for 1 hour. MIC was determined based on the color change in wells. Viable cells were recognized with the development of red color in the wells.

2.3. Growth Conditions

B. subtilis 168 cells were grown in NB medium at 37°C and 180 rpm and treated with silibinin, berberine, or silibinin-berberine combination as OD_{600} reached 0.45-0.55. Control cells were only treated with equal volume of DMSO. The growth of the cells was monitorized spectrophotometrically by measuring OD_{600} values in 1-hour intervals.

2.4. Ethidium Bromide Accumulation Assay

A modified version of the previously reported method was used to perform ethidium bromide (EtBr) accumulation assay (Jin *et al.*, 2011; Steinfels *et al.*, 2004).

After overnight growth, *B. subtilis* 168 cells were inoculated into a tube containing 5 mL fresh NB and grown at 37ºC and 180 rpm until OD_{600} reached 0.5. Cells were then centrifuged for 4 min at 2,000 *g* and 4ºC. Cell pellets were suspended in 2 mL of 0.35 M sodium chloride (NaCl). 180 µL of the cell suspension was mixed with 50 mM KP_i, 5 mM magnesium sulfate ($MgSO_4$), and 25 mM glucose. 10 µM of EtBr (Invitrogen, California, USA) was added to the mixture immediately after the addition of glucose. Silibinin treatment (25 and 50 μgmL^{-1}) was made prior to the addition of glucose. Control samples were supplied with an equal volume of DMSO. Fluorescence intensities were monitored for 20 min via Synergy HTX Multi-Mode Reader Reader (BioTek Instruments, Inc., Winooski, VT, USA) with excitation at 540 nm and emission at 590 nm (Serçinoğlu *et al.*, 2020).

3. RESULTS and DISCUSSION

Antibiotic resistance development against available antimicrobials has reached alarming rates which causes a bottleneck during the fight against bacterial infections. Since the novel antibiotics with different targets are limited, the discovery of new antimicrobial classes has a high priority. Plant-derived molecules may be considered as potential alternatives to existing drugs with their various biological activities and multi-target properties. In spite of that, the limited information about their mechanisms restricts their use.

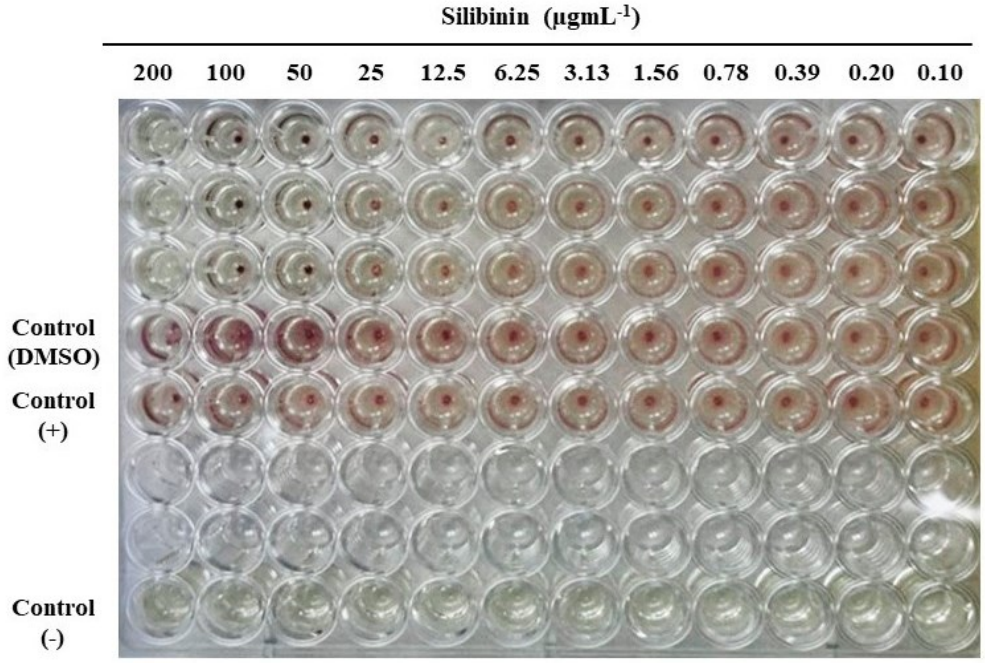
Silibinin has been reported to display diverse biological activities including antimicrobial, antioxidant, anticancer, anti-inflammatory, free radical scavenging, and membrane stabilizing properties under *in vitro* and *in vivo* conditions (Cai *et al.*, 2017; de Oliveira *et al.*, 2015). It has also been reported that silibinin inhibits P-glycoprotein-mediated cellular efflux (Zhou *et al.*, 2004) and is involved in bacterial resistance through drug efflux (Wang *et al.*, 2018). In the light of these recent findings, EPI property of silibinin was evaluated on the Gram-positive model organism *B. subtilis* 168. Following the analysis of cellular growth in the presence of silibinin alone and in a combinatorial treatment, the contribution of silibinin to intracellular EtBr accumulation was monitored.

3.1. Berberine and Silibinin Combinatorial Treatments

Broth micro-dilution assay was carried out to find that the MIC of silibinin lied between 100-200 μgmL^{-1} (Figure 1). In order to study the EPI property of silibinin, a working concentration

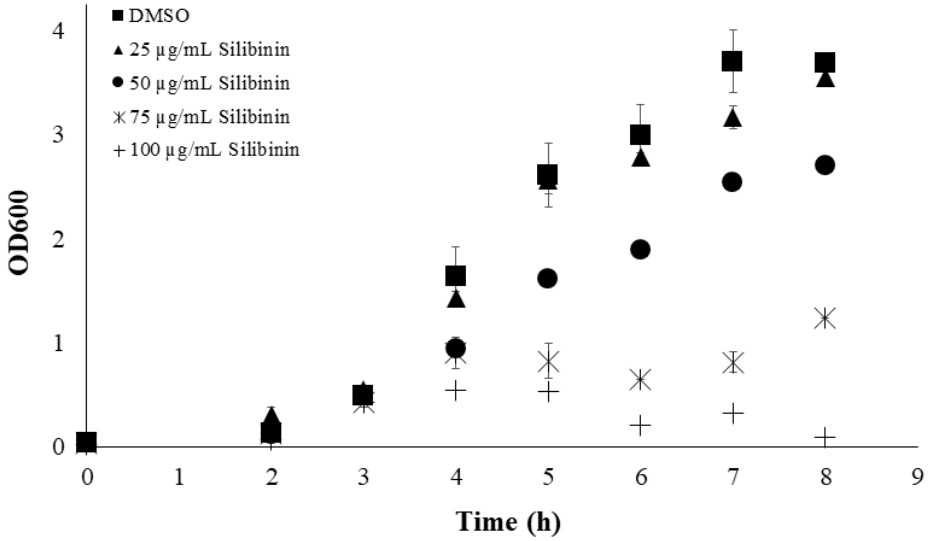
that is well below its MIC value was selected. Thus, microbial growth studies were carried out in the presence of increasing silibinin concentrations from 25 to 100 µgmL^{-1} (Figure 2).

Figure 1. Minimum inhibitory concentration (MIC) determination for silibinin.



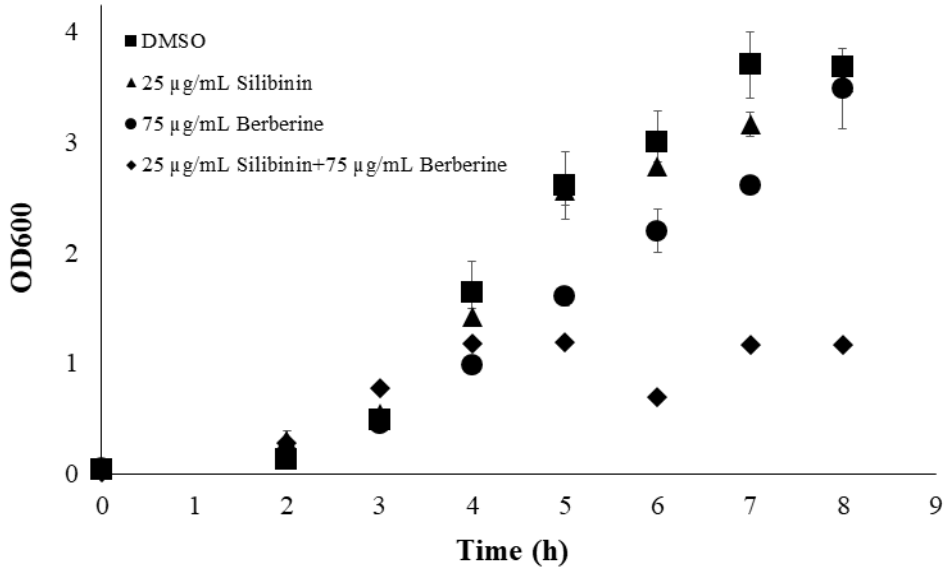
Treatment of the cells with 25 µgmL^{-1} silibinin barely altered the growth profile of *B. subtilis* 168 cells whereas treatment with 50 µgmL^{-1} silibinin led to a slight retardation in growth. When silibinin concentration was raised to 75 µgmL^{-1}, growth was severely affected. With 100 µgmL^{-1} there was no growth at all. Growth curves obtained with increasing silibinin concentrations have shown that 25 µgmL^{-1} silibinin is suitable for testing its EPI feature since it did not alter growth.

Figure 2. Microbial growth under increasing silibinin concentrations.



The antimicrobial berberine is widely known to be a substrate of a number of drug efflux pumps therefore its combination with different natural pump inhibitors may offer a way to enhance its efficacy (Avci *et al.*, 2019; Stermitz *et al.*, 2000). In order to evaluate the EPI property of silibinin, its combination with berberine has been tested on *B. subtilis* cells. Berberine working concentration was determined based on previous work (Avci *et al.*, 2019). In our previous work, 75 μgmL^{-1} berberine has been shown to only slightly affect *B. subtilis* 168 growth. Thus, 25 μgmL^{-1} silibinin was combined with 75 μgmL^{-1} berberine (Figure 3). Although either 25 μgmL^{-1} silibinin or 75 μgmL^{-1} berberine has no significant effect on cell growth alone, their combination killed the cells. Since plant-derived natural products are known to have multiple targets to cause death, EtBr accumulation test was further carried out to verify that silibinin's EPI property was responsible for the observed behavior.

Figure 3. Effect of the berberine and silibinin combination on microbial growth.



3.2. Effect of Silibinin on Efflux Pumps of *B. subtilis* 168

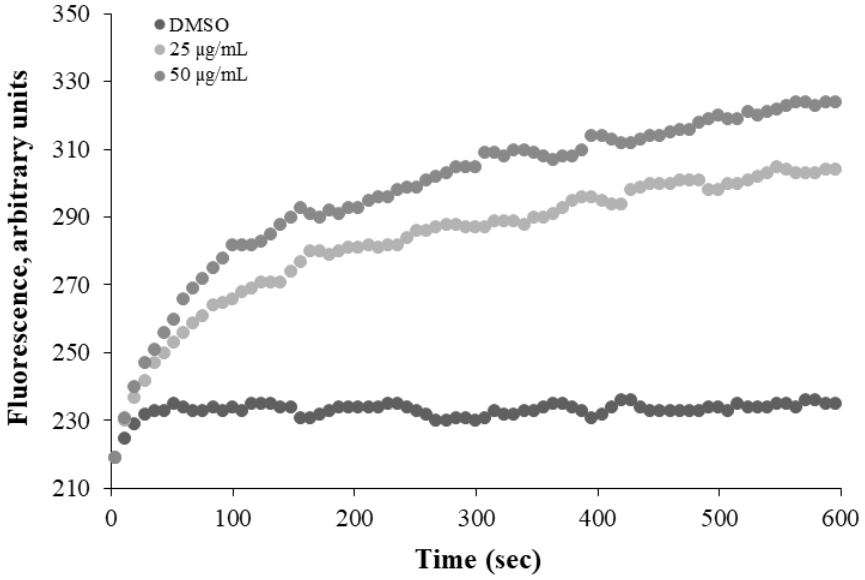
EtBr accumulation is a commonly used assay to monitor the activity of efflux pumps. EtBr passes the cell membrane and binds by intercalating between DNA base pairs. Therefore, its intracellular presence can be detected by fluorometry (540 nm as excitation and 590 nm as emission) (Steinfels *et al.*, 2004). However, free EtBr is also continuously effluxed through efflux pumps. Thus, in the presence of an EPI, the increase in fluorescence would be expected to be higher.

To this end, EtBr accumulation in *B. subtilis* 168 cells in the presence of silibinin was investigated to assess its EPI potential. The selected concentrations were believed to facilitate EtBr accumulation, while not causing cell death. Based on the growth results obtained in the presence of increasing concentrations of silibinin (Figure 2), this assay was carried out with 25 and 50 μgmL^{-1} silibinin.

In the control cell sample, which was treated with DMSO (the solvent of silibinin), fluorescence intensity was pretty constant after approximately 50 s. This showed that EtBr was inside the cells but with the action of the pumps, its concentration remained at its steady state value. The increase in fluorescence intensity due to EtBr accumulation was highest in the presence of 50 μgmL^{-1} silibinin. Since this concentration had a considerable effect on cell growth (Figure 2), this observed behavior in fluorescence could as well be a result of released genetic material due to cell death. When silibinin concentration was dropped to 25 μgmL^{-1}, the

recorded fluorescence was only slightly affected which demonstrated that silibinin displayed an EPI character (Figure 4).

Figure 4. EtBr accumulation in *B. subtilis* in the presence of silibinin.



Due to the significant genomic abundance compared to other Gram-positives, *B. subtilis* transporters have been extensively studied (Lorca *et al.*, 2007; Neyfakh *et al.*, 1991). Blt and Bmr (Ahmed *et al.*, 1995; Baranova *et al.*, 1999; Woolridge *et al.*, 1997) of MFS, YerP (Tsuge *et al.*, 2001) of RND superfamily, EbrAB (Masaoka *et al.*, 2000) of SMR family, and BmrA (Steinfels *et al.*, 2004) of ABC superfamily are some of the well-characterized efflux pumps in *B. subtilis*. Among these pumps, EtBr has been reported to be a substrate for BmrA, Blt, Bmr, and EbrAB (Ahmed *et al.*, 1995; Masaoka *et al.*, 2000; Neyfakh *et al.*, 1991; Steinfels *et al.*, 2004). The results strongly suggest that the silibinin binds and inhibits the efflux pump(s) of *B. subtilis* 168 so that EtBr uptake rate is higher than its efflux rate in the presence of silibinin. This leads to continuous EtBr accumulation in the cells.

Silibinin has been reported to inhibit the efflux through the mammalian P-glycoprotein (Zhou *et al.*, 2004) and since BmrA of *B. subtilis* cells is a homologue of P-glycoprotein (Steinfels *et al.*, 2004), it could be proposed that the ABC transporter BmrA could be a target of silibinin. However, plant derived molecules are commonly known as multi-target molecules: thus, silibinin could be binding the other pumps in *B. subtilis* for which EtBr is a substrate.

4. CONCLUSION

Within the scope of this study, silibinin was assessed as a candidate for inhibiting efflux mechanisms in *B. subtilis* 168 cells. Here, it demonstrated a significant efflux pump inhibitor activity in the EtBr accumulation test and enhanced the activity of berberine. Because of the multi-target properties of the plant-derived molecules, it was not possible to determine the exact target of the silibinin. Further studies that will involve the purified efflux pump proteins are necessary. Although *B. subtilis* 168 is not a pathogenic microorganism, it is a well-known model organism for low G+C Gram-positives including many pathogenic bacteria, which means that silibinin could have comparable effects on these pathogenic bacteria. For future work, the synergistic antimicrobial effect of silibinin with available drugs or antibiotics could be assessed to overcome the bacterial defense mechanisms.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Fatma Ece Altinisik Kaya: Design of the study, performing the experiments, writing. **Basak Atas**: Design of the study, performing the experiments, writing. **Fatma Gizem Avci:** Design of the study, writing, editing, validation. Fatma Ece Altinisik Kaya and Basak Atas contributed equally to this work.

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

Evaluation of the Extract Obtained from Various Medicinal and Aromatic Plants "Antibacterial and antioxidant" in the Wood Industry

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Abstract: Throughout the history of mankind, various plants (medicinal aromatics etc.) have been used naturally to prevent all diseases, or they have been used in a wide range of fields, especially in the pharmaceutical industry, by cultivating in agriculture/greenhouse areas. However, as a result of the protection and coloring of the wood material by chemically, especially the indoor contamination causes negative effects on human health. The main objective of this study is to use the wooden product in wooden child toys, hospitals, sterile areas, pharmacies, wood-based materials used in the kitchen (fork, knife, serving plates and chopping boards etc.), playgrounds, dining table surfaces, nursery and kindergarten furniture, beehives etc. In addition, it will be recommended to use on all surfaces where there is an antibacterial / antioxidant effect, as well as on the surfaces with collective contact such as door handles, cabinet handles, elevator buttons and cash dispenser keys. In research, extracts of Evelik (*Rumex patientia* L.) and Çakşır (*Ferula comunis* L.) herbs (1% concentration) from medicinal aromatic plants were prepared and spruce and mahogany wood were used as wood type. According to ASTM D 143-76 principles, retention and bending resistance properties were determined by impregnation according to the results of the experiment, the highest retention (0.55%) and bending strength (100.20 N/mm^2) were determined in Evelik plant and the optimum increase was determined when the results were compared with the control sample.

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1. INTRODUCTION

With It is estimated that there are approximately 1000000 plants in the world today. Nearly 500000 of these species have been identified and named, and as a result of the researches conducted by the World Health Organization (WHO), it has been determined that they consist of medicinal plants used for treatment. The amount of medicinal plants used for treatment in our country is at least 500 (Baytop, 1984). From existence of mankind until recently, human beings provided almost all their needs such as clothing, shelter, food and fuel from within the boundaries of forests. But today, the development of technology, forestry method and forest management has focused the needs of people obtained from forests only on wood raw materials.

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In our country, this understanding has started to prevail day by day and started to be limited to wood (log, lumber, etc.) production. However, it is an incomparable resource for a healthy life, in which many economic and cultural activities such as many herbaceous plants, wild animals, water resources, recreation areas can be carried out together, except for the tree wealth within forest areas (Özkan & Akbulut, 2014). It is a very old tradition that medicinal plants started to be used in cure of diseases with the settlement of mankind. In many developing countries, herbal medicines constitute an important part of the culture and traditions in rural communities. Plants have a wide variety of chemical substances that have important biological activities on humans (Njume *et al*., 2010).

In addition to compounds with antioxidant activity in plants, it is known that there are biological activities in essential oils and herbal extracts obtained from various plants. In scientific researches, it has been proven that the antimicrobial substances found in herbal extracts are able to preserve the food safety at a high rate and because of these properties, plants can be used as natural antimicrobials (Souza *et al.*, 2005). Many herbs known as medicinal plants have been found by the public through trial and error. The use of such wild plants in the treatment of diseases in the world and in our country lies back to ancient times (Yiğit & Benli, 2005). It has been reported that wood is subjected to impregnation with some vegetable oil structure and as a result, there are increases in weight and density values (Bazyar *et al.*, 2010). Since the use of toxic component structure in wood preservation has caused the increase of important environmental pressures and prohibitions, it has been necessary to create/develop new environmentally friendly materials (Tomak, 2011).

Rapid reduction in forest existence and exposure of human beings to synthetic/chemical effects in the environment they live, cause serious threats. Natural plants are used for various purposes (medical, cosmetic, food, spice, agriculture, animal husbandry, spice, paint industry, etc.); The antioxidant/anti-bacterial properties of the organic wood were determined by obtaining various concentrations (1%) of plant extracts and the impregnation feature and some technological properties, as well as a hygienic structure in wood.

2. MATERIAL and METHODS

2.1. Wood Material and Plant Type

Spruce grown in our country and Mahogany wood which is an imported wood type were used in the study. Processes were carried out by cutting in radial direction according to the principles of TS 2470 (TSE, 1976[a]). Çakşır (*Ferula comunis* L.) and Evelik (*Rumex patientia* L.) plants , whose antibacterial/antioxidant properties have been determined in the literature, were chosen (Çetin, 2017).

2.2. Experiment Sample Preparation

While the samples were being prepared, the smoothness of the fibrous structure of the wood was considered and it was prepared for the sapwood (TS 2471) without any cracks, knots and color defects. Air-dried samples were prepared according to the principles of TS EN 2474 for flexure resistance (TSE, 1976[b], TSE, 1976[c]).

2.3. Impregnation Process

The impregnation process was applied in accordance with the conditions in "ASTM-D 1413-76" (1984). Experimental samples were prepared in the dimensions of 20x20x300 ± 1mm and subjected to 45 minutes vacuum/45 minutes diffusion process. In order to prevent impregnated.

2.4. Obtaining Plant Extract

The sample weight determined for the experiment is put in 200 mL of hot distilled water or water at least equal to this purity and heated at a temperature below the boiling point in the

refluxing apparatus for 1 hour by mixing at certain intervals, after filtering in the previously prepared porous capsule with vacuum, no sample will remain in the balloon. The process was continued to be washed several times with distilled water and the insoluble part was completely left inside the porous capsule. Finally, the residue was washed with 200 mL of hot distilled water and after the residue was dehydrated by a pump or another device that would serve as a suction, the porous capsule and its contents were dried by keeping it in an oven set at 103°C for 16 hours, then cooled in a desiccator and weighed with 0.001 g precision (Ceylan, 1997).

2.5. Retention Amount (% Rate)

1.0 g After the impregnation process, the remaining substance (tcoao-% retention) compared to the complete dry wood was calculated (1) from the formula (Baysal, 1994).

$$R(\%) = ((Wfdia-Wfdib)/ Wfdib)x100 \quad (1)$$

Wfdia = Sample full dry weight after impregnation (g)
Wfdib = sample full dry weight before impregnation (g)

2.6. Bending Strength

Bending Strength is based on TS 2474/1976 standard. The samples were prepared in 20x20x360 mm dimensions. The samples were sanded and air-conditioned (20±2 ºC/65±5% relative humidity) to 12% humidity. Before the experiments, all samples were air dried and values were taken by measuring both thicknesses (radial/tangent) with a digital caliper with ±0.01 mm precision. Then, the speed of the loading mechanism of the universal testing machine was adjusted to break in 1.5 ± 0.5 minutes. The flexural resistance was calculated (2) with the help of the equation given below (Çıtak, 2012).

$$\text{Bending strength } (N/mm^2): \delta_\theta = (3 \times P_{max} \times L_s) / (2 \times b \times h^2) \quad (2)$$

Pmax: Force at break (N), Ls: Clearance between abutments (mm), b: Sample width (mm), h: Sample thickness (mm).

3. RESULTS and DISCUSSION

3.1. Solution Properties

Solution properties are given in Table 1.

Table 1. Solution Properties of *Ferula comunis* L. and *Rumex patientia* L.

Concentration	Extract	Solvent	Temperature (ºC)	pH		Density (g/mL)	
				BI	AI	BI	AI
%1	Rumex patientia L.	Distilled Water	22ºC	6.07	6.07	0.987	0.987
	Ferula comunis L.			7.17	7.17	0.987	0.987

AI: After impregnation, BI: Before impregnation

When the table is examined, no significant changes were determined before and after impregnation. It is known that the pH factor being close to acidic character creates a negative structure on the mechanical properties.

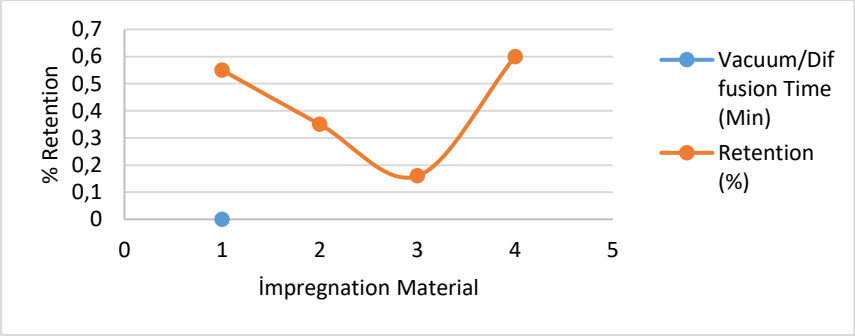
3.2. Retention Amount (% Retention)

The net dry impregnation material (retention) remaining amount as (%) is given in Table 2 and Figure 1.

Table 2. % Retention of Çakşır (*Ferula comunis* L.) and Evelik (*Rumex patientia* L.)

Wood Type	Concentration	Extract	Vacuum/Diffusion Time (Min)	Retention (%)
Spruce Wood	%1	*Rumex patientia* L.	45 min	0.55
		Ferula comunis L.		0.35
Mahogany Wood	%1	*Rumex patientia* L.		0.16
		Ferula comunis L.		0.60

Figure 1. Change in adhesion amount in impregnated and control groups (%).



The highest adhesion was detected in mahogany wood in Çakşır plant (*Ferula comunis* L.) (0.60%) and the lowest in mahogany wood in Evelik plant (*Rumex patientia* L.) (0.16%). The level of adhesion can vary according to the wood type, anatomical structure, impregnation method and impregnation material; This feature (adhesion) can reveal different interactions in technological properties. The retention amount varies according to the wood type and other factors (thickness, wood type, anatomy, moisture, impregnation method, impregnation material). Similar results are observed when the results are compared with the literature. Bal (2006) reported that the process performed with ACQ in wood is effective on the mechanical properties and retention is positively affected and provides rapid penetration. Alkan (2016) impregnated the scotch pine wood with boron compounds and kebracodan, and reported that the highest retention occurred at 1% concentration. Özçifçi *et al*. (2009) reported in their study that the highest % retention value was found in the samples treated with pressure-vacuum method in scotch pine, the highest % retention value was in the pressure-vacuum method (6.42%) in the yellow pine, and the lowest in beech immersion (0.30%). Dişli (2018) reported that the highest % retention was on $(Al_2SO_4)_3$ as (9.90%) and the lowest % retention was on Ba as (1.07%) in scotch pine wood, and the increase in solution concentration increased the amount of retention. In terms of flexural resistance, the flexural resistance value of plant extracts (extract) increased in both types of wood. The results reflect a positive structure when compared with the literature.

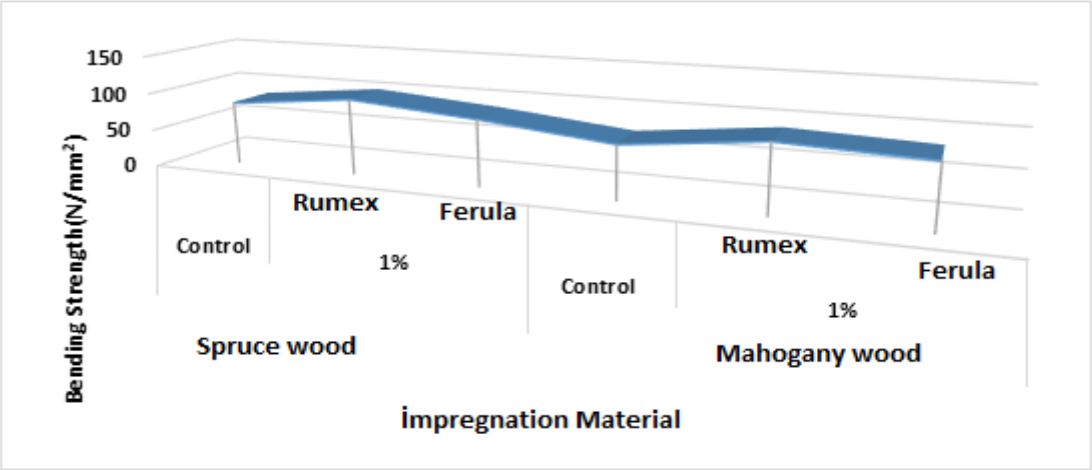
3.3. Retention Amount (% Retention)

The bending strength change is given in Table 3 and Figure 2. Highest bending strength is in evelik plant extract from spruce wood (100.20 N/mm^2), the lowest was observed in çaşır plant extract from mahogany wood (82.22 N/mm^2).

Table 3. Bending Strength Change of Çakşır (Ferula comunis L.) and Evelik (Rumex patientia L.) Plants (N/mm^2).

Wood Type	Concentration (%)	Extract	Vacuum/Diffusion Time (min)	Bending Strength (N/mm^2)
Spruce	%1	Control sample	45 min	83.15
		Rumex patientia L.		100.20
		Ferula comunis L		87.26
Mahogany	%1	Control sample		70.10
		Rumex patientia L.		88.80
		Ferula comunis L.		82.22

Figure 2. Bending strength change in impregnated and control groups (N/mm^2).



Ertürk (2011) impregnated some types of wood and the flexural resistance according to chemicals were determined; Imersol Aqua (98.177 N/mm^2), Boric acid (95.623 N/mm^2), Tanalith-E (94.708 N/mm^2) and Borax (85.926 N/mm^2). In the F test, which was carried out to determine the flexural resistance of the ash, leafy rowan massive wood materials impregnated with various preservatives; He reported that bending resistance values according to tree species showed statistically significant differences. Çıtak (2012) impregnated eastern beech (*Fagus orientalis* L.) wood with 2.5% boric acid and borax solution, and determined that the decrease in the flexural resistance was higher in experimental samples impregnated with borax. It was determined that the elastic modulus values of the experimental samples that were not subjected to impregnation were lower than the non-impregnated test samples.

Çakır (2012) chipped the bond pruning residues and subjected them to impregnation with boron compounds (1-4%) and it was reported that the impregnation process with boron compounds and the increase in the solution concentration in general caused decreases in the flexural resistance and elastic modulus of the test samples.

4. CONCLUSION

Healthy life in the human-environment relationship is provided by the wooden equipment used in the indoor and outdoor spaces where it lives. The natural strength of wood in simple use is not long lasting. This causes huge losses in terms of the country's economy and forest resources. Many of the wood preservatives are of chemical origin, which required orientation towards organic / natural preservatives. Suggestions have been made by determining the various technological features of the Evelik plant, which has an important place in terms of healthy life, and also has antioxidant / antibacterial properties, in order to determine the level of adhesion and usage areas in wood.

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Authorship Contribution Statement

Huseyin Peker: Laboratuary work, the methodology and statistical analysis of results, the writing of original draft. **Hatice Ulusoy:** The collection of plant materials and the other performing technological tests.

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Research Article

Antibacterial Activities of Methanolic Extracts of Different Seaweeds from Iskenderun Bay, Turkey

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Abstract: Antibacterial activities of the methanolic extracts of *Stypopodium schimperi* (Kützing) Verlaque and Boudouresque, *Halopteris filicina* (Grateloup) Kützing, *Dictyota dichotoma* (Hudson) J.V.Lamouroux, *Gracilaria bursa-pastoris* (S.G.Gmelin) P.C.Silva, *Ulva intestinalis* Linnaeus species from the Iskenderun Bay, Turkey against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhimurium and Proteus vulgaris* were evaluated by using the microdilution method. Results of the study showed that all of the extracts had an antimicrobial effect on the tested bacteria. A minimum inhibitor concentration and minimum bactericidal concentrations of the extracts ranged from >125 to <0.39 mg mL^{-1}. Furthermore, this is the first report on antibacterial effects of the methanolic extracts of *S. schimperi* (Kützing) Verlaque and Boudouresque and *G. bursa-pastoris* (S.G.Gmelin) P.C.Silva species. The present findings revealed that all studied seaweed species could act as a natural source of bioactive compounds for the treatment of infectious diseases.

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1. INTRODUCTION

For centuries, human beings have suffered from infectious diseases caused by various pathogenic microorganisms. The discovery of antibiotics used to control infectious diseases has been a beacon of hope to reduce the deaths caused by these diseases. Today, however, people are in danger of losing this powerful weapon due to resistant strains caused by the improper use of antibiotics. For this reason, researchers have been examining many natural resources for the discovery of new antibiotics (Mohr, 2016; Zaman *et al*., 2017). One of these natural resources is seaweed, which has many important medical properties due to the valuable components it contains (Shelar *et al*., 2012). Seaweeds also known as macroalgae are photosynthetic eukaryotic organisms that are essential components of the living resources of the sea. Seaweeds consist of three main classes; namely, Rhodophtya (red algae), Chlorophyta (green algae) and Phaeophyta (brown algae) (Wang *et al*., 2017). In some studies to date, it has been observed

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that many species of algae can synthesize antimicrobial metabolites such as bromophenols, indolocarbazoles, cycloeudesmol, laurinterol, and elatol to kill or inactivate the pathogen microorganisms (Parsaeimehr and Lutzu, 2016).

There is a very rich variety of seaweed in Turkey, which has coasts in the Mediterranean, Black Sea and Aegean Sea, as well as an inland sea called the Marmara Sea (Aysel and Erdugan, 1996; Ribera *et al*., 1992; Taskin, 2014). There are some studies on the biochemical composition (total protein, carbohydrate, phenolic, chlorophyll-a and carotene contents) of some brown, green and red seaweeds from Iskenderun Bay in the Mediterranean Sea in the south of Turkey (Ozgun *et al*., 2015; Turan *et al*., 2015). On the other hand, there are no studies in the literature showing the antimicrobial effects of the methanolic extracts of *Stypopodium schimperi* (Kützing) Verlaque and Boudouresque and *Gracilaria bursa-pastoris* (S.G.Gmelin) P.C.Silva species.

The aim of this study is to determine the antimicrobial effect of methanol extract of seaweeds; namely, *Stypopodium schimperi* (Kützing) Verlaque and Boudouresque (Phaeophyta), *Halopteris filicina* (Grateloup) Kützing (Phaeophyta), *Dictyota dichotoma* (Hudson) J.V.Lamouroux (Phaeophyta), *Gracilaria bursa-pastoris* (S.G.Gmelin) P.C.Silva (Rhodophyta), and *Ulva intestinalis* Linnaeus (Chlorophyta) collected from the Iskenderun Bay, on various bacteria.

2. MATERIAL and METHODS

2.1. Sample Collection

Antimicrobial activity analysis was performed on *Stypopodium schimperi*, *Halopteris filicina*, *Dictyota dichotoma*, *Gracilaria bursa-pastoris,* and *Ulva intestinalis* species (Table 1). Sampling studies were carried out in June 2018 at 0-20 m depth from free dives on the Iskenderun Gulf coast, Hatay, Turkey. The seaweeds were collected underwater in gathered mesh bags. The collected seaweeds were washed with water to remove the epiphytes, rocks, sand and mud that could be present. In the laboratory, the washed materials were dried in a shaded environment without sun exposure for further study. Some of the collected seaweeds were stored in jars with a 4-6 % neutralized formaldehyde solution prepared with seawater for identification. The identification of the seaweeds was carried out using the Olympus brand Ckx41sf model stereo inverted light microscope.

Table 1. Macroalgae species used in antimicrobial activity test.

Seaweed species	Class
Stypopodium schimperi (Kützing) Verlaque and Boudouresque 1991	
Halopteris filicina (Grateloup) Kützing 1843	Brown algae
Dictyota dichotoma (Hudson) J.V.Lamouroux 1809	
Gracilaria bursa-pastoris (S.G.Gmelin) P.C.Silva 1952	Red algae
Ulva intestinalis Linnaeus 1753	Green algae

2.2. Preparation of the Extracts

The dried algal samples were extracted by maceration in 1.4 (w/v) biomass/solvent ratio with methanol for 2 weeks at room temperature in a dark environment. The obtained methanolic extract was filtered through filter paper. After the filtration, the solvent was evaporated at 50 °C under reduced pressure in a rotary evaporator (Heidolph, Germany), and deposited at +4 °C before further usage. For antimicrobial analysis, the extracts were dissolved in DMSO at a concentration of 250 mg mL^{-1} and sterilized by a 0.45 mm pore sized syringe filter.

2.3. Microorganisms and Growth Conditions

Seven bacterial strains have been used to detect the antimicrobial activities of the extracts. Bacterial strains were as follows: Gram positive bacteria, *Bacillus cereus* NRRL B-371, *Staphylococcus aureus* ATCC 25923, gram negative bacteria, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* RSKK 96029. All the bacterial cultures were incubated in Tryptic soy agar at 37 °C for 24h.

2.4. Broth Microdilution Assay

The minimum inhibitory concentrations (MIC) of the extracts were evaluated by the broth microdilution assay in 96-well microtiter plates according to CLSI reference methods for bacteria M07 (CLSI, 2018). Serial dilutions of the samples were made in Mueller Hinton broth at a concentration range of 125–0.39 mg mL^{-1} on 96-well microtiter plates. The antibiotics ampicillin and chloramphenicol (Sigma) were used as positive control. Microorganism inoculums were prepared from a 24-hour culture, suspensions in 0.9 % of NaCl were adjusted to 0.5 McFarland standard turbidity ($1.5x10^8$ cfu mL^{-1}), and this microbial suspension was diluted 10^{-1} in 0.9 % of NaCl solution. Five microliters of the diluted microbial suspension had been added to all wells. The plates were then incubated for 24 h at 37°C. After the incubation, the minimum inhibitory concentration (MIC) values of the extracts were determined by absence of visual turbidity. The minimum bactericidal concentration (MBC) values were determined by subculturing ten microliters from non-turbid wells and spot inoculated onto an appropriate growth medium. After the incubation, the growth was recorded and MBCs were defined as the lowest concentration resulting in the death of 99·9% of the inoculum compared to the initial viable counts. The assay was repeated at least three times and the mean values of MIC and MBC were selected.

3. RESULTS and DISCUSSION

Antimicrobial activity of the seaweed extracts was evaluated by using the microdilution method. MIC and MBC values of five methanolic seaweed extracts against tested bacteria are given in Table 2 and Table 3, respectively. According to the obtained results MIC and MBC values ranged from <0.39 to >125 mg mL^{-1}. All extracts were found to have a strong bactericidal and bacteriostatic effect on the studied bacteria.

Table 2. Minimum inhibitory concentration (MIC) values of the extracts (mg mL^{-1}).

	Gram positive			Gram negative			
	S. aureus	*B. cereus*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. typhimurium*	*P. vulgaris*
Stypopodium schimperi	3.13	3.13	12.5	3.13	1.56	6.25	12.5
Halopteris filicina	15.63	15.63	125	31.25	31.25	31.25	31.25
Dictyota dichotoma	50	1.56	50	<0.39	12.5	50	12.5
Gracilaria bursa-pastoris	50	6.25	50	1.56	12.5	50	25
Ulva intestinalis	12.5	1.56	25	12.5	12.5	50	25
Ampicillin	62.5	31.25	>125	>125	125	62.5	>125
Chloramphenicol	125	125	>125	>125	15.63	125	125

Table 3. Minimum bactericidal concentration (MBC) values of the extracts (mg mL^{-1}).

	Gram positive			Gram negative			
	S. aureus	*B. cereus*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. typhimurium*	*P. vulgaris*
Stypopodium schimperi	6.25	3.13	12.5	3.13	6.25	6.25	12.5
Halopteris filicina	62.5	>125	>125	62.5	>125	125	62.5
Dictyota dichotoma	50	1.56	50	<0.39	50	50	12.5
Gracilaria bursa-pastoris	50	25	50	1.56	50	50	25
Ulva intestinalis	25	1.56	50	12.5	50	50	25
Ampicillin	62.5	>125	>125	>125	125	62.5	>125
Chloramphenicol	>125	125	>125	>125	15.63	125	>125

Among the bacteria tested, extracts were found to demonstrate the highest antimicrobial effect on *B. cereus*. The lowest MIC and MBC values were recorded for the *D. dichotoma* extract against *P. aeruginosa* (<0.39 mg mL^{-1}). Methanolic extract of *S. schimperi* showed a higher antimicrobial effect against the tested bacteria than the other algal species.

Ballesteros *et al*. screened the antibacterial, antifungal, antiviral, cytotoxic, and antimytotic properties of seventy-one different macroalgae species collected from the Central Mediterranean (Ballesteros *et al*., 1992). They said that antifungal activity (against *Candida albicans* and *Aspergillus niger*) was more common than antibacterial activity (against *E. coli* and *B. subtilis*) among these species and *H. filicina* did not have antibacterial effects. Val *et al*. studied the antibacterial and antifungal activities of the methanol extracts from 44 species harvested from Gran Canaria (Canary Islands, Spain) against *B. subtilis* MB964, *Enterococcus faecium* MB5571, *S. aureus* MB5393, *P. aeruginosa* MB979, *Serratia marcescens* MB252, *Mycobacterium smegmatis* MB2233, *Candida albicans* MY1055, *Saccharomyces cerevisiae* W303, *Aspergillus fumigatus* MF5668 (Val *et al*., 2001). They found that *H. filicina* extract did not have any antimicrobial effects against these microorganisms. However, Alghazeer *et al*. evaluated the antibacterial activity of crude methanolic and water extracts of 19 seaweed species collected from the western coast of Libya against *S. aureus*, *B. subtilis*, *Bacillus* spp., *Staphylococcus epidermidis*, *E. coli*, *Salmonella typhi*, *Klebsiella* spp., and *P. aeruginosa* (Alghazeer *et al*., 2013). They found that methanol extract of *H. filicina* was less effective than the positive control antibiotic, ciprofloxacin. Taskin *et al*. studied the antibacterial activity of methanol extracts of six marine algae from the North Aegean Sea (Turkey) against *S. aureus*, *Micrococcus luteus*, *Enterococcus faecalis*, *E. coli*, *Enterobacter aerogenes* and *E. coli* O157,H7 (Taskin *et al*., 2007). They determined that *H. filicina* methanol extract had a moderate antibacterial effect only on *S. aureus* among the bacteria studied. In our study, it was observed that *H. filicina* extract was less effective on the tested bacteria than the other extracts. This difference between the results could have been due to the difference in preparation methods such as concentration of extract, type of the solvent and extraction technique or some ecological factors such as the location and season of sampling.

D. dichotoma extract showed an antibacterial effect in variable values against studied bacteria. This was in consistence with the finding of Ibraheem *et al*. and Demirel *et al*. (2009)

who found that the extracts of *D. dichotoma* obtained from Hurghada on the Red-Sea coast of Egypt and Aegean Sea had antimicrobial properties on both gram positive and gram negative bacteria (Ibraheem *et al*., 2017; Demirel *et al*., 2009).

Like other species, *U. intestinalis* extract has been found to have a fairly high antimicrobial effect, similar to previous studies (Abdel-Khaliq *et al*., 2014; Berber *et al*., 2015; Sahnouni *et al*., 2016; Srikong *et al*., 2017; Srikong *et al*., 2015). Berber *et al*. tested the antimicrobial activities of the methanolic extracts of *Cystoseira crinita* Duby and *U. intestinalis* from the coastal region of Sinop, Turkey against 18 bacteria and 3 yeasts. According to their results, *U. intestinalis* extract had a moderate antibacterial effect on 10 bacteria out of 18 species (Berber *et al*., 2015). Srikong *et al*. evaluated the antibacterial activities of methanol, ethanol, dichloromethane, and hexane extracts of *U. intestinalis* harvested from Thailand on *Bacillus cereus* TISTR 687*, Enterococcus faecalis* ATCC 29212*, Listeria monocytogenes* DMST 4553*, S. aureus* ATCC 29213 *and* methicillin-resistant *S. aureus* 001 R (MRSA 001 R), *E. coli* ATCC 25922*, K. pneumonia* PSU 1*, Proteus mirabilis* PSU 1*, P. aeruginosa* ATCC 27853*, Salmonella typhi* PSU 1*, Vibrio alginolyticus* PSU VA 1*, V. harveyi* PSU 4109 *and V. parahaemolyticus* PSU 5124. They found that only the hexane extract had a significant antibacterial activity against Gram-positive bacteria but not against Gram-negative bacteria (Srikong *et al*., 2017). Moreover, Sahnouni *et al.* studied *Ulva rigida* C. Agardh and *U. intestinalis* methanolic extracts for antimicrobial activities on *E. coli*, *Salmonella* sp., *Shigella dysentriae*, *P. aeruginosa*, multi-drug resistant *Proteus mirabilis,* sensitive *Streptococcus pyogenes,* methicillin-resistant *S.aureus* (MRSA), *Staphylococcus epidermidis*, *K. pneumoniae*, vancomycin resistant *Enterococcus faecalis*, *C. albicans*, *A. niger* and *Cryptococcus neoformans*. They showed that *E. coli*, *S. pyogenes* and *S. epidermidis* bacteria were more susceptible to *U. intestinalis* extract than the other bacteria studied (Sahnouni *et al*., 2016).

There are no studies on the antimicrobial effects of the *S. schimperi* and *G. bursa-pastoris* species. Both of the species have been found to inhibit the growth of all bacteria studied. Therefore, this initial data is very important as it reveals the antimicrobial potential of these species.

4. CONCLUSION

Findings obtained from this study suggest that methanolic extracts of *S. schimperi*, *H. filicina*, *D. dichotoma*, *G. bursa-pastoris* and *U. intestinalis* species from Iskenderun Bay are potential natural source of antibacterial agents. In particular, the lack of any study showing the antimicrobial effectiveness of the *S. schimperi* and *G. bursa-pastoris* species has made this study the first research to reveal the antimicrobial properties of these species. Further studies will pave the way for the use of active substances contained in these algae in the field of pharmacy and medicine. Therefore, detailed research should be carried out for the detection of their active components.

Declaration of Conflicting Interests and Ethics

The author declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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Research Article

Productivity of medicinal raw materials by different genotypes of *Matricia chammomila L.* is affected with pre-sowing radiation exposure of seeds

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Abstract: One of the key tasks of modern pharmacology is complete and diverse use of natural raw materials - microorganisms and plants. So, different approaches of metabolism redirection were applied. Studying of plant protective reactions indicated a possibility to use various stress factors for the metabolism reorientation. One of the most effective approaches is to use ionizing and UV-C exposure. Thus, there is a shift in metabolic processes towards the formation of secondary metabolism substances with antioxidant, anticancer, immunomodulatory and anti-inflammatory effects. Biotechnological use of radiation exposure is based on the systemicity of radiobiological reactions, including protective and adaptive reactions in non-exposed organs («abscopal effect») and even in non-exposed organisms that are found in the same environment as exposed ones («by stander effect»). The products synthesized in these structures are some medicine materials and directly used by human. Radiation exposure affects developing, blocking of primary and secondary metabolism, so must be improved the selection of varieties with initial high productivity of medicinal raw materials, the choice of exposure and optimal doses inducing an increasing yield of the target metabolite and do not reduce the yield of medicinal raw materials. Effect of UV-C and X-ray pre-sowing exposure of seeds to the productivity of inflorescence formation of eight genotypes of *Matricia chammomila* L. was studied. There were indicated genotypes with increasing yield of inflorescence only under one or two exposure types and the variety with stimulation of flowering under UV-C exposure and absence of the marker under X-ray one.

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1. INTRODUCTION

With the increasing use of plants in official medicine, the variety of approaches to obtain more medicinal substances from the natural raw material expands (García-Granados *et al.*, 2019; DellaPenna, 2001). Along with the search for new species with healing properties and the derivation of more productive varieties, metabolism redirection is used to increase the productivity of the substances necessary for practice (Dmytriev *et al.*, 2018).

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One of the effective approaches is the application of both ionizing and non-ionizing radiation exposure (Klein *et al.*, 2018; Jan *et al.*, 2012; Alothman *et al.*, 2009; Vardhan, & Shukla, 2017; Nocchi *et al.*, 2020). It was shown metabolic shift toward the formation of secondary metabolites, mostly radioprotectors, having antioxidant, anticancer, immunomodulatory, and anti-inflammatory effects under acute and chronic radiation exposure (Dai, & Mumper, 2010; Kaur, & Mondal, 2014; Unuofin, & Lebelo, 2020; Pisoschi *et al.*, 2016).

The development of any biotechnology inevitably faces some key issues, the solution of which should determine the reproducibility of the results. Under radiation exposure variability and plasticity of living things are found through all levels of their organization not limited to rearrangement only one metabolic direction, necessary for the practice. Reaction to the exposure covers the processes of growth and development, various blocks of primary and secondary metabolism, significantly changing their dynamic characteristics (Dmytriev *et al.*, 2018; Jan *et al.*, 2012). Thus, the development of technology to increase medicinal raw material under various radiation exposure types, in practice have to include:

a) Selection of varieties with high and stable productivity of medicinal raw material;
b) Choosing both type of radiation exposure and estimation of dose curves; an indication of optimal doses that do not decrease the yield of medicinal material and induce a higher level of the target metabolite;
c) Assessment of time coincidence of the yield of both pharmaceutical raw material and required metabolite with the total effect of the exposure.

The paper presents the results of studying both plant growth and development processes of different genotypes of chamomile, including the yield of crop formation of pharmaceutically significant raw materials under two types of radiation exposure: UV - C and X-ray.

2. MATERIAL and METHODS

Chamomile is a wide-spread, long-day plant. It is a mesophyte, used in different countries in pharmacology and cosmetology during the treatment of inflammatory processes with various etiologies. The inflorescences of the plant are medicinal raw material (Matricaria chamomilla, 2021). Chamomile is a convenient experimental object.

The research was done using 8 genotypes of *Matricaria chamomilla*. 6 certified varieties of different origin were used: 1 - generative generation of the mutant Perlyna Lisostepu (treated with herbicide RaudAr in concentration 10); 2 – variety Kvedlinburg (Germany); 3 – variety Goral (Slovenia); 4 – variety Azulena (Russia); 5 – variety Zlatyi lan (Poland); 6 – variety Perlyna lisostepu (Ukraine). Non-varietal material, in fact, edaphic ecotypes, were included in the study: 7 – from manufacturer Gold Garden (Ukraine) further – ecotype Gold Garden; 8 – from manufacturer Seed era (Ukraine) further - ecotype Seed era. The experiment was repeated three times.

Dry seeds were exposed with dose 10 Gy, dose rate 1.42×10^{-2} Gy/sec using X-ray installation RUM-17 (Russia). UV-C exposure with dose 10 kJ/m^2, dose rate 3.4 W/m^2 was carried out using installation OBM-150 M (Ukraine) with two lamps Philips Special TUV 30 W (the Netherlands) maximum radiation under wavelength of 253.7 nm.

Determination of pre-sowing exposure dose of dry seeds was based on wide-known effects in the field of "small" doses that lead to the stimulation effect of growth processes in parallel with DNA damage. According to the research, the optimal dose range for pre-sowing X-ray exposure of chamomile seeds was 5-10 Gy. The results were assigned with the patent (Shylina *et al.*, 2018).

The choice of UV-C exposure dose was based on both previous research of the exposure effects equivalent to chromosome aberration yield as a marker of low-intense radiation exposure with 10 Gy dose (Dmytriev *et al.*, 2018) and dose curve of antioxidant yield.

The plants were grown in pots containing 1.5 kg of soil under the conditions of the vegetation experiment. According to the ecological characteristics of *Matricaria chamomilla*, sod-podzolic loamy soil was modeled mixing Polissky soil with sand in the ratio 1:2.

During the experiment, the physiological effects of ionizing and UV-C radiation exposure (germination, growth, a transition to flowering, flowering dynamics, the total yield of the target product) were studied. The inflorescences were collected at the stage of their full disclosure. The drying of raw materials was carried out in a dark room at a temperature of 25-27° C. Statistical analysis of experimental findings – the mean value and variance value were calculated by traditional methods.

3. RESULTS and DISCUSSION

As shown in Figure 1, the stimulation of germination, in other words priming effect was not observed for all genotypes of the experiment. Priming effect, i.e. germination stimulation and synchronization with various stressors is intensively studied up-to-date. In addition to the phenomenon, there are unstable next stages of the production process, which brings the effect of priming with other hormesis effects (Kravets, & Sokolova, 2017).

Hormesis effects during germination were observed for Goral variety, the mutant of Perlyna Lisostepu, ecotypes Golden Garden and Seed Era. They have maximum germination yield among variants exposed with UV-C. Along with high germination yield for non-varietal plants SeedEra, there was significant mortality yield during the transition to flowering under UV-C exposure. Both varieties Perlyna lisostepu and Zlatyi lan demonstrated germination stimulation under X-ray exposure. In general, germination dynamics for different chamomile varieties during the growing season had a multiphase nature. There were no common differences among the germination rate of exposed and control variants.

A significant effect of different radiation exposure types was shown during plant pass to flowering (Figure 2). There were distinct maximums of inflorescences under X-ray pre-sowing exposure for Kvedlinburg, Goral, and Azulena varieties. In contrast, other varieties did not show such reactions.

There is time congruence between start flowering time of both control and exposed with UV-C variants for mutant Perlyna lisostepu (Figure 2). However, the exposed demonstrated clearer maximum in contrast to the fairly "smooth" dynamics of inflorescences in control. There was a 10-day flowering delay for the variety under X-ray exposure. Also, the same delay with the maximum flowering index between 20th and 30th day was indicated for the Kvedlinburg variety. The maximum of the index for both UV-C and control variants appeared with a 10-day delay.

There was a clear maximum of the index for Goral variety with the same transition terms to flowering under X-ray exposure and a relatively "smooth" flowering formation dynamics for both control and UV-C exposed variants. All the variants of the Azulena variety characterized by the simultaneous transition to flowering. Moreover, flowering stimulation was indicated under X-ray exposure.

Figure 1. Dynamics of germination of different chamomile genotypes. Confidence interval, p=0.95.

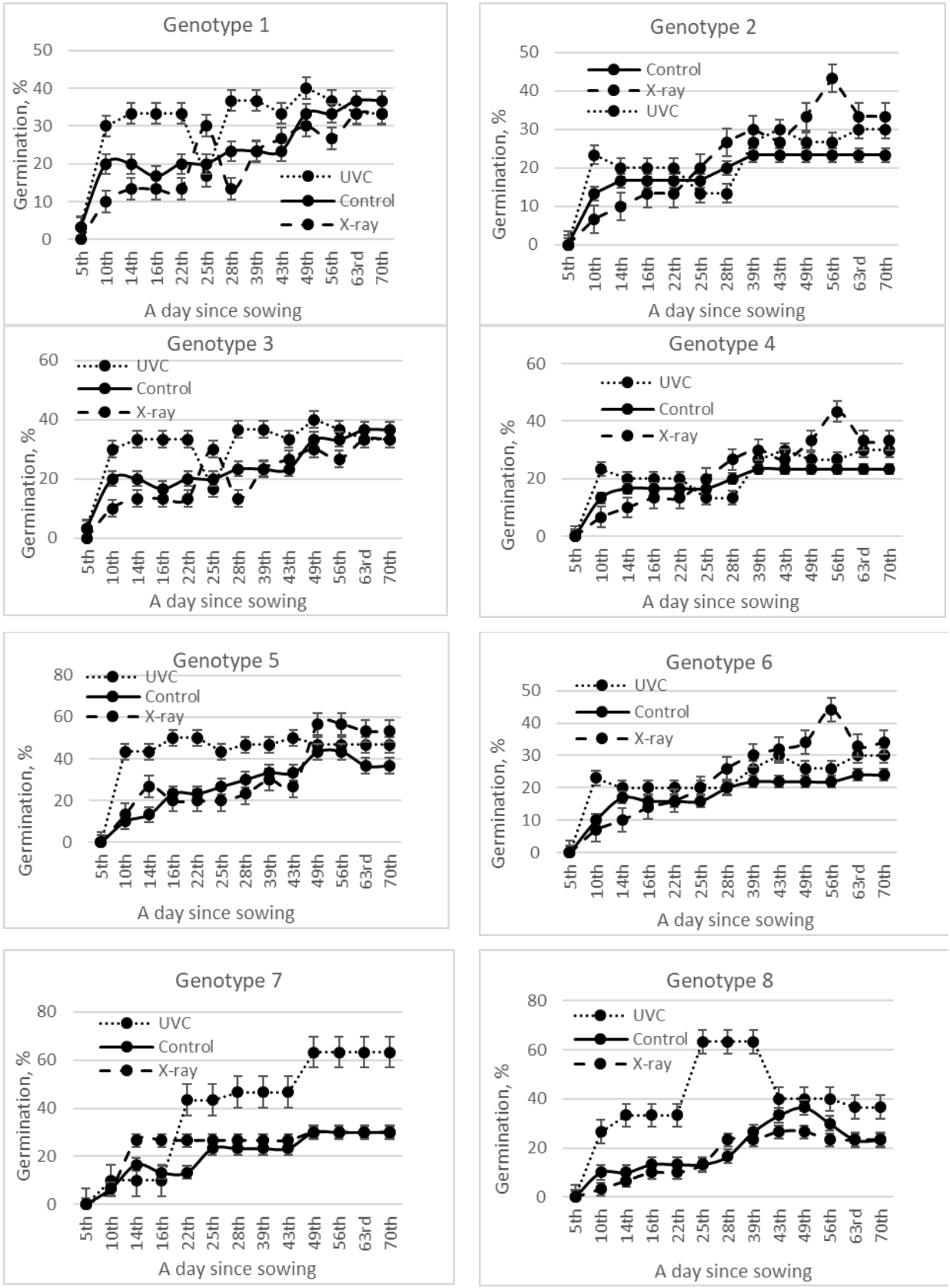


Figure 2. Dynamics of inflorescence formation by different chamomile genotypes. Confidence interval, p=0.95.

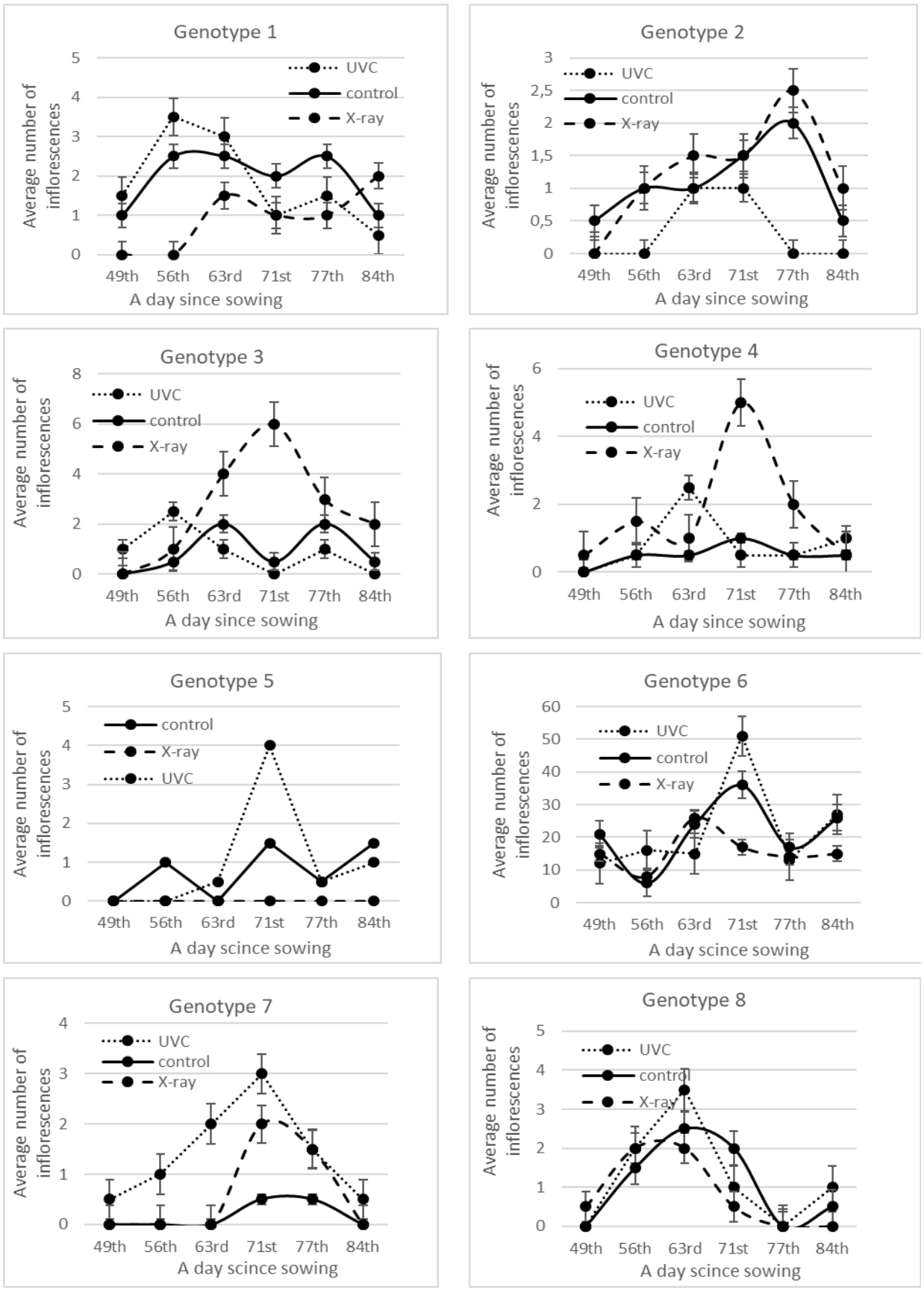
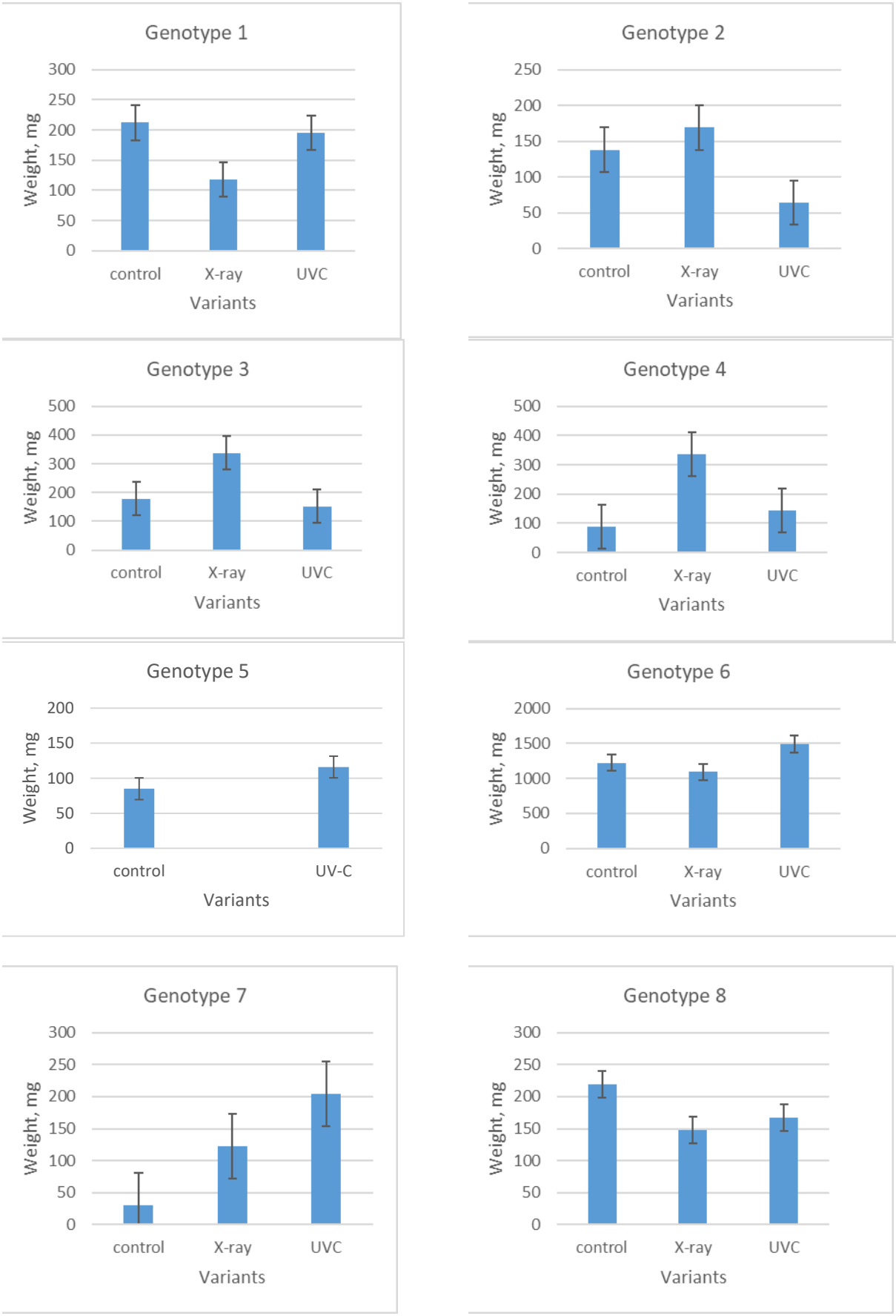


Figure 3. The total harvest of inflorescence collected during the season. Confidence interval, p=0.95.



The total yield of pharmaceutical raw material, in this case – inflorescences, is an important technological marker. According to the research, all analysed genotypes differed with initial harvest yield. The highest yield of the inflorescences in control was indicated for Perlyna Lisostepu, the lowest one – for non-varietal ecotype Golden Garden.

Typically, start flowering time and its kinetics through different varieties exposed with various types of radiation were distinct among each other and control.

Obtained data showed various effectiveness of two radiation exposure types through different genotypes. Major stimulation of inflorescence yield from the one plant under X-ray exposure was indicated for most varieties: Kvedlinburg, Goral, Azulene. Stimulation with pre-sowing UV-C exposure of seeds was shown for both Perlyna Lisostepu (variety of Ukrainian selection) and Zlatyi Lan (variety of Poland selection). Eightfold increased harvest yield was indicated for non-varietal plants Golden Garden and mutant Perlyna Lisostepu.

Comparison of the effects of priming and increasing the target productivity indicates the possibility of both coincidence and divergence of these markers through the genotypes under different types of radiation exposure (Figure 3). Stimulating both germination and harvest of the pharmaceutical products was indicated for Kvedlinburg and Asulena varieties under X-ray exposure. It was shown minor germination hormeziz effect and major stimulation of medicinal raw materials' formation for Perlyna Lisostepu variety under UV-C exposure. There was indicated germination stimulation under UV-C exposure and increasing yield of inflorescences' harvest under X-ray exposure for Goral variety. For Zlatiy lan variety the stimulation of germination under UV-C exposure coincided with the increasing yield of pharmaceutical raw material. However, these plants did not pass to flowering under X-ray exposure.

There is a difference between the primary "targeted" effects of UV-C and ionizing radiation exposure and, at the same time, the similarity of the effects of oxidative stress and the mechanisms of reparative processes under these two types of exposure (Dmytriev *et al*., 2018; Sinha, & Hader, 2002). According to ecology and phytocoenology, it is known that increasing both the formation of generative organs and fertility, in general, is one of the forms of plant adaptive strategy (Rabotnov, 1983). Results concerning the difference through inflorescences' harvest of eight genotypes under the exposure indirectly indicated the formation otherness of the plant stress reactions under various types of radiation exposure. The diversity could include both primary damage processes associated with different compaction of plant chromatin of different genotypes and effectiveness of reparative, protective and antioxidant processes.

4. CONCLUSION

The results of studying the production process shown various reactions to a different type of radiation exposure through eight genotypes of chamomile. There were established genotypes in which increasing yield of inflorescence appeared only under one type of radiation exposure, variety in which stimulation of inflorescence yield was shown under two types of exposure and variety where flowering stimulation appeared under UV-C exposure and was absent under X-ray exposure. The results indicating the difference in the yield of inflorescences of different genotypes under UV-C and X-ray exposure point to a difference through the formation of plant stress reactions at the organismal level.

Considering the applied focus of the study and the complexity of the plant organism's stress metabolic response, including different efficiency of enzymatic and non-enzymatic antioxidant systems, the next step in research aimed at increasing pharmaceutically important substances is to study the production of low molecular weight antioxidants as markers of stimulation of secondary metabolism.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Daryna Sokolova: Investigation, Methodology, Visualization, Software, Formal Analysis and Writing original draft. **Alexandra Kravets**: Investigation, Methodology, Visualization, Supervision, Validation and Writing original draft. **Vladyslav Zhuk**: Investigation, Methodology, Visualization, Software, Formal Analysis. **Volodymyr Sakada**: Methodology. **Ludmila Glushenko**: Resources. **Mykola Kuchuk**: Supervision.

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Review Article

Phytochemicals, Traditional Uses and Pharmacological Activity of *Rhamnus prinoides*: A Review

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Abstract: *Rhamnus prinoides* L'Herit belongs to Rhamnaceae family widely distributed in India, Eastern, Central and Southern Africa. In Ethiopia it is commonly known as Gesho (Amharic, Tigrigna and Afan Oromo), Gishe (Guragegna) and Geshu (Agewgna). Decoction of the leaves is recommended as remedy for treatment of variety of diseases such as back pain, malaria, pneumonia, sexually transmitted disease, skin infections, wounds, blood purifiers, water borne diseases and as ethnoveterinary medicine. Various secondary metabolites such as flavonoids, alkaloids, tannins, terpenoids, saponins, steroids and anthraquinones have been reported from the genus of which polyphenols were abundant with tremedousantioxidant, wound healing and antiinflammatory activities. The plant also serve as hopping agent, making traditional alcoholic beverages like tella and tej (in Ethiopia), animal feed, medicine, nectar for bees, soil conservation, ornamental, shade and dyes in textiles. This review presents chemical profile as well as biological activities of the species which confirmed that the plant is a good source natural polyphenols and provided valuable information in support of its use as an alternative medicine for future healthcare practice.

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1. INTRODUCTION

Traditional and complementary medicine is an essential and often underestimated health resourcewith many applications, especially in prevention and management of lifestyle-related chronic diseases. Many countries are expanding coverage ofessential health services at a time when consumer expectations for care are rising, costs are soaring andmost budgets are either stagnant or being reduced contributing to the revival of alternative traditional medicine. In developing contries, traditional medicine is the main source of healthcare and sometimes the only source of care, due to its closeness to the ordinary rural communities and its accessibility and affordability in view of the rising healthcare costs (Abebe *et al*., 2003).

Rhamnus prinoides (Figure 1), known as Gesho (Amharic, Tigrigna and Afan Oromo), Gishe (Guragegna) and Geshu (Agewgna) in Ethiopia, belongs to *Rhamanceae* family (Tesema *et al*., 1993) widely distributed in India, East, Central and South African countries (Dlamini and Turner, 2002). The only two *Rhamnus* species that occur in Africa are *R. prinoides* and *R.*

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Staddo (Abegaz *et al*., 1999). The genus name '*Rhamnus*'is derived from an ancient Greek word *rhamnos* for blackthorn meaning a 'tuft of branches' and the species name *prinoides* means 'resembling prion' which is an ever green oak. The genus *Rhamnus* comprises about 150 species of shrubs and small trees occurs both intemperate and tropical countries (Chen and Schirarend, 2007). *R. prinoides* also known as *R. pauciflorus* Hochst. ex A. Rich or *R. celifollius* Kallogg is a climbing shrub or small tree that grows up to 4 m high (Schmidt *et al*., 2002). In Ethiopia, *R.prinoides* (Gesho) is used to add flavour to the local drinks, tella and tej brewed from fermented barley, sorghum or finger millet(d'Avigdor *et al*., 2014; Tesema *et al*., 1993). In Angloa, the bark is used to induce vomiting (Dlamini and Turner, 2002).In South Africa, a decoction of the root is taken to cleanse the blood by the Zulu and to treat pneumonia by the Sotho(Coates-Palgrave, 1988).The boiled soup of the root is used for the treatment of common cold, leaves are used for chest pain and leaves as well as stems are used for the treatment of tonsil in central Kenya(Njoroge & Bussmann, 2006).In a related study in Kenya, it was reported that the leaves are used in preparation of the "muteta″soup used as an appetizer (Mwangi, 2005).

The decoction of the leaves is recommended as remedy for treatment of variety of diseases such as back pain, malaria, pneumonia, sexually transmitted disease, skin infections, wounds, blood purifiers, water borne diseases and as ethnoveterinary medicine (Berhanu, 2014; Enyew *et al*., 2014; Fratkin, 1996; Megersa *et al*., 2013; Muthee *et al*., 2011; Njoroge & Bussmann, 2006; Prozesky *et al*., 2001; Sobiecki, 2002).The leaves are maily used in traditional medicine followed by roots (Araya *et al*., 2015; Diallo *et al*., 2002; Giday *et al*., 2010; Seid & Aydagnehum, 2013). Various class of secondary metabolites such as flavonoids, alkaloids, tannins, terpenoids, saponins, steroids and anthraquinones have been reported from the genus of which polyphenols were abundant withtremendous antioxidant and antiinflammatory activities (Chen *et al*., 2020). The plant also serve as an important hopping agent, making traditional alcoholic beverages like tella and tej (in Ethiopia), animal feed, medicine, nectar for bees, soil conservation, ornamental, shade and dyes in textiles. This review presents a comprehensive overview of the chemical profile as well as biological activities of the plant underlining remarkable activities demonstrated by various parts of the plant supporting its potential use asan alternative medicine for future healthcare practice.

Figure 1. Photo of *Rhamnus prinoides* (Amabye, 2015; Molla, 2015, respectively).

1.1. Botanical Distribution

The plant grows up to 8 m in height and has globular, glossy and serrated leaves. The leaves size varies from 10-125 mm (Vetter, 1997). It is commonly cultivated between altitudes of 1400 to 3200 m above sea level (Nielsen, 1992). The flowers are light yellow-green, solitary up to 20 mm (Orwa *et al*., 2009). The plant is commonly distributed in African countries such as Tanzania, Uganda, exotic to Kenya, Ethiopia, Eretria, Angola, Malawi, Mozambique, Zambia,

Zimbabwe, Cameron, Democratic Republic of Congo and South Africa (Abegaz, 1996; Dlamini & Turner, 2002; Edwards, 1991; Orwa *et al*., 2009).

1.2. Ethnomedicinal uses

The leaves, fruits, roots, seeds, shoots and barks are reported to possess diverse traditional medicinal uses to heal various human as well as animal diseases (Table 1). In Tanzania, a root decoction of *R. prinoides* is mixed with bark of *Erythrina abyssinica* Lam. ex DC as remedy for colics (Chhabra *et al*., 1984).In Ethiopia, the leaf often mixed with the root of *Rubus apetalus* Poir., is boiled, decocted and drunk before meal as remedy for sexually transmitted diseases (Tuasha et al., 2018). The leaf decoction is used for treatment of animal diarrhea and intestinal parasites (Bekele & Musa, 2009), hepatitis (Yineger *et al*., 2007) and leech infestation (Bekele *et al*., 2012).

1.3. Economic Uses

R. prinoides has remarkable importance for nutrition, medicine, or other religious purposesin Africa. The leaves and stems are used to add flavor for the preparation of traditional alcoholic beverages like tella and tej (in Ethiopia) by mixing with other components responsible for the bitter taste. It maintains acidic pH during tella fermentation, so it is used to modify the nature of mesh growth of micro-flora and inhibits the growth of undesirable microorganisms (Kebede, 1994; Van Vuuren *et al*., 1979). It is estimated that close to 5 million people consume it every day (Abegaz & Kebede, 1995; Ashenafi, 2006; Hayeshi *et al*., 2004). The leaves and stems also serve as a commercial hopping agent in the brewery industry that can be used as an alternative substitute hop(Berhanu, 2014). In a related study, the leaves are also proved to serve as potential dye with direct affinity to cotton fabrics (Kechi *et al*., 2013; Tewachew *et al*., 2018). Farmers also use the plant to protect soil erosion and retaining soil on sloping land (Fernandes *et al*., 1984).

1.4. Nutritional Content Analysis

Nutritional content analysis of *R. prinoides* showed that it has protein (8.5%), fiber (25.6%), ash (9.5%), carbohydrate (70.5%), moisture (9.5%) and fat (3.5%) in support of its nutritional significance (Amabye, 2015)

Table 1. Ethnomedicinal uses of *R. prinoides*in Africa.

Disease treated	Plant part	Mode of preparation and administration	Country practiced	Reference
Sexually transmitted diseases, arthritis, flu/cold, back pains, stomachache, pneumonia, brucellosis, strength/nutrient supplement, enhancing/facilitating digestion	Tree	-	Kenya	(Kiringe, 2006)
Ear, nose and throat (ENT) diseases (chest pain, common cold and Tonsil)	Roots, Leaves, Stems	Roots, Leaves and Roots/ stems boiling taking orally respectively	Kenya	(Njoroge & Bussmann, 2006)
Tinea capitis, Itching/skin rash	Seeds, Leaves	Rubbed seeds on the affected part and Burn leaves in oven, crush, mix it with butter and apply on the skin	Ethiopia	(Hishe & Asfaw, 2014; Teklay *et al*., 2013)
Tonsilities	Leaves	Leaves infusion Gargle to rinse the throat	Ethiopia	(Kidane *et al*., 2014; Teka *et al*., 2020)
Skin infection	Leaves	Fresh leaves rubbed it the affected part	Ethiopia	(Tamene, 2020)
Wound	Leaves	Crush the leaf and apply to the wound till the wound cures.	Ethiopia	(Bitew *et al*., 2019)
Tonsillitis	Leaves	Mothers chewing the leaves and spit to mouth of their children whereas young ones chew it for themselves	Ethiopia	(Araya *et al.*, 2015; Tegene, 2018; Teklehaymanot & Giday, 2007)
Eczema	Leaves	Leaves crushed, mixed with pure butter and dressing the affected part		
Scabies	Leaves	The leaf is crushed, powdered, mixed with butter and creamed Wounds and exposing the sunlight for few minutes.	Ethiopia	(Gebeyehu, 2016)
Tonsillitis	Fruits & Shoots	Three fruits and shoots are crushed with three fruits of malt barley, squeezed and droplets of juice are taken through the nose.		
Ring worm	Seed	Adding the grinded powder	Ethiopia	(Abdeta e*t al*., 2020)

Table 1. *Continued.*

Fever in children	Leaves	Adding the leaf in to water, stay for a while and give the drop of this Concoction to the child	Ethiopia	(Asmare *et al*., 2018)
Sedative, gonorrhea, blood purifier, colics	Leaves, roots	Decoction of leaves is sedative; decoction of roots drunk against gonorrhoea and rheumatism; its decoction mixed with that of the bark of *Erythrinaabyssinica*Lam. ex DCalleviates colics; decoction of the roots is considered blood purifier and plant liniment is used for sprains	Tanzania	(Chhabra *et al*., 1984)
Malaria	Bark, roots	-	South Africa	(Cock *et al*., 2019)
Blood cleaning, pneumonia, rheumatism, sprains, gargle, skin complaints, respiratory infections, sexually transmitted disease, arthritis, stomach ache, headache	Fruit, leaves, stems, seeds, roots	-	South Africa	(Dzoyem *et al*., 2016)
Sexual Transmitted Infection	Root	A 3 finger thick pieces of root palm length are boiled in 2 liters of water. 200mls is taken 3 times daily for 7 days	Kenya	(Gakuya *et al*., 2015)
Malaria, backaches	Root bark	Root bark boiled all day and drunk with goat fat for malaria, backaches. Drink only one cup, one time	Kenya	(Koch *et al*., 2005)

Table 1. *Continued.*

Sexually transmitted diseases (STDs)	Leaves	The leaf, often with the root of *Rubusapetalus*Poir., is boiled, decocted, and drunk before meal	Ethiopia	(Tuasha *et al*., 2018)
Tuberculosis	Leaves	-	Uganda	(Tabuti *et al*., 2010)
Dandruff	Leaves	-	Ethiopia	(Mesfin *et al*., 2013)
Waterborne and related diseases	Leaves		Ethiopia	(Siyum & Woyessa, 2013)
Animal diarrhea and intestinal parasites	Leaves	Chopped leaves mixed little water and then filtered then taken orally	Ethiopia	(Bekele & Musa, 2009)
Animal hepatitis	Leaves	Crushed and powdered leaves of *Cheilanthes farinosa*Forsk. Kaulf.and *Rhamnus prinoides*; and whole parts of *Crepis rueppellii* are brushed over the body	Ethiopia	(Yineger *et al*., 2007)
Leech infestation	Leaves	Pounded leaves applied nasally	Ethiopia	(Bekele *et al*., 2012)
Psychoactive agent	Bark	Ground bark that is administered as snuff for mental disorders	South Africa	(Sobiecki, 2002)

1.5. Mineral Content

Mineral contentanalysis of leaves and stem revealed the presence of Ca, Mg, Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd and Pb of which the concentration of Ca and Mg was reported to be high (Gebre and Chandravanshi, 2012, Table 2). In a related study potassium, sodium and magnesium were the most abundant elements from the leaves and stems samples collected from the low-altitude (1500-1670 m above sea level) and medium-altitude (1670-2000 m above sea level) areas (Nagari & Abebaw, 2013).

Table 2. The mineral content of *R. prinoides.*

Constituents (mg/Kg)	Leaf (mg/kg)	Stem (mg/kg)
Ca	6304-22236	3601- 5675
Mg	3202-5706	2635 - 5568
Cr	5.08 - 20.6	ND - 16.3
Mn	8.12 - 17.9	2.16 - 3.98
Fe	47.9 - 187	22 - 124
Co	22.2 -42.1	18.7 - 91.7
Ni	12.8 - 27.3	9.68 - 19.2
Cu	6.5 - 73.0	16.8 - 233
Zn	12.2 - 43	17.4 - 28.2
Cd	0.81 - 3.10	ND - 1.56
Pb	11.7 - 25	ND

ND – Not detected

1.6. Chemical Constituents

Various class of secondary metabolites including alkaloids, triterepenes, saponins, tannins, phenols, cardiac glycosides, anthraquinones, polyphenols and flavonoids were reported from different parts of *R.prinoides* (Amabye, 2015; Co *et al*., 1990; Lin *et al.*, 1991; Lin *et al*., 1990; Molla *et al.*, 2016; Ozipek *et al*., 1994) as summerized here below (Figure 2, Table 3).

Campbell and his co-workers (2019) reported the presence of numerous essential oils from the leaves of *R. prinoides* of which 4-hydroxy-4-methyl-2-pentanone (15, Figure 3) and ethyl 4-ethoxybenzoate (16, Figure 3) account for more than 85% and exhibited significant antibiofilm activity. In a related study, it has been reported that the essential oils have antibacterial, antifungal, antioxidant and anti-diabetic activties (Sut *et al*., 2020).

Figure 2. Structures of compounds isolated from *R. prinoides*.

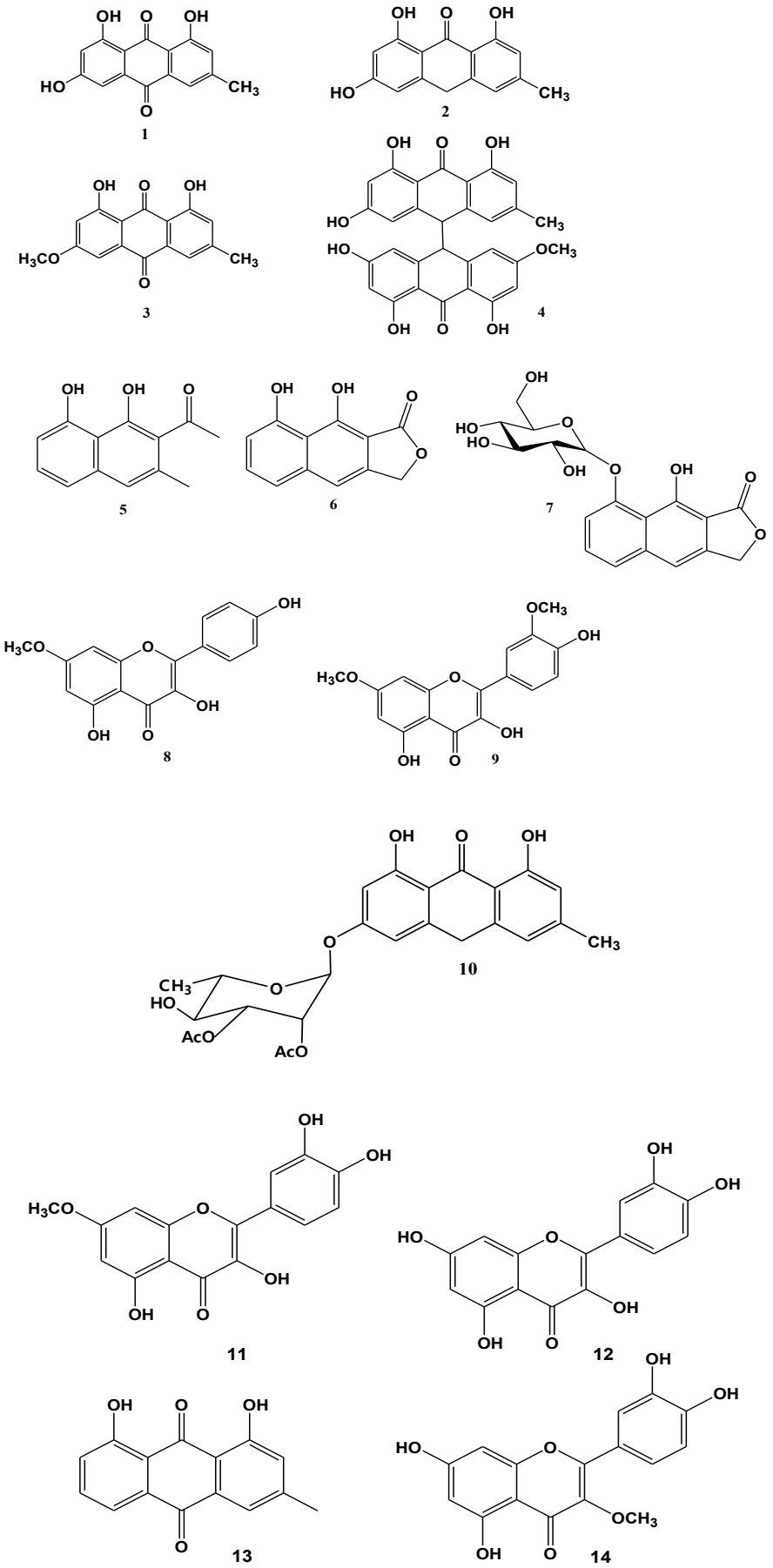
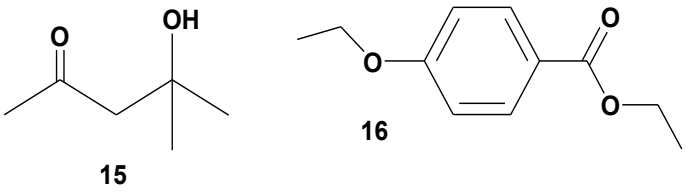


Table 3. Secondary metabolites isolated from *R. prinoides*.

Compound	Plant Part	Reference
Emodin (1)	Fruit, Leaves, stems	(Abegaz & Dagne, 1988; Abegaz & Kebede, 1995; Gebru, 2010)
Emodinanthrone (2)	Leaves, stems, Fruit	(Abegaz & Dagne, 1988; Abegaz & Peter, 1995)
Physcion (3)	Fruit, Leaves, stems	(Abegaz & Dagne, 1988; Abegaz & Kebede, 1995; Gebru, 2010)
Emodinbianthrone (4)	Fruit	(Abegaz & Dagne, 1988; Abegaz & Peter, 1995)
Musizin (5)	Leaves	(Abegaz & Kebede, 1995)
β-sorigenin (6)	Leaves, roots	(Abegaz & Kebede, 1995; Gebru, 2010)
Geshoidin (P-Sorigenin-8-*O*-β-D-glucoside (7)	Leaves, stems, roots	(Abegaz & Kebede, 1995; Gebru, 2010)
Rhamnocitrin (8)	Leaves	(Abegaz & Kebede, 1995)
Rhamnazin (9)	Leaves, roots	(Abegaz & Kebede, 1995; Gebru, 2010)
Prinoidin (10)	Fruits	(Abegaz & Kebede, 1995; Abegaz & Peter, 1995)
Rhamnetin (11)	Leaves, stems	(Abegaz & Kebede, 1995)
Quercetin (12)	Leaves	(Abegaz & Kebede, 1995)
Chrysophanol (13)	Leaves, stems, roots	(Abegaz & Kebede, 1995; Gebru, 2010)
3-*O*-Methylquercetin (14)	Leaves, Stems	(Abegaz & Kebede, 1995)

Figure 3. Structure of major constituents of essential oils from leaves of *R. prinoides*.



1.7. Biological Activity

1.7.1. *Antioxidant activity*

It was reported that *n*-hexane, chloroform, ethyl acetate, and methanolic extracts of the leaf and stem bark of *R.prinoids* possess antioxidant activities by DPPH assay with IC_{50} values of >3000, >3000, >3000, 950.42, ~1500, 710.50, ~1000 and 902.78 µg mL^{-1}, respectively (Pillai *et al*., 2019). The documented antioxidant activities of *R.prinoides*are probably due to flavonoids and polyphenols reported from leaves, stems and roots(Amabye, 2015; Molla *et al*., 2016; Pillai *et al*., 2019).

1.7.2. *Antiinflammatory activity*

Semi purified ethanolic stem and stem bark extracts of *R.prinoides* with higher contents of polyphenols and flavonoids displayed anti-inflammatory activity through reducing the Nitric

Oxide production at the dosage of 11.11-100 µg/mL and the COX-2 inhibitory activity with an IC_{50} value at 20.61 ±0.13 µg/mL (Chen *et al*., 2020).

1.7.3. *Antibiofilm activity*

Leaf and stem ethanol extracts of *R. prinoides* showed significant inhibition of *Staphylococcus aureus* Rosenbach, *Bacillus subtilis* and *Streptococcus mutans* Clarke biofilm formation up to 99.9% and reduced planktonic cell growth up to 10 log units relative to untreated controls(Campbell *et al*., 2019). In a related study, the stem ethanol extracts disrupted *S. mutans* and *C. albicans* co-culture synergism, with 98% less polymicrobial biofilm formation than the untreated control (Campbell *et al*., 2020).

1.7.4. *Antibacterial activity*

Antibacterial activity of methanol and chloroform leaves extracts of *R. prinoides* against *Staphylococcus aureus*, *Streptococcus pyogenes* Rosenbach, *Streptococcus pneumoniae* revealed minimum inhibitory concentration ranging from 8.13-32.5 mg/mL and 8.13-16.25 mg/mL, respectively, compared to a positive control cefotaxin (0.03 mg/mL) and ampicillin (0.01 mg/mL) ranged from 23.67- 28.00 mg/mL in both fractions, respectively (Molla *et al*., 2016).

1.7.5. *Antimalarial activity*

The chloroform root bark extracts of *R.prinoides* were active against chloroquine sensitive *Plasmodium falciparum* strain with IC_{50} value of 3.53 µg/mL (Koch *et al*., 2005). In a related study, *n*-hexane and dichloromethane extracts were demonstrated to have antiplasmodial activity with IC_{50} values of 19.9 µg/mL and 30.3 µg/mL, respectively, with no toxicity in the brine shrimp test (Bosire, 2003). The *in-vitro* antiplasmodial activities of dichloromethane/methanol (1:1) crude extracts and the isolated compounds were performed against the chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *Plasmodium falciparum.* The naphthalenic derivative Geshoidin (7) displayed IC_{50} value of 4.0 ± 0.9 pM and 0.4 ± 0.2 pM against the chloroquine sensitive (D6) and chloroquine resistant (W2) strains of *Plasmodium falciparum* (Gebru, 2010). *In vivo* antimalarial activity of aqueous extracts from leaves and root barks against a blood-induced CQ-resistant rodent parasite in mice showed high chemosuppression in the range of 51-75% (Muregi *et al.*, 2007) suggesting the promissing application of leaves, stem bark and roots of *R.prinoides* to treat malaria.

1.7.6. *Antimycobacterial activity*

The acetone leaf extracts of *R.prinoides* demonstrated antimycobacterial activity with minimum inhibitory concentration (MIC) values ranging from 0.625 to >2.5 mg/mL aganist three fast-growing mycobacteria species *i.e. Mycobacterium smegmatis* Trevisan*, Mycobacterium aurum* Tsukamura and *M. fortuitum* Da Costa Cruz and one pathogenic *M. tuberculosis* strain (Dzoyem *et al*., 2016).

1.7.7. *Wound healing activity*

In vivo study in mice revealed that the hydroalcoholic extracts of the leaves of *R. prinoides* possess wound healing activity established by a significant rate of wound contraction and shorter epithelization period. In this study, ten percent of 80% methanol leaves extract showed significant wound contraction against the control and rate increased in significant level with number of days $p < 0.05$, $p < 0.01$, and $p < 0.001$ on days 2 to 4, 8 to 10 and 12 to14, respectively (Tessema *et al.*, 2021). Biological activities of different parts of *R. prinoides* are summerized here below (Table 4).

Table 4. Biological activity of different parts of *R. prinoides*

Plant part	Activity	Reference
Flowers, leaves, stems	Antimutagenic	(Wall *et al*., 1988)
Leaves	Antibacterial	(Chhabra & Uiso, 1991; Chhabra *et al*., 1984; Molla *et al*., 2016)
	Insecticide	(Van Puyvelde et al., 1985)
Root bark	Cytotoxic	(Koch *et al*., 2005)
Leaves, roots, bark root	Antimalarial	(Muregi *et al*., 2007)
Leaves	Wound healing	(Tessema *et al*., 2021)

2. CONCLUSION

The present review presented chemical constituents and biological activity of *R. prinoides* native to Africa and India. Various secondary metabolites such as flavonoids, alkaloids, tannins, terpenoids, saponins, steroids and anthraquinones have been reported from the genus of which polyphenols were abundant with tremedous antioxidant, wound healing and anti-inflammatory activities. Economically, the leaves and stems have been used as hopping agent in the brewery industry, making traditional alcoholic beverages like tella and tej (Ethiopia), animal feed, medicine, nectar for bees, soil conservation, ornamental, and dyes in textiles. Considering diverse class of secondary metabolites as well as wide spectrum of biological activities of the plant, it is believed that plant is a good natural source of polyphenols and can be used as an alternative medicine for future healthcare practice.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

GN, MA, SN, FI, YW, MT: literature compilation, review and analysis. **GN & ME**: manuscript write up and review. All authors have read and approved the manuscript.

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Research Article

Growth performance, tolerance and vigor dynamics of *Salvia candidissima* subsp. *occidentalis* Hedge against heavy metal contamination

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Abstract: In this study, ecotoxicological and ecophysiological effects of some different heavy metal compounds ($CdCl_2$, $PbCl_2$, and $CuCl_2$) on *Salvia candidissima* subsp. *occidentalis* Hedge (Lamiaceae) were examined. Seeds of this plant were exposed to three different concentrations of $CdCl_2$ (2, 6, 10 ppm), $PbCl_2$ (50, 100, 500 ppm), and $CuCl_2$ (20, 60, 150 ppm). The results indicated that increasing $CdCl_2$ and $PbCl_2$ concentrations had no specific inhibitory impacts on seed germination rates, growth performance, biomass, and seedling vigor index, but increasing concentrations of $CuCl_2$ had significant inhibitory effects on these parameters. The metal tolerance index of all applications showed that all heavy metal treatments reduce this value.

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1. INTRODUCTION

Genus *Salvia* L. has 100 different species in Turkey, *Salvia candidissima* Vahl is a species which belongs to Group D and has two subspecies (Davis, 1985; Sen-Utsukarci et al., 2019). *Salvia candidissima* subsp. *occidentalis* Hedge is one of these subspecies. It differs from the other subspecies with smaller leaves, thick indumentum and white flowers (Figure 1 a, b). This taxon is widespread and common for Inner Anatolia, Turkey (Davis, 1985).

Genus *Salvia* has rich chemical compounds like the other members of its family. *Salvia* species is important as medicinal plants. There are some pharmacological studies about *S. candidissima* (Ulubelen and Topcu, 1998), but not any other studies about its ecological or ecophysiological characteristics. From the aboveground parts of *S. candidissima*, 3-oxosalvipisone, was acquired with 11β-hydroxymanoyl oxide, 8,13-diepimanoyl oxide, spathulenol, salvigenin, crysoeriol, diosmetin and *o*,*p*-dimethoxybenzoic acid (Ulubelen *et al.,* 1995). From the roots of *S. candidissima* subsp. *candidissima*, new diterpenes, new steroidal ester and α-amyrin acetate were insulated (Ulubelen *et al.,* 1997). In addition to diterpenoids, 11-hydroxy-12-methoxyabieta-8,11,13-trien and 1-oxosalvipisone, 14-oxopimaric acid, ferruginol, horminone, 7-acetylhorminone, cryptanol, montbretyl 12-methyl ether,

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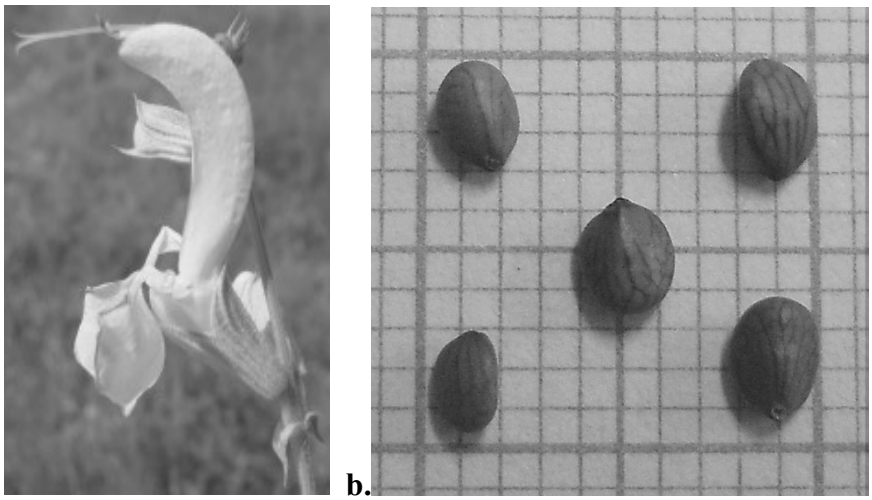
microstegiol, 1-oxoaethiopinone and salvipisone were obtained (Ulubelen *et al.,* 1992a), a new diterpene, candidissiol was insulated from the underground parts of *S. candidissima* (Ulubelen *et al.,* 1992b).

In consequence of increasing industrial human actions, releasing heavy metals to the nature (water, air and soil) has become a scientific matter. All components of the ecosystem are affected of heavy metal contamination in different levels. Heavy metal contamination of agricultural areas is a worldwide issue (Angelova *et al.,* 2017). Heavy metals are not degradable by biological pathways and permanent in the ecological environment continually (Singh & Prasad, 2011). Heavy metals can join the food chain after they are taken from the soil by plants. A deep understanding of metal uptake, transport, and defense processes in plants is required to improve metal uptake under metal-limiting conditions to increase vegetative productivity. (Gangwar *et al.,* 2014).

For plant species, cadmium is not an essential but a toxic element that can be actively extracted from the soil, high concentrations of cadmium can have adverse effects on the production and standard of the crop, as well as harm humans via the food chain (He *et al.,* 2008). Lead is an inherently consisting heavy metal element and main human origin basis of Pb contains metal mining activities, gem-dressing, and molding; pile fabrication; removal of lead containing outcomes without control processes, such as used up piles and computer products, etc. (Scheuhammer *et al.,* 2008). Copper element is an essential micronutrient for the life of plants, but it is thought to have a toxic effect by causing abiotic stress at high concentrations (Gill *et al.,* 2012).

The aims of this study were to observe the reactions of *S. candidissima* subsp. *occidentalis* (Fig 1) against pollution factors such as different heavy metal concentrations, like germination characteristics, shoot and root growth and production as biomass, seedling growth, seedling vigor index and metal tolerance index.

Figure 1. a. Flower and, **b.** Seeds of *Salvia candidissima* subsp. *Occidentalis.*



2. MATERIAL and METHODS

The plant samples were collected from study area (Borabey Pond environs, Eskisehir Technical University) which is located at Eskisehir, Turkey. *S. candidissima* subsp. *occidentalis* seeds were used in this study. The seed germination experiments were performed out in MLR-350 Model Sanyo (Japan) plant growth chamber. During germination experiments, a photoperiod with 8 hours light/16 hours dark and stable temperature (+25°C) were applied (Yucel, 2000). Based on literature data and hazard levels, different concentrations of $CdCl_2$ (2, 6, 10 ppm), $PbCl_2$ (50, 100, 500 ppm) and $CuCl_2$ (20, 60, 150 ppm) were applied. Germination experiments

were carried out in petri dishes (9 cm diameter) with two layers of circular filter papers and 25 seeds were sowed at each petri dish. Seed beds were filled with 9 ml of solutions including different heavy metal concentrations. Control groups were prepared with distilled water. The seed sowed beds kept into plant growth cabinet for 10 days. In order to accept the seed as germinated, the root tip must contact the germination bed (Yucel & Yilmaz, 2009). In the course of the experiments, controls were made every day at a specific time. End of the tenth day, hypocotyl and radicle lengths were measured by using rulers, then hypocotyls and radicles were separated to weigh each of them. For observing wet amounts of hypocotyl and radicle, fresh shoots and roots were measured directly without any application by ruler and for dry amounts of hypocotyl and radicle, shoots and roots were dried at 105°C, for 48 hours at sterilizer. Seedling vigor index (SVI) was calculated with respect to Murthy and Tejavathi (2016). Metal tolerance index (MTI) is calculated according to Turner and Marshall (1972).

The data obtained from experiments were tested statistically with the SPSS Statistics 20 package program, One-Way ANOVA Test with a p-value less than 0.05 considered statistically significant.

3. RESULTS

Seed germination experiments showed that increasing $PbCl_2$ and $CdCl_2$ concentrations has no significant effect on seed germination percentage (F=0.681; df=9.20; p>0.05), seedling growth (For hypocotyl F=10.079; df =9.20; p>0.05, for radicle F =11.558; df =9.20; p>0.05), biomass (For hypocotyl F =1.452; df =9.20; p>0.05, for radicle F =1.824; df=9.20; p>0.05) and SVI (F =1.815; df=9.20; p>0.05) value of *S. candidissima* subsp. *occidentalis,* but increasing $CuCl_2$ concentrations had an inhibitory effect of all ecophysiological parameters of *S. candidissima* subsp. *occidentalis* (Table 1).

Table 1. *Ecophysiological parameters observed after different heavy metal applications.*

Application		% Germination	Hypocotyl length (cm)	Radicle length	Hypocotyl biomass (mg)	Radicle biomass	SVI	MTI
Control		31	2.52	5.35	7.50	2.27	83.48	1.000
$CdCl_2$	2 ppm	39	2.80	4.99	5.77	2.63	113.67	0.708
	6 ppm	35	2.04	2.74	5.93	1.90	73.81	0.523
	10 ppm	44	2.84	3.70	12.13	4.03	126.19	0.586
$PbCl_2$	50 ppm	36	2.41	5.15	5.53	2.50	92.72	0.814
	100 ppm	44	2.25	4.10	9.50	2.90	104.46	0.760
	500 ppm	39	2.35	2.71	8.27	2.23	93.47	0.518
$CuCl_2$	20 ppm	44	2.12	1.49	11.57	2.97	93.62	0.233
	60 ppm	24	1.73	1.14	6.03	0.93	42.53	0.170
	150 ppm	24	1.02	0.60	5.77	0.87	26.70	0.097

Biomass data observed from this research showed that increasing $PbCl_2$ and $CdCl_2$ concentrations had no significant effect on biomass of *S. candidissima* subsp. *occidentalis,* but increasing $CuCl_2$ concentrations had an inhibitory effect on biomass of *S. candidissima* subsp. *occidentalis* (Table 1).

Results about root and stem growth of seedlings showed that increasing $CdCl_2$ concentrations had no significant effect on root and stem growth of seedlings of *S. candidissima* subsp. *occidentalis,* but increasing $PbCl_2$ and $CuCl_2$ concentrations had an inhibitory effect on root and stem growth of seedlings of *S. candidissima* subsp. *occidentalis* (Table 1).

MTI values of all heavy metal applications showed that all treatments decreased metal tolerance index value (F=48.845; df=9.30; p<0.05). MTI parameter is more critical than other ecophysiological data. The sensitivity of experiments can be examined through the metal tolerance index.

4. DISCUSSION

In this study, an examination of the ecophysiological effects of various concentrations of heavy metals which are $CdCl_2$ (2, 6, 10 ppm), $CuCl_2$ (20, 60, 150 ppm) and $PbCl_2$ (50, 100, 500 ppm) on *S. candidissima* subsp. *occidentalis* were investigated. Determining the "seed germination behavior" is essential to protect the natural habitats and gene pools of plant species, and supplying the incrasing request for plant-based outcomes can only be probable by conserving and growing natural plants (Yucel & Yilmaz, 2009).

It is known that elements and different solutions introduced to seeds have an impact on germination and seedling growth, especially, some macro or micro nutrition elements such as potassium or boron stimulates it. However, seed germination is lagged and detentioned concerning some toxic element concentrations like high percentages of iron or sulphur (Katkat and Kaçar, 2009).

Petrescu *et al.* (2014) observed that the cadmium decreased seed germination in *Salvia officinalis*, germination percentages were 81.00% (10 ppm), 59.66% (50 ppm) and 21.33% (100 ppm), compared to control 93.33%. Contrary to that, our observations showed that increasing $CdCl_2$ (2, 6, 10 ppm) and $PbCl_2$ (50, 100, 500 ppm) concentrations had no significant effect on seed germination of *S. candidissima* subsp. *occidentalis,* but increasing concentrations of $CuCl_2$ (20, 60, 150 ppm) had a significant inhibitory effect on seed germination of *S. candidissima* subsp. *occidentalis.* We can say that *S. candidissima* subsp. *occidentalis* could tolerate effect of $CdCl_2$ (up to 10 ppm) and $PbCl_2$ (up to 500 ppm) during seed germination stage.

The observations about biomass showed that increasing $CdCl_2$ (2, 6, 10 ppm) and $PbCl_2$ (50, 100, 500 ppm) concentrations had no significant effect on biomass increase of *S. candidissima* subsp. *occidentalis* seedlings*,* but increasing concentrations of $CuCl_2$ (20, 60, 150 ppm) had a significant inhibitory effect on biomass increase of *S. candidissima* subsp. *occidentalis* seedlings*.* We can claim that *S. candidissima* subsp. *occidentalis* could tolerate effect of $CdCl_2$ (up to 10 ppm) and $PbCl_2$ (up to 500 ppm) on biomass increase during seedling stage.

Petrescu *et al.* (2014) observed that cadmium solutions applied to *S. officinalis* seeds increased radicle development at concentrations of 10 ppm, but decreased at concentrations of 50 ppm and 100 ppm. Although our findings about seedling growth showed that increasing $CdCl_2$ (2, 6, 10 ppm) concentrations had no distinct impact on plant development of *S. candidissima* subsp. *occidentalis* seedlings*,* but increasing concentrations of $PbCl_2$ (50, 100, 500 ppm) and $CuCl_2$ (20, 60, 150 ppm) had significant inhibitory effects on seedling growth of *S. candidissima* subsp. *occidentalis* seedlings*.* There is an interesting result about effect of $CdCl_2$ concentrations on root growth. Different $CdCl_2$ concentrations had significant inhibitory effect on root growth of *S. candidissima* subsp. *occidentalis* seedlings.

Bini *et al.* (2012) reported that *Taraxacum officinale* is a bioindicator plant and also has ethnobotanical usage for liver disease and cooking, but its heavy metal accumulation ability is harmful for human health. *S. candidissima* subsp*. occidentalis* is also tolerant to both cadmium and lead (at a certain level) and is consumed as herbal tea by the local people, according to the information transmitted orally during field studies. therefore we can assert negative effects with *T. officinale* is valid for *S. candidissima* subsp. *occidentalis.* However, Carman Sosa *et al.* (2016) revealed that *Tagetes minuta* plants growing in areas polluted with lead, while lead was not found in essential oil and some ingredients were generated more than control group.

Novo *et al.* (2013) reported concerning the capacity of *Salvia verbenaca* to bear trace metals and actively replied to the following oxidative stress. *S. candidissima* subsp. *occidentalis* Hedge is also like *S. verbanaca* in terms of tolerance to heavy metal stress.

Chand *et al.* (2016) stated that when the essential oil is extruded through hydrodistillation, the heavy metals in the plant do not migrate to the essential oil, so the *Pelargonium graveolens* plant is suitable for the volatile oil even if it is grown in places exposed to heavy metal pollution. *S. candidissima* subsp. *occidentalis* has also valuable volatile oil content, its tolerance to Pb and Cd makes this plant appropriate for grown in polluted areas for volatile oil production.

Similarly, to findings of this study, Torun (2019) observed that *Salvia officinalis* was showed no significant difference comparatively water ingredient and chlorophyll fluorescence under salt and cobalt stress, but relative growth rate was raised after salt+cobalt application and it can be said that the mixture of salt and cobalt is suitable for improving toleration of *S. officinalis* for stress (Torun, 2019). Heavy metals can stimulate plant growth performance at a certain level (Arif et al., 2016).

Duka *et al.* (2015) stated that *Salvia officinalis* plant should be checked for heavy metal ingredients before being processed for human consumption. Similar to the findings of Duka *et al.* (2015), we can say that it is necessary to be careful in consuming *Salvia candidissima* subsp. *occidentalis* as it is tolerant to heavy metals such as cadmium and lead, if the gathering areas of the plant in question are exposed to cadmium and lead pollution, we can say that the plant can be harmful to people consuming it due to heavy metal accumulation.

5. CONCLUSION

S. candidissima subsp. *occidentalis* is a perennial plant that can be used in park and garden landscaping because of its white and interesting flowers. In addition, it can be grown for medical purposes owing to its chemical substances such as diterpenoids. According to the results, it can be said that *S. candidissima* subsp. *occidentalis* can be grown in habitats contaminated with both cadmium and lead due to its tolerance ability against heavy metals. It has been revealed that ecophysiological parameters such as% Germination, Hypocotyl and radicle lengths, biomass, SVI and MTI are the values that must be taken into consideration when examining the effects of heavy metals on the plant species in question.

Declaration of Conflicting Interests and Ethics

The author declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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Research Article

Characterization of Bioactive and Antioxidant Composition of Mountain Tea (*Sideritis montana* ssp. *montana*): Microwave-Assisted Technology

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Abstract: The use of synthetic antioxidants has been restricted and even prohibited in some countries due to their toxic effects. This study aims to perform the biochemical assays representing the bioactive compounds in *Sideritis montana* L. ssp. *montana* L. known as mountain tea. The study specifically investigates the potential application of mountain tea in the food industry as a natural antioxidant. Different extraction procedures using various solvents are carried out to obtain phenolic compounds and antioxidant activities in plant matrices. In this study, the maceration method was used for the extraction procedure. Furthermore, the microwave-assisted extraction method was applied to shorten the extraction time and decrease the solvent quantity. Methanol was used as a solvent in both conventional and microwave-assisted extraction procedures. The samples were evaluated in terms of the total phenolic compounds, total flavonoids, antioxidant activities, and metal chelating capacity. The obtained results for classical extraction and microwave-assisted extraction methods were found to be 51 and 55 mg/g for TPC, 16.3 and 22 mg/g for TFC, 93 and 103 mg/g for FRAP, and 284 and 282 mg/g for MCC, respectively. The microwave-assisted extraction method produced higher concentrations of bioactive molecules as compared to the classical method. Microwave-assisted extraction was found to be superior to the conventional method in terms of effectiveness, extraction time, and solvent quantity. Besides, *Sideritis montana* ssp. *montana* is suggested in the food industry as a natural antioxidant instead of synthetic ones to prevent health-damaging effects.

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1. INTRODUCTION

Numerous studies have shown a high correlation between the consumption of medicinal plants in the prevention and treatment of various health problems including cancer, cardiovascular and neurodegenerative diseases. Phenolic compounds in medicinal plants and foods are known to significantly decrease the adverse effects of chemically reactive species on normal physiological functions in humans. The genus *Sideritis* L. (Labiatae/ Lamiaceae) comprises more than 150 species and several taxa are distributed in Turkey and other Mediterranean countries (Bilginoğlu, 2017). Many species that belong to *Sideritis* are traditionally applied to

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prepare herbal teas (Semiz & Özel, 2017), commonly known as "Mountain tea", which is widely consumed around the world due to its therapeutic properties. Many biological effects including anxiolytic, diuretic, antimicrobial, antibacterial, antioxidant, and anti-inflammatory effects of *Sideritis* species have been reported (Irakli *et al*., 2018) (Ognyanov *et al*., 2021). *Sideritis* species have a pleasant aroma making their consumption appealing to be consumed as herbal tea (Zyzelewicz *et al*., 2020). Also, many species of *Sideritis* have been shown to induce neuroprotective and memory-enhancing properties (Axiotis e*t al*., 2020).

The physiological roles of phenolic compounds include their protection against disorders associated with oxidative stress such as cancer, atherosclerosis, cardiovascular, and neurodegenerative diseases. Regulating the endogenous antioxidant defense system, polyphenolic compounds participate as the scavengers of free radicals (Arruda *et al*., 2020). Antioxidants could be either natural or synthetic. Synthetic antioxidants have phenolic arrangements of many degrees of alkyl replacement, while natural antioxidants can be phenolic compounds including a- tocopherols, flavonoids, and phenolic acids, b- nitrogen compounds such as alkaloids, chlorophyll derivatives, amino acids, and amines, and c- carotenoids as well as ascorbic acid (Xu *et al*, 2021). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene, propyl gallate, and tert-butylhydroquinone (TBHQ) have been used in the food industry attributable to their low cost and tasteless flavor. Synthetic preservatives are applied to the foods to prevent them from oxidants during processing, packaging, transportation, and storage. However, long-term excessive intake of the chemical residues of such synthetic preservatives has been reported to induce toxicity even carcinogenicity (Martelli & Giacomini, 2018). Therefore, some countries such as China, Japan, Canada, and Europe have been restricted and even prohibited the use of synthetic antioxidants. Recently, BHA has been removed from the generally recognized as safe (GRAS) list of compounds. Natural products are considered safe alternatives to synthetic ones with many beneficial properties. Increasing reports on "green" preservation reagents called food-grade antioxidants are based on natural substances. Besides, phenolic compounds have also been shown to possess significant antibacterial activity *(Ceylan et al.*, 2020). Therefore, application of natural antioxidants instead of synthetic ones could be a good candidate in food industry (Rodrigues *et al*., 2020).

Replacement of *Sideritis*-based antioxidants such as chlorogenic acid, quercetin, naringin, verbascoside, luteolin, and apigenin derivatives reported to be responsible for the antioxidant, anti-inflammatory, and anticarcinogenic effects with synthetic antioxidants may be suggested. Thus, *Sideritis* species could also be a good candidate in food industry instead of synthetic preservatives including TBHQ and the aforementioned ones *(González-Burgos et al.*, 2011). Other possible applications of *Sideritis* for their higher phenolic profiles, range from food preservatives to cosmetics and also a product of interest for the pharmaceutical industry (Marchev *et al.*, 2021). Due to its terpenoid constituents, *Sideritis* species could also be a good candidate as flavoring agents in the food industry (González-Burgos *et al.*, 2011). The whole bioactive molecules of *Sideritis* may have synergistic effects with many other health-benefit properties.

Various plant extraction techniques could be divided into two main groups; namely, conventional and novel techniques. Conventional or classical extraction method includes maceration, Soxhlet, soaking, turbo-fast blending, and solvent permeation. On the other hand, the novel or so-called modern extraction processes include ultrasonic-assisted, subcritical, supercritical CO2, pressure-assisted, enzyme-assisted, and microwave-assisted extraction (MAE) methods. The conventional methods have great disadvantages due to their long extraction time and high solvent requirement. Therefore, a reliable, easy, quick, and eco-friendly plant extraction technique is significantly important to determine phenolic compounds and antioxidant activities (Chemat *et al*., 2009).

In this study, total phenolic content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP), and metal chelation capacity (MCC) of *Sideritis montana* L. ssp. *montana* L. were determined. These parameters were suggested to be related to the food preservative as well as their contribution to health benefits. Their measurements were carried out using both conventional and MAE methods with a purpose to develop an alternative method taking into consideration less time and solvent-consuming procedure together with high efficiency.

2. MATERIAL and METHODS

2.1. Chemicals and Materials

Aluminum chloride, sodium acetate, potassium ferricyanide, iron (III) chloride, 1,10-phenanthroline monohydrate, potassium hydrogen phosphate, sodium hydroxide, sodium carbonate, hydrogen phosphate, trichloroacetic acid, iron sulfate monohydrate, and Folin-Ciocalteu's phenol reagent used in this study were purchased from Merck (Germany). Quercetin, gallic acid, and *n*-hexane were purchased from Sigma-Aldrich (Germany), Iso Lab (Eschau, Germany), and Carlo Erba (Sabadell, Spain), respectively. A rotary evaporator (Buchi, Rotavapor R-100) coupled with a heating bath (B-100) and vacuum bath (V-100) was used to evaporate solvent for sample preparations; a microwave oven (Mars Express) was used for the extraction. UV Spectroscopy measurements were performed with UV-VIS (Isolab, Germany) spectrophotometer.

2.2. Plant material

Sideritis montana ssp. *montana* samples (as whole aerial parts) were purchased in 2020 from a local producer in Elazig and identified and confirmed. The leaves of the plant samples were removed and air-dried in a shady room at room temperature, and grounded in a high-speed household blender.

2.3. Conventional solid/liquid extraction under stirring (Maceration)

Plant samples (10 g) were placed in a balloon with 75 ml methanol. At room temperature, the balloon was continuously stirred by a magnetic stirrer for 24 hours. Then the samples were filtrated by Whatman filter paper (102 Medium, 125 mm, S-H Labware). This procedure was repeated 3 times. Finally, all extracts were combined and evaporated via a rotary evaporator at 40°C under the pressure of 150 mbar. After the evaporation, the extract was defatted by hexane. Then, the extract was first air-dried and completely dried in a vacuum oven (Nuve 180). From the extract, 0.1 g was dissolved in 8 mL methanol and used for the biochemical assays.

2.4. Microwave-assisted extraction

MAE was performed using a closed vessel microwave apparatus. The dried sample (0.1 g) was placed into polytetrafluoroethylene vessel of 70 mL and was extracted under different conditions with 8 mL of solvent (70% methanol in water). The temperature of the system was set as 60°C, 70°C, and 85°C. Three microwave powers were used (200, 300, and 400 W). The ramp time was adjusted to 10 minutes; the hold time was as 8, 14, and 20 minutes. The extraction conditions of MAE are described in Table 1. After the extraction, the vessels were allowed to cool, and then they were centrifuged at 3000 rpm for 10 minutes. The supernatant was taken for biochemical analyzes. All procedures belonging to biochemical assays are given in Figure 1.

2.5. Determination of Total Phenolic Content

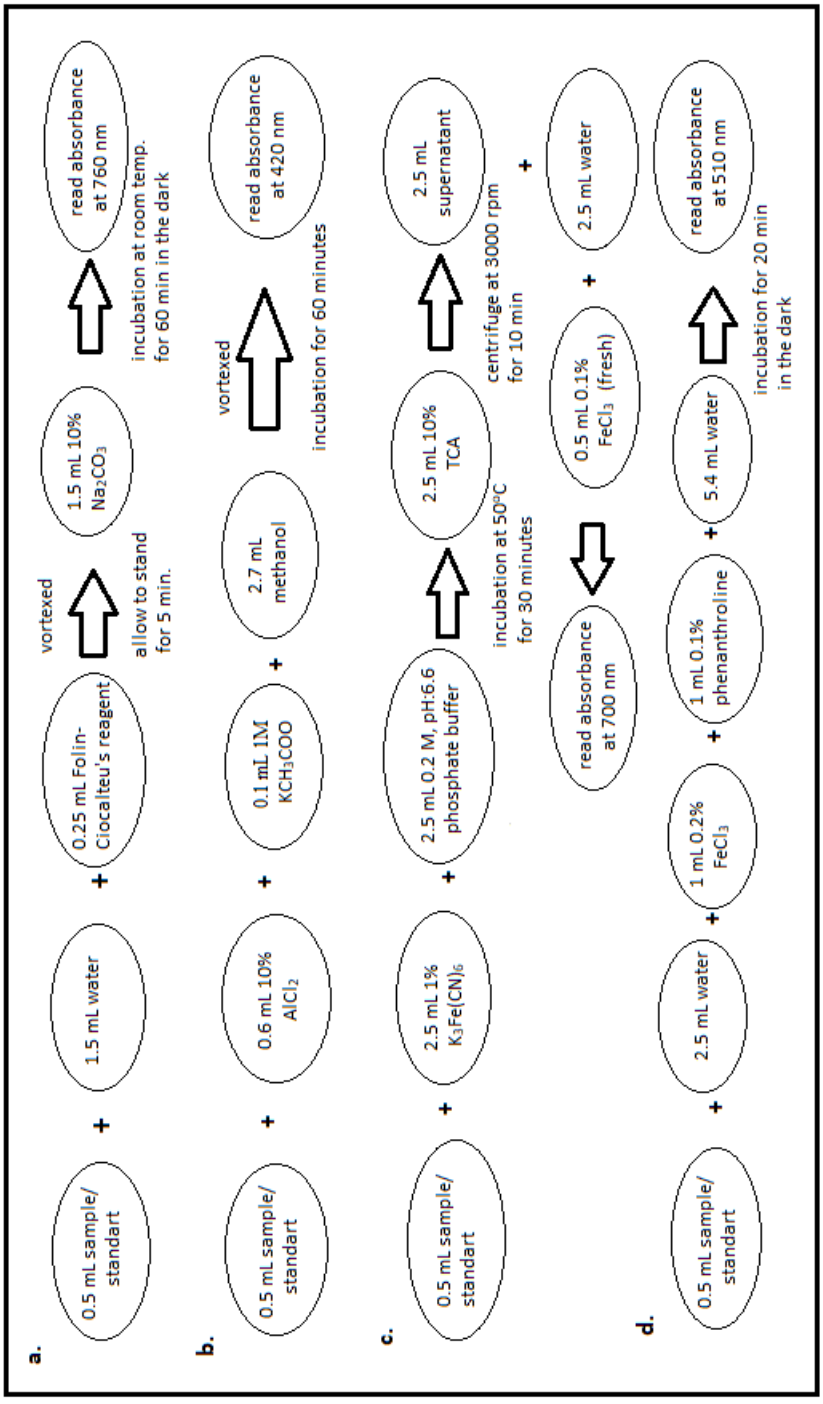
The Folin–Ciocalteu assay was used for the determination of the total phenolic content (TPC) in this study. The mechanism of this assay is based on the reduction of the Folin–Ciocalteu reagent by phenolic compounds (Magalhaes *et al.*, 2008). The procedure of the total phenolic

content assay is given in Figure 1a. Briefly, 0.5 mL sample or standard solutions (gallic acid) were

Table 1. The applied conditions of the microwave-assisted extraction method.

Temperature (ºC)	Time (minutes)		
60	8	14	20
70	8	14	20
85	8	14	20

Figure 1. The experimental procedures of a. total phenolic content, b. total flavonoid content, c. ferric reducing antioxidant power, d. metal-chelating capacity assays.



placed in test tubes with 1.5 mL water and 0.25 mL Folin–Ciocalteu reagent. After vortexing, the tubes were allowed to stand for 5 minutes. Then, 1.5 mL 10% Na_2CO_3 was added prior to the incubation at room temperature in the dark for 1 hour. The absorbances of all samples were measured at 760 nm using a UV-Vis Spectrophotometer. The construction of the calibration curve (5, 10, 20, 50, 100, and 200 ppm) was obtained with gallic acid. The results are expressed as gallic acid equivalents/ the dry plant (mg GAE/gdw).

2.6. Determination of Total Flavonoid Content

The total flavonoid content was determined according to the aluminum chloride-colorimetric method. 0.5 mL sample or standard solutions (quercetin) were added in test tubes. 0.6 mL 10% $AlCl_2$, 0.1 mL 1M KCH_3COO and 2.7 mL methanol were added followed by the incubation for 1 hour (Figure 1b). The construction of a calibration curve (5, 10, 20, 50, 100, and 200 ppm) was obtained with quercetin. The absorbances were measured at 420 nm. The results are expressed as quercetin equivalents/ the dry plant (mg QE/gdw).

2.7. Ferric Reducing Antioxidant Power Assay

Reducing power (FRAP) of the methanolic *Sideritis montana* extracts was determined based on their antioxidant principles to form a colored complex with potassium ferricyanide (Berker *et al.*, 2010). 0.5 mL sample or standard solutions (quercetin) were added in test tubes with 2.5 mL 1% $K_3Fe(CN)_6$ and 2.5 mL 0.2 M phosphate buffer (pH: 6.6). After the incubation for 30 min. at 50°C, 2.5 mL 10% TCA was included into the tube followed by the centrifuge for 10 min at 3000 rpm. Then 2.5 mL supernatant was pipetted into a new tube with 2.5 mL water and 0.5 mL 0.1% freshly prepared $FeCl_3$ (Figure 1c). The calibration curve was obtained by using different quercetin concentrations between 5-200 ppm. The absorbances were measured at 700 nm. The results are expressed as quercetin equivalents/ dry plant (mg QE/gdw).

2.8. Metal Chelating Capacity

Metal chelating activity of *Sideritis* was carried out using the 1,10-phenanthroline method. 0.5 mL sample or standard solutions (iron II sulfate) were added in test tubes. Then, 2.5 mL water, 1 mL 0.2% $FeCl_3$, 1 mL 0.1% phenanthroline and 5.4 mL water were added. After vortexing thoroughly, the tubes were incubated for 20 min in the dark (Figure 1d). The results are expressed as iron II sulfate equivalents on the dry plant basis (mg Fe2+/gdw). The calibration curve was obtained by using different Fe^{2+} concentrations between 50-800 ppm. The absorbance was measured at 510 nm.

2.9. Statistical Analyzes

All tests were conducted in triplicate. The mean ± standard deviation was determined according to the results of each test. These results were statistically compared with One-way ANOVA and Tukey's post hoc test for multiple comparisons. $p < 0.05$ was considered as the significance value.

3. RESULTS and DISCUSSION

Phenolic compounds, as potent antioxidants and have been known to induce many health benefits against degenerative diseases. Typically, a phenolic compound carries one or more hydroxyl groups on an aromatic ring. Phenolic compounds can be found in plants in two ways: free or bound form. The phenolic compounds which are in bound form are linked to cell wall components via ester bonds (Gupta & De, 2017).

Novel extraction techniques include soxhlet extraction, ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and accelerated solvent extraction. The advantages and disadvantages of these extraction techniques depend on physical and chemical conditions in the procedures. The main drawbacks of soxhlet extraction include the

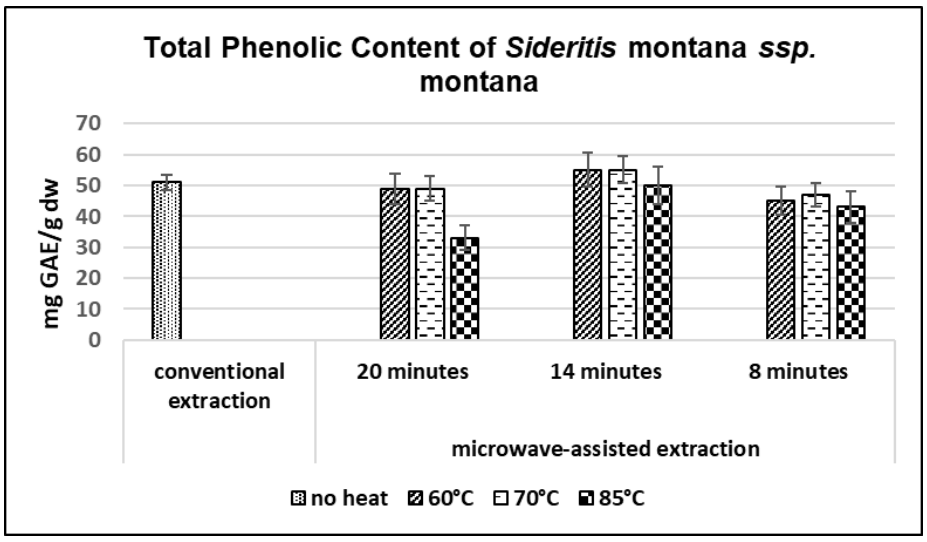
requirement of time, and solvent quantity, the potential for light, and thermal degradation of the extract. On the other hand, supercritical fluid extraction requires high costs with trained personnel to figure out the complex configuration of the system and monitor the extraction. Furthermore, supercritical carbon dioxide is not efficient for the extraction of more polar phenolic acids, while the ultrasound-assisted extraction method has many weaknesses such as large volume of solvent requirement and potential degradation of the extract by light and oxygen (Al Jitan *et al.*, 2018).

When compared with the other techniques mentioned above, it has been shown that bound phenolic acids can also be extracted via MAE. Thus, MAE provides superiority for the studied parameters including TPC, TFC, FRAP, and MCC. The other advantages of MAE include rapid and inexpensive processes, short extraction time, the ability to conduct many extractions simultaneously, and less solvent requirement. The main drawback of MAE is the work of relatively low quantities.

In this study, total phenolic content, total flavonoid content, total antioxidant and metal chelating capacity of *Sideritis montana* ssp. *montana* were evaluated using classical and microwave-assisted extraction methods.

The concentrations of phenolic compounds obtained in *Sideritis montana* ssp. *montana* extract via MAE (55 mg/g) was found to be slightly higher than the conventional (51 mg/g) method (Figure 2). On the other hand, the phenolic compounds obtained via MAE were determined to be significantly low (33 mg/g, $p<0.05$) at 85°C for the 20 minutes procedure, suggesting the total phenolic components to be degraded at high temperature. On the contrary, this situation was not observed at the same temperature for 8 and 14-minutes, suggesting that the degradation of phenolics is also related to the extraction time.

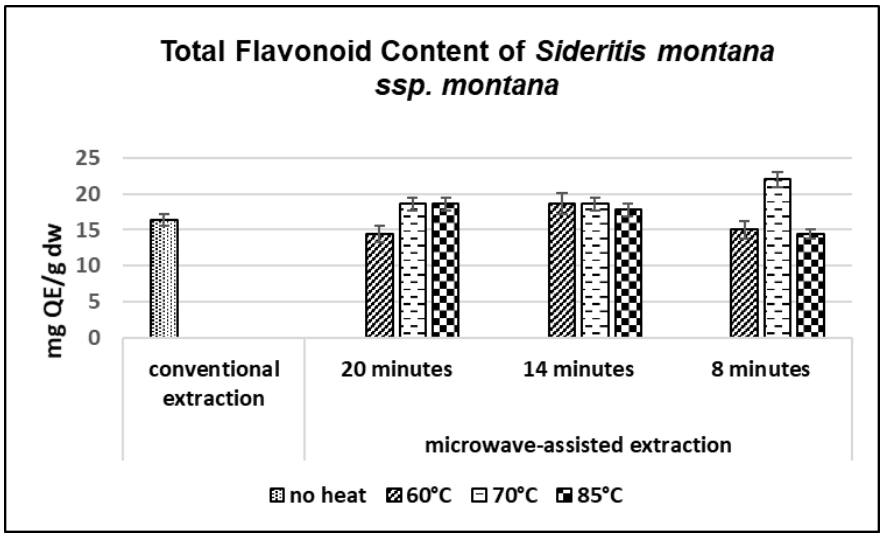
Figure 2. Total phenolic content of *Sideritis montana* ssp. *montana* extract. All values are expressed as mg GAE/g dry plant samples.



In the literature, a wide range of values (from 3.9 to 209 mg/g) of TPC for *Sideritis* species (Table 2) were reported (Axiotis *et al.*, 2020; Ertas & Yener, 2020; Celep *et al.*, 2019), attributable to the solvent type, extraction technique, time, and temperature, particularly boiling water. Higher TPC values were reported using infusion (boiling water) as shown in Table 2. The higher values for TFC were also found for the same samples shown in Table 2 supporting this explanation.

Flavonoids are common and larger secondary metabolism compounds that occur naturally in plants. They are considered quality indicators of fruits and medical plants (Sammani *et al.*, 2021). In this study, the concentration of flavonoids (22 mg/g) using MAE (70°C, 8 min) was found to be significantly higher than (p<0.05) the values (16.3 mg/g) obtained from the maceration (Figure 3). Many of the values obtained using MAE were found to be higher or close to the values obtained by the classical method.

Figure 3. Total flavonoid content of *Sideritis montana* ssp. *montana* extract. All values are expressed as mg QE/g dry plant samples.



The TFC values reported in the literature were in wide range of 1.5-101 mg QE/g dw (Irakli *et al.*, 2018; Kara *et al.*, 2014; Sarikurkcu *et al.*, 2020; Bardakci *et al.*, 2020). As is shown in Table 2, these TPC values which are higher than our results were obtained from the infusion of the sample with boiled water. The other TPC values were lower than our results. Similarly, Table 2 shows that the TFC values in the literature which are higher than our TFC values were obtained using heat.

In this study, the antioxidant power of *Sideritis montana* extract obtained via MW-assisted extraction (103 mg/g) was obtained to be significantly higher (p<0.05) than that of the extract obtained via the conventional method (93 mg/g). In the MW-assisted extraction procedure, on the other hand, lower FRAP values were obtained at 60°C and 70°C for 20 minutes. The lower values at 60°C for 20-minutes may be attributed to insufficient temperature. This situation implies that the molecules contributing to FRAP in mountain tea are significantly sensitive to temperature. Therefore, the maximum concentration was obtained at 85°C for 20-minutes of extraction. Furthermore, at the 8-minutes of extraction, the highest degree of antioxidant power was found at 70°C, suggesting that the antioxidant molecules contributing to FRAP assay are highly susceptible to extraction time and temperature (Figure 4).

The FRAP method measures the ability of antioxidants to perform as reducing agents. Potassium ferricyanide has been used as the ferric reagent in the FRAP assay. Transition metal ions are known to stimulate lipid oxidation via Fenton reaction and also by decomposing lipid hydroperoxides into more reactive peroxyl and alkoxyl radicals. By Fenton reaction, the ferrous ions produce •OH radicals, which are highly reactive, and contribute appreciably to oxidative stress.

$$Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^-+OH^{\cdot}$$ Fenton reaction

The resulting hydroxy radicals cause damage to proteins, carbohydrates, cellular lipids, and nucleic acids leading to cellular damage. Numerous metal ions such as Cu^{+}, Ti^{3+}, Cr^{2+}, and Co^{2+} and their complexes in their lower oxidation states similarly react with H_2O_2 as Fe^{2+}, and the mixtures of these metal ions with H_2O_2 are named "Fenton-like" reagents. The antioxidants, powerful metal chelators, can easily deactivate prooxidant metal ions and thus, prevent or retard metal ion-induced lipid oxidation. Phenolic compounds act as metal-chelators and scavengers of hydroxyl- and other radicals formed from iron-mediated Fenton reactions. In other words, polyphenols sacrificially reduce ROS/RNS, such as •OH, O2•-, NO•, or OONO- after generation, preventing damage to biomolecules or formation of more reactive ROS. Typically, chlorogenic acid (CA) in *Sideritis montana* is known to be intercalated in Fenton reaction as follows (Zhou *et al.*, 2019):

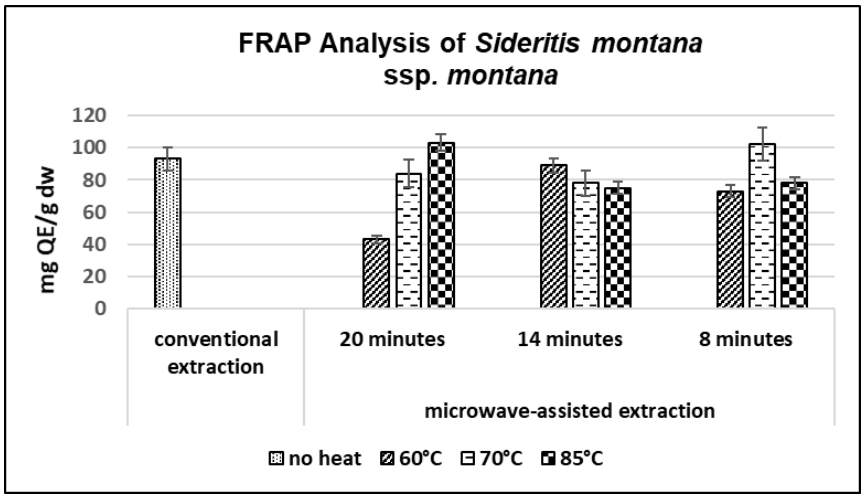
$$CA–Fe(III) + H_2O_2 \rightarrow CA – Fe(II) + HOO\bullet + H^{+}$$

$$CA– Fe(II) + H_2O_2 \rightarrow CA – Fe\ (III) + OH^{-} + \bullet OH$$

$$Fe^{3+} + CA \rightarrow CA – Fe(III)\ (chelate)$$

$$CA – Fe(III) + CA \rightarrow CA – Fe(II) + [CA]_{ox}$$

Figure 4. FRAP analysis of *Sideritis montana* ssp. *montana* extract. All values are expressed as mg QE/g dry plant samples.



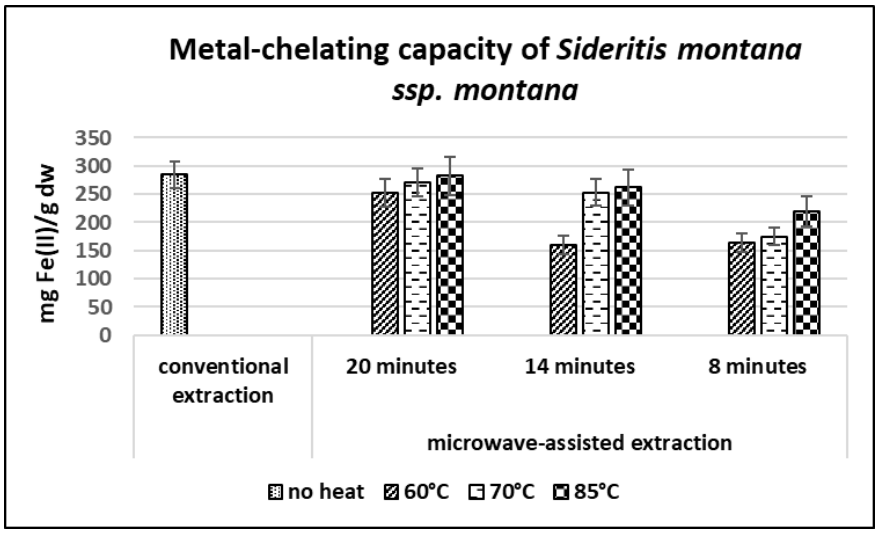
As seen above, CA representative of phenolic acids eliminates the production of more harmful radicals such as peroxyl and alkoxyl produced by metals. The antioxidant property of metal chelators is assessed when a complex is formed between the antioxidant and the metal, in such a way that metal ions can no longer act as an initiator of lipid oxidation. Polyphenols can chelate transition metal ions through their multiple OH groups and carbonyl moiety (Perron & Brumaghim, 2009). Based on the feature and structure of CA, the major phenolic compound of *Sideritis*, the adjacent couple OH groups might chelate ferric iron ions to form a Fe^{3+}-CA complex. Therefore, metal chelation capacity is also used as an indicator of antioxidant activity (Miah *et al.*, 2020).

In this study, for the metal chelating capacity, the values obtained for 20-minutes for all extraction temperatures are higher or closer than the values found for the other extraction times using MAE. No significant differences were obtained with MAE for 20-minutes at 85ºC and the classical method (Figure 5). As a result, the molecules with metal chelating capacity in *Sideritis montana* ssp. *montana* extract was not found to be interconnected to the time and temperature conditions of the MAE method except for 60ºC for 14 min and 60ºC and 70ºC for 80ºC.

4. CONCLUSION

In this study, conventional and microwave-assisted extraction methods were compared in terms of obtaining TPC, TFC, FRAP, and MCC as well as extraction time and solvent quantity for mountain tea. The microwave-assisted extraction method was found to be superior to the classical method in *Sideritis montana* ssp. *montana* extraction. Phenolics and flavonoid compounds in mountain tea were found to be extremely sensitive to the temperature and extraction time in microwave-assisted extraction. The obtained results for classical extraction

Figure 5. The metal-chelating activity of *Sideritis montana* ssp. *montana* extract. All values are expressed as mg Fe(II)/g dry plant samples.



and MAE were found to be 40 and 43 mg/g for TPC, 12.4 and 22 mg/g for TFC, 37 and 49 mg/g for FRAP, and 133 and 134 mg/g for MCC, respectively. In conclusion, microwave-assisted extraction of *Sideritis montana* ssp. *montana* has many advantages as its effectiveness, extraction time, and solvent quantity. Besides, *Sideritis montana* ssp. *montana* is suggested to be applied in the food industry as a natural antioxidant instead of synthetic ones to prevent health-damaging effects.

Table 2. Literature values for the studied parameters of *Sideritis* species.

Species	Ext type	Solvent	Ext time	Samp/solv ratio (g/mL)	TPC (mg GAE/g)	TFC (mg QE/g)	FRAP (mg QE/g)	Reference
S. scardica	INF	water	10 m 99.8ºC	1:100	45.43	47.45 mg CATE/g	-	(Irakli *et al.*, 2018)
S. condensate	INF	water	5 m 60ºC	2.5:60	12.17	-	-	(Kara *et al.*, 2014)
S. perfoliata	SOX	water	5 h	250 ml	52.18 mg/g ext	29.13 mg/g ext	-	(Sarikurkcu *et al.*, 2020)
S. perfoliata	SOX	met	5 h	250 ml	41.64 mg/g ext	40.90 mg/g ext	-	(Sarikurkcu *et al.*, 2020)
S. congesta	INF	water	ni	2:100	209	101	1.37	(Bardakci *et al.*, 2020)
S. sipylea	UAE	met	ni	1:40	13.3	-	-	(Axiotis *et al.*, 2020)
S. sipylea	UAE	50% met	ni	1:40	15.8	-	-	(Axiotis *et al.*, 2020)
S. libanotica	MACE	met	72 h	10:25	6.5	2.4	-	(Ertas & Yener, 2020)
S. libanotica	MACE	water	72 h	10:25	3.9	1.5	-	(Ertas & Yener, 2020)
S. thirkei	MACE	met	72 h	10:25	8	2.9	-	(Ertas & Yener, 2020)
S. thirkei	MACE	water	72 h	10:25	6.9	3.4	-	(Ertas & Yener, 2020)
S. trojana	INF	water	30 m	2:100	85	36	-	(Celep *et al.*, 2019)
S. trojana	MACE	80% Eth	30 m 45ºC	2:100	113	59	-	(Celep *et al.*, 2019)
S. trojana	UAE	water	45ºC	2:100	53	25	-	(Celep *et al.*, 2019)
S. montana	MACE	met	72 h	10:75	51	16.3	93	This study*
S. montana	MAE	70% met	20 m	0.1:8	55	18.6	103	

*RSD for this study ranges between 7-12%. UAE: ultrasound-assisted extraction, MACE: maceration, MAE: microwave-assisted extraction, INF: infusion, SOX: soxhlet assisted extraction, met: methanol, m: minutes, h: hours, ni: no information

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Declaration of Conflicting Interests and Ethics

The author declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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Research Article

Achievements in Genetic Engineering of *Amaranthus* L. Representatives

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Abstract: Despite the fact that in the modern world more than a thousand edible plants are used for food, only 3 staple cereal crops are grown worldwide: wheat, rice, and maize. Growing a limited number of crops often causes many problems: ranging from the loss of biodiversity, due to the constant cultivation of the same monocultures in the same areas, to the deterioration of soil quality. A way out of this situation is the selection of new untraditional and neglected plants that could grow in a wide range of temperatures, produce high yields and at the same time have a balanced amino acid composition. Pseudocereals of the genus *Amaranthus* L. meet these criteria. Amaranth grain and plant raw materials are used in many industries: food, medicine, cosmetics.
Modern technologies do not stand still. Along with traditional methods of plant breeding, the rapid pace of development involves genetic engineering of plants, which allows the process of creating improved plants to be speeded up several times.
The purpose of this study is to analyze and systematize the achievements in the field of regeneration and genetic transformation of representatives of the *Amaranthus* genus. The results can be used for a practical application: the genetic transformation of species of the genus *Amaranthus* and other close genera of plants.

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1. INTRODUCTION

Amaranth is a high-yielding plant. From 1 plant it is possible to obtain more than 5,000 seeds. Moreover, amaranth has a uniquely balanced amino acid composition that ensures easy digestion. Amaranth is a rich source of protein and essential amino acids, deficits of which cannot be compensated by traditional agricultural crops.

Furthermore, amaranths are used in medicine. Amarantin substance ($C_{29}H_{31}N_2O_{19}$) was derived from some species of amaranth (*A. caudatus* L., *A. tricolor* L., *A. cruenthus* L.) (Yaacob *et al*., 2012). Amarantin relates to alkaloids-betalains. It has useful antioxidant properties in the human organism (Burd, 2006).

Due to the fact that amaranths are indifferent to the type of soil and are drought-resistant, they are grown as a grain crop in countries with a temperate climate (Western Europe), as well

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as in hot-climate countries, where many traditional crops grow poorly: Mexico, the USA, African countries, India.

Given that amaranth is one of the main food crops in India and Africa, has a unique rich amino acid composition with a high nutritional value, and can serve as a source of biologically active substances for further use in medicine, amaranth plants have undergone improvements for many decades using hybridization, selection and mutagenesis methods.

In recent years, the chemical composition of plants and some agronomic properties have begun to improve using biotechnological methods, namely genetic engineering. Genetic engineering methods make it possible to improve not only the useful properties of a plant, but also to provide additional useful characteristics during plant transformation.

Since it is known that the percentage of *Agrobacterium* - mediated transformation of plants is often low, usually even before this transformation possible ways of obtaining a large number of transformed plants from a single parent plant are consequently worked out. One of the optimal methods of rapidly increasing the number of plants is considered to be direct regeneration of plants *in vitro* conditions.

Therefore, we first consider the main achievements related to obtaining regenerants of amaranths *in vitro*.

2. ACHIEVEMENTS IN REGENERATION OF *AMARANTHUS L.* SPECIES

To date, there have been many studies on the regeneration and callus formation of amaranth. Basically, the researchers who obtained calluses, had as primary objective their use as a source of secondary metabolites and other valuable substances. In this connection, the largest number of studies devoted to amaranths have had a biochemical orientation.

Amin and colleagues verified the possibility of obtaining the *Amaranthus gangeticus* L. callus. The leaves, stems and roots were used as initial explants. The scientists observed the formation of calluses in 99.7% ± 0.2% of explants which were derived from stem calluses on MS medium supplemented with 2.0 mg/l NAA(α-Naphthalene acetic acid) + 1.0 mg/l BA (6-benzylaminopurine) (Amin *et al*., 2015).

The group headed by Bennici studied the morphogenesis and growth of calluses. As an object of investigation, they chose lines of several species: *A. caudatus* L., (PI490458, AMES15114, AMES5461), *A. cruentus* L. (434, 622, AMES2248, AMES2247, PI511731, PI777913), *A. hybridus* L. (1047), *A. hypochondriacus* L. (1221, 718, 674, 722, 412, PI540446). The stem segments derived from 15-day sprouts were used for explants (Bennici *et al*., 1997).

Callus tissue was obtained from the explants of the lines *A. caudatus* L. (Bennici *et al*., 1997), *A. cruentus* L. (Bennici *et al*., 1997) and *A. hypochondriacus* L. (Bennici *et al*., 1997) on MS medium with the addition of 2.3 μM 2.4-D (2,4-dichlorophenoxyacetic acid) + 2.3 μM KIN (kinetin); NAA from 0.5 μM to 5.4 μM + BA from 0.4 μM to 13.3 μM.

The callus formation was observed in 100% of explants, with the exception of two lines of *A. caudatus* L. and three lines of *A. cruentus* L. and *A. hypochondriacus* L.. Different concentrations of NAA + BA did not induce callus formation on the *A. caudatus* explants line AMES5461, while 5.4 μM NAA + 13.3 μM BA caused callus formation only in 43% of PI490458 *A. caudatus* L. explants. *A. cruentus* L. lines formed calluses in percentage ratios of less than 100%: AMES2247, 71% on MS medium, with addition of 5.4 μM NAA + 4.4 μM BA; PI511731, 60% on MS medium with addition of 2,4-D + KIN and 67% on MS medium with addition of 5.4 μM NAA + 13.3 μM BA; PI477913 – 75% on 2,4-D + KIN and 79% on MS medium with addition of 5.4 μM NAA + 4.4 μM BA.

Plant regenerants were obtained for *A. hybridus* L. (line 1047) and for *A. hypochondriacus* L. (line 674). The rate of regeneration was low – 8.5% (*A. hybridus* L.) and 14.3% (*A. hypochondriacus* L.). Regenerants were also obtained for *A. hybridus* L., *A. hypochondriacus* L., *A. cruentus* L. on MS medium with addition of 2.7 μM NAA + 2.5 μM 2iP (N^6-(2-isopentenyl)adenine), 2.7 μM NAA + 2.3 μM KIN. The regenerants of *A. cruentus* L. line 434 and 1034 were obtained on MS medium with addition of 2.7 μM NAA + 4.4 μM BA. The general conclusion of the authors was as follows: the absolute majority of species and lines of amaranths are able to form calluses on most media tested by the authors (almost 100% of callus formation). There was no clear connection between regeneration of shoots and the use of growth regulators. This is due to the strong influence of the genotype of plants on organogenesis. Amaranths have high levels of cytokinins (auxins), which inhibit regeneration processes. The authors believe that the best stimulator of amaranth regeneration was BA.

Mousumi Biswas and colleagues conducted experiments aimed at obtaining calluses for further isolation of betacianins from them (Biswas *et al*., 2013). The biggest volumes of callus synthesizing betacianins were obtained from explants of stem origin on MS medium supplemented by NAA (0.25 mg/l) + BA (2 mg/l). In addition, researchers found red-purple amaranthine pigment in the callus lines, 2 new yellow pigments and 18 other biologically active phenylpropanoids. A new betaxanthin has been identified and a methyl derivative of arginine betaxanthin was also identified. Pigments were purified by size exclusion chromatography (Biswas *et al*., 2013).

Flores and colleagues studied the formation of callus and regeneration for the *A. hypochondriacus* L., *A. cruentus* L. and *A. tricholor* L. species. They observed a rapid growth of calluses and abnormal roots on *A. hypochondriacus* L. and *A. cruentus* L. leaf disks on MS medium in the presence of 0.1–1.0 mg/l of 2.4-D. At higher levels (1.0–10.0 mg/l) of 2,4-D, embryo-like structures formed from the surfaces and veins of the leaf discs. Shoots were formed from hypocotyl derivative callus on the medium B5 + 0.1 mg/l NAA and 0.1–1.0 mg/l ZEA (zeatin). Lower ratios of ZEA/NAA stimulated the formation of roots from hypocotyl segments (Flores *et al*., 1982).

Gajdošová, with a team of researchers, selected the ideal conditions for the regeneration and cultivation of *Amaranthus cruentus* L. 'Ficha' and *Amaranthus hybridus* (Gajdosova *et al*., 2007; Gajdosova *et al*., 2013) 'K-433'. As explants, they used epicotyls with the first pair of leaves, hypocotyls, roots and segments of the leaves of 10-day seedlings. For both species studied, the most effective media for direct regeneration from epicotyls were MS_{30}, supplemented with 5 mg/l BA + 0.01 mg/l NAA, MS_{30} supplemented with 1 mg/l TDZ (thidiazuron), MS_{30} supplemented with 3mg/l TDZ + 0.01 mg/l NAA. The most effective medium for induction of callus was MS_{30} with 6 mg/l NAA + 0.1 mg/l BA (for *Amaranthus cruentus* L. 'Ficha') and MS_{30} + 2 mg/l 2.4 D + 0.5 mg/l BAP (for *Amaranthus hybridus* L. "K-433"). The authors made the following conclusions: in order to obtain regenerants, it is necessary to use mediums with a high cytokinin content: auxins; amaranths are characterized by a high callus forming ability, almost 100% on all tested mediums; regenerants were obtained only from epicotyl segments; the ability to regenerate strongly depends on the genotype, age of plants and used types of explants; the overall regeneration frequency was low (Gajdosova *et al*., 2007; Gajdosova *et al*., 2013).

Flores and colleagues investigated the regeneration ability and the callus formation of the following species: *A. hypohondriacus* L., *A. cruentus* L., A. *tricolor* L.. Parts of the hypocotyls were used as explants. The regeneration was indirect (first, callus tissue was obtained). The scientists concluded that the optimal medium for regeneration is B5 supplemented with 0.1mg/l NAA + 0.1–1.0 mg/l ZEA. The callus tissue was obtained from leaf discs of *A. hypohondriacus* L. and *A. cruentus* L. Intensive growth of the callus was observed on MS_{30} medium with 0.1–1

mg/l 2,4-D. However, after addition to the MS_{30} medium of 0.2 mg/l BA + 2 mg/l NAA and 10% coconut water, they observed shoot induction from callus tissue (Flores & Teutonico, 1986).

The team of researchers headed by Bennici intended to obtain regenerants for the following species: *A. hypohondriacus* L., *A. cruentus* L., *A. hybridus* L., *A. caudatus* L. As explants, hypocotyls were used. Regeneration was obtained for 2 species as a result: *A. hypochondriacus* L. (MS_{30} + 3 mg/l BA + 1 mg/l NAA), *A. caudatus* L. (MS_{30} + 3 mg/l KIN + 0.3 mg/l IAA (indole-3-acetic acid). The percentage of regeneration was low (26%). At the same time as the main objective of obtaining regenerants, researchers obtained a callus tisssue. Rapid and intensive callus formation from hypocotyl explants was observed for *A. cruentus* L. (6 mg/l NAA + 0.1 mg/l BA) and *A. hybridus* L. (6 mg/l 2.4-D + 0.1 mg/l KIN (Bennici *et al*., 1992). Arya and colleagues chose *A. paniculatus* L. as an object of research. Parts of the inflorescence were used as explants. When transferring the explants on the MS_{30} medium with 8–15 mg/l KIN or MS_{30} + 5–10 mg/l BA, secondary inflorescences were formed from stems and leaves of the primary inflorescence buds (Arya *et al*., 1993). Bui van Le and colleagues received regenerants of *A. edulis* L. from thin cell layers. For experiments, they used thin slices (0.2–0.4 mm) of cotyledons, hypocotyls, roots, tissues from the apical and sub-apical areas. Explants were obtained from 7-day seedlings (Bui van Le *et al*., 1998). Regenerants were obtained solely from tissues taken from the apical and sub-apical zone. Only callus tissue was obtained from all other types of explants.

Initially, embryonic buds were formed from the tissues of the apical and sub-apical zone on a medium of MS_5 + 2 μM TDZ + 10 μM of CPPU (forchlorfenuron). These embryonic buds were then transferred on MS_5 + 10 μM BAP for elongation of stems (Bui van Le *et al*., 1998). Tisserat and Galletta obtained only callus tissue for *A. gangenticus*, *A. hypochondriacus*, *A. caudatus* L., *A. viridis* L., *A. retroflexus* L. (Tisserat & Galletta, 1988). Callus tissue was obtained by Yaacob and colleagues. Callus was obtained for further extraction of biologically active substances using leaves, stems, roots on MS_{30} + 1.5 mg/l IAA + 0.5 mg/l of ZEA or MS_{30} + 1 mg/l IAA medium (Yaacob *et al*., 2012).

A team of researchers headed by Bagga, studied the regeneration ability and callus formation of *A. paniculatus* L. The hypocotyls were used as the explants. Regeneration of 1-2 shoots from one end of the hypocotyls explants was obtained on medium B_5 + 1ppm KIN + 1 ppm NAA; on medium B_5 + 0.5 mg/l KIN + 0.1 mg/l NAA numerous buds formed (10–14 pieces), from which stems developed later. Intensive callus growth was observed on medium B_5 + 1 mg/l GA_3 (gibberellic acid) + 1 mg/l KIN + 1mg/l 2,4-D (Bagga *et al*., 1987).

Jofre-Garfias and co-authors obtained embryos from the cotyledons of *A. hypochondriacus* L. cv. Azteca on medium MS_3 + 10% coconut milk and MS_3 + 10μM 2.4-D (Jofre-Garfias *et al*., 1997). Pal and colleagues obtained *A tricolor* regenerants from hypocotyls and epicotyls of 7-day seedlings on MS_{30} + 13.2 μM BA +1.8 μM NAA (Pal *et al*., 2013 a). In another study, Pal argued that he and his colleagues received regenerants of *A. spinosus* from the culture of "hairy" roots. Regenerants were obtained on MS_{30} medium without growth regulators (spontaneous regeneration) and on MS_{30} medium + 2 mg/l ZEA (Pal *et al*., 2013 a).

Swain and his colleagues obtained *A. tricolor* regenerants from the culture of "hairy" roots. Regenerants were obtained (on MS_{30} medium without growth regulators (spontaneous regeneration) and on MS_{30} medium + 2 mg/l ZEA (Swain *et al*., 2009; Swain *et al*. 2010).

For clarity, the achievements in the field of callus formation and regeneration is presented in tabular form (Table 1).

Table 1. Achievements in amaranth regeneration.

Species of amaranth, cultivar, hybrid, line	Most effective medium for regeneration	Type of explants, age	Authors, year of publication Title
A. cruentus L. 'Ficha', *A. hybridus* 'K-433'.	MS_{30} + 5 mg/l BA + 0.01 mg/l NAA	epicotyls with 1st pair of leaves	(Gajdošová *et al*., 2013)
A. cruentus L. 'Ficha', *A. hybridus* 'K-433'.	MS_{30} + 1 mg/l TDZ, MS_{30} + 3mg/l TDZ + 0.01mg/l NAA	epicotyls of 10-day seedlings	(Gajdošová *et al*., 2007)
A. hypohondriacus L., *A. cruentus* L., *A. tricolor* L.	B_5 + 0.1mg/l + 0.1-1.0 mg/l ZEA	hypocotyls	(Flores *et al*., 1982)
A. hypohondriacus L., *A. cruentus* L.	MS_{30} + 2mg/l NAA + 0.2 mg/l BA + 10% coconut water	hypocotyls (non-direct regeneration), leaf discs	(Flores & Teutonico, 1986)
A. caudatus L., *(PI490458, AMES15114, AMES5461),* *A. cruentus* L., *434, 622, AMES2248, AMES2247, PI511731, PI477913)* *A. hybridus* L. *1047,* *A. hypochondriacus* L.)*, 1221, 718, 674, 722, 412, PI540446)*	MS_{30} + 2.7µM NAA+ 2.5µM 2iP, 2.7µM NAA + 2.3µM KIN). 2.7 µM NAA + 4.4 µM BA	stems	(Bennici *et al*., 1997)
A. caudatus L., *A. hypochondriacus* L.	MS_{30} + 0.3 mg/l IAA + 3mg/l KIN; MS30 + 1mg/l IAA + 3mg/l BA; MS_{30} + 6mg/l 2,4-D + 0.1 mg/l KIN; MS_{30} + 6mg/l NAA + 0.1 mg/l BA	hypocotyls (non-direct regeneration)	(Bennici *et al*., 1992)
A. paniculatus L.	MS_{30} + 8-15 mg/l KIN or 5-10 mg/l BA; MS_{30} + 0.5 – 10mg/l 2.4-D+ 0.5 – 10 mg/l NAA	inflorescence	(Arya *et al*., 1993)

Table 1. Continued.

A. edulis L.	MS30 + 2 µMTDZ, MS30 + 10µM CPPU	thin cell layers, obtained from the apical and sub-apical meristems of 7-day seedlings	(Bui van Le et al., 1998)
A. paniculatus L.	B5 KIN (0.5 ppm) and NAA 0.1 ppm), B5 + 1 mg/l GA3 + 1 mg/l KIN + 1 mg/l 2,4-D.	hypocotyls	(Bagga *et al*., 1987)
A. hypochondriacus, cv 'Azteca' L.	MS_{30} + 13.2 µM BA+1.08µM NAA	epicotyls and hypocotyls 7 day seedlings	(Jofre-Garfias *et al*., 1997)
A. spinosus L.	MS_{30}, MS_{30} +2mg/l ZEA	"hairy" roots	(Pal *et al*., 2013 b)
A. tricolor L.	MS_{30} + 13.2 µM BA +1.8 µM NAA	epicotyls and hypocotyls 7-day seedlings	(Pal *et al*., 2013 a)
A. tricolor L.	MS_{30}, MS30 +2mg/l ZEA	"hairy" roots	(Swain *et al*., 2009; Swain *et al*. 2010)
A. gangenticus L.	MS_{30} + 2 mg/l NAA + 1 mg/l BA	Leaves, stems, roots	(Amin *et al*., 1993)
A. cruentus L.	MS_{30} + 1.5 mg/l IAA + 0.5 mg/l ZEA; MS_{30} + 1 mg/l IAA	Leaves, stems, roots	(Yaacob *et al*., 2012)

3. ACHIEVEMENTS IN THE TRANSFORMATION OF AMARANTHUS SPECIES AND FUTURE PROSPECTS

The next step after obtaining regenerated plants is genetic transformation. The number of studies devoted to genetic transformation of *Amaranthus* is rather small.

So far, it is reported that genetically transformed parts or whole plants of amaranth have been obtained by two different methods: *Agrobacterium*-mediated transformation and transformation using the "floral-dip" method.

The *Agrobacterium* – mediated transformation method was developed on the basis of a natural process. Wild soil bacterium *Agrobacterium rhizogenes* or *tumefaciens* is able to infect plants, causing the appearance of "hairy" roots (*A. rhizogenes*) or tumors – crown galls (*A. tumefaciens*). At the same time as the infection process, the transfer and integration of two groups of genes into the plant genome occurs. Genetically modified *Agrobacterium* transfers the genes of interest or selective genes needed by humans into the plant's genome.

The first experiments on the transformation of amaranths were unsuccessful (De Cleene & De Ley, 1976). At present, it has been proved that transgenic amaranth plants can be obtained through *Agrobacterium*-mediated transformation. But still there are very few studies devoted to amaranth transformation.

Transgenic roots were obtained for *Amaranthus tricolor* L. (Swain *et al*., 2010) and *A. spinosus* L. (Pal & Swain, 2013). Transgenic plants were obtained for *A. hypochondriacus* L. and *A. tricolor* L. (Pal & Swain, 2013; Swain *et al*., 2009; Swain *et al*., 2010), *A. retroflexus* L. (Taipova & Kuluev, 2015), *A. viridis* L. (Taipova & Kuluev, 2015), *A. cruentus* L. (Taipova & Kuluev, 2015).

There is no information on the transformation of *A. caudatus*, varieties of which are also used in agriculture.

Transgenic roots were obtained for *A. tricolor* L. plants by Swain and colleagues (Swain *et al*., 2010) and for *A. spinosus* L. by Pal and colleagues (Pal & Swain, 2013). The transformation of amaranths was carried out using a wild strain of *Agrobacterium rhizogenesis* A4. Research group Taipova, Kulaev and others obtained transgenic roots for *A. cruenthus* L. from epicotil segments (Taipova *et al*., 2019 a; Taipova *et al*., 2019 b).

Positive results were also obtained in the transformation of amaranth species using strains of *Agrobacterium tumefaciens*. Jofre-Garfias and co-authors transformed the Azteca variety of *A. hypochondriacus* L. They used the vector from *Agrobacterium tumefaciens* with marker genes (Jofre – Garfias *et al*., 1997). Transgenic *A. tricholor* L. was obtained by two different groups of scientists – Swain and colleagues and Pal with co-authors (Swain *et al*., 2009; Pal *et al*., 2013). A team of researchers headed by Pal used a vector with marker genes.

Taipova and Kulaev obtained regenerated transformed plants from epicotil explants after *Agrobacterium*-mediated transformation (Taipova *et al*., 2019 b; Taipova & Kuluev, 2018).

Castellanos-Arévalo with colleagues obtained transgenic *A. hypochondriacus* L. and *A. hybridus* L. from "hairy" roots culture after transformation by *A. rhizogenes* strains ATCC 15834, A4 and HRI. They obtained transgenic plants with *rolB*, *bar*, *gfp*, *uid*A genes (Castellanos-Arévalo *et al*., 2020).

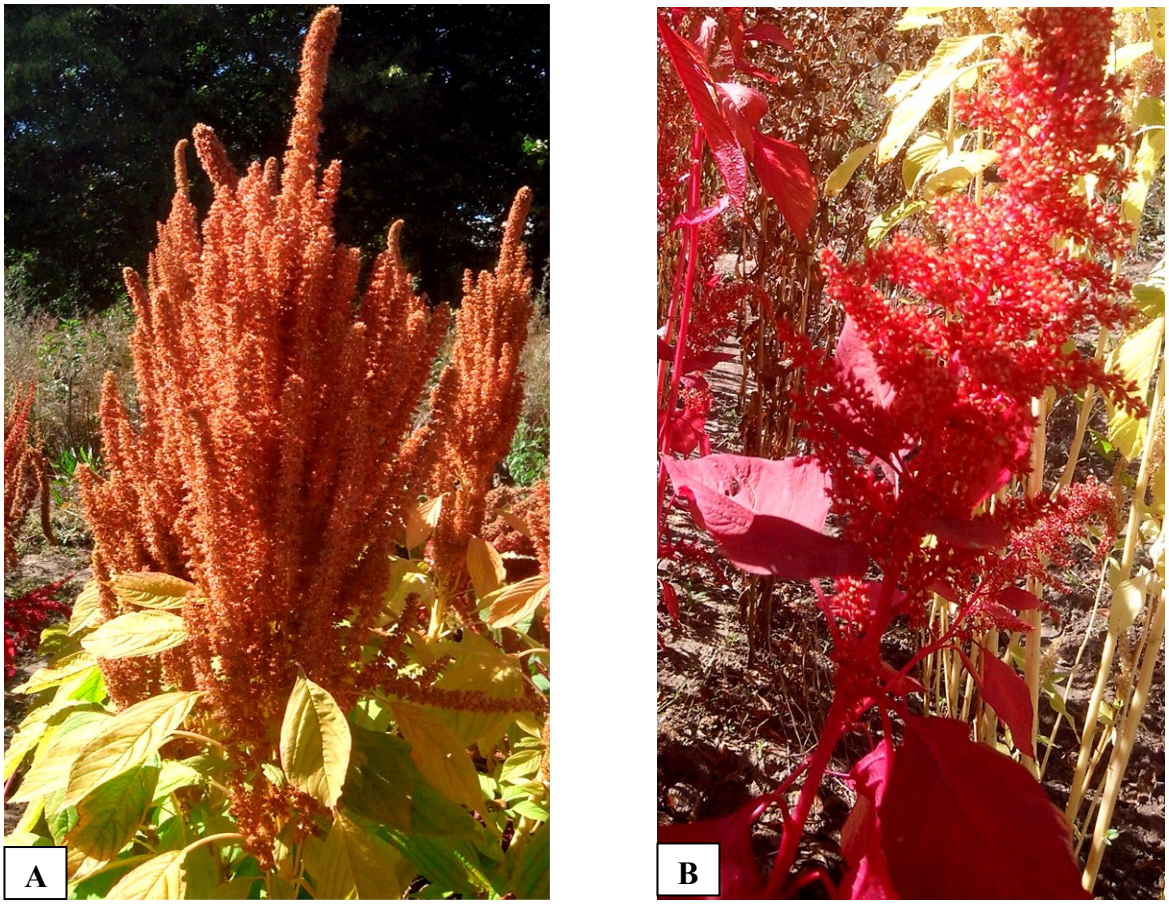
There are also 3 studies devoted to amaranth transformation through inflorescences by the "floral-dip" method – Umaiyal Munusamy and co-authors. They used a vector with selective genes (Munusamy *et al*., 2013).

Another group of researchers – Taipova and Kuluyev – carried out experiments on the transformation of *A. retroflexus* L. (Kuluev *et al*., 2017; Taipova & Kuluev, 2015; Taipova *et al*., 2019 a; Taipova *et al*., 2019 b), *A. viridis* (Kuluev *et al*., 2017; Taipova & Kuluev, 2015;

Taipova *et al*., 2019 a; Taipova *et al*., 2019 b), *A. cruentus* L. (Kuluev *et al*., 2017; Taipova & Kuluev, 2015; Taipova *et al*., 2019 a; Taipova *et al*., 2019 b). They used inflorescences of immature plants for transformation by the "floral-dip" method.

Yaroshko, Kuchuk and co-authors obtained transgenic plants of *A. caudatus* L. local cultivars Karmin and Helios with *bar* gene, after transformation by the "floral-dip" method (Yaroshko *et al*., 2018; Yaroshko & Kuchuk, 2018) (Figure 1).

Figure 1. *Amaranthus caudatus* L. cultivars Helios (A) and Karmin (B).



"Floral-dip" method protocols are described in detail in the articles of several authors (Curtis, 2005; Martins *et al*., 2015). This method was first successfully applied to *Arabidopsis thaliana* transformation (Clough & Bent, 1998; Bent, 2006; Harrison *et al*., 2006; Zhang *et al*., 2006). In addition, successfully transformed by this method were *Brassica rapa via* (Hu *et al*., 2019), *Setaria* (Saha & Blumwald, 2016; Sood & Prasad, 2017; Van Eck, 2018; Van Eck & Swartwood, 2015), rice (Ratanasut *et al*., 2017), *Schrenkiella parvula* (Wang *et al*., 2019), sugarcane (Mayavan *et al*., 2015), tomato (Sharada *et al*., 2017), *Eustoma grandiflorum* (Fang *et al*., 2018). The researchers from the Umaiyal Munusamy group, as well as Taipova and Kuluyev, assured that they had obtained viable transgenic seeds.

Yaroshko and Kuchuk obtained transgenic plants of *A. caudatus* L. and hybrids *A. caudatus* L.x *A. paniculatus* L. after floral-dip transformation (Yaroshko & Kuchuk, 2018). The researchers Murugan and Sathishkumar obtained only transgenic callus for *A. trisis* (Murugan & Sathishkumar, 2016), after transformation of parts of leaves with the *Agrobacterium tumefacies* strain EHA 105 harboring pCAMBIA 1301 (Murugan & Sathishkumar, 2016). The achievements in the field of amaranth transformation are presented in tabular form below (Table 2).

Table 2. Achievements in amaranth transformation.

Species of amaranth, cultivar	Parts of plants used for transformation	Strain of *Agrobacterium* used for transformation	Result	Authors, year of publication
A. hypochondriacus L. *"Azteca"*	germs and cotyledons	*A.tumefaciens* pgv2260 (pEsc4 with genes of *npt* II (neomycin phosphotransferase gene) - kanamycin resistance and *uid*A (gene of *β*-glucuronidase)	transgenic plants	(Jofre – Garfias *et al*., 1997)
A. tricolor L.	internodes and leaf blades	*A. rhizogenes* A4	transgenic plants	(Swain *et al*., 2009)
A. tricolor L.	internodes and leaf blades	*A. rhizogenes* A4, LBA9402	"hairy" roots, transgenic plants***	(Swain *et al*., 2010)
A. spinosus L.	internodes and leaf blades	*A. rhizogenes* LBA9402	"hairy" roots, transgenic plants***	(Pal & Swain, 2013)
A. tricolor L.	epicotyls	*A. tumefaciens EHA 105, LBA 4404 (p35SGUSINT* with genes of *npt II* - kanamycin resistance and *uid*A for each strain)	transgenic plants	(Pal *et al*., 2013)
Amaranthus L.*	inflorescence of adult plants	*A. tumefaciens AGL1 (p5b5, p5d9, p5f7* with gene of *hph* (gene codes hygromycin-B-phosphotransferase protein)	transgenic plants	(Munusamy *et al*., 2013)
A. trisis Willd. (trisis is the synonym of *Amaranthus dubius Mart. ex Thell.*	segments of leaf explants	*A. tumefacies strain EHA 105 harbouring pCAMBIA 1301*	transgenic callus	(Murugan & Sathishkumar, 2016)

Table 2. Continued.

A. retroflexus L.	inflorescence of adult plants	*A. tumefaciens* strain AGL0, which contained gene construction in the vector pCAMBIA 1301 (with ARGOS-like gene from *A. thaliana* (ARL)	transgenic plants	(Kuluev *et al*., 2017)
A. cruentus L.	epicotyls	*A. tumefaciens* strain AGL0, which contained gene construction in the vector pCAMBIA 1301 (with ARGOS-like gene from *A. thaliana* (ARL)	transgenic plants	(Taipova *et al*., 2019)
A. caudatus L.cv. Karmin, cv. Helios	inflorescence of adult plants	*A. tumefaciens* strain GV3101 (with *uid*A and *bar* (phosphinothricin N-acetyltransferase) genes)	transgenic plants	(Yaroshko *et al*., 2018)
A. caudatus L.	hypocotyls	*A. rhizogenes* A4	"hairy" roots	(Yaroshko & Kuchuk, 2018)

Note: * – name of amaranth species not stated;
** – name of *Agrobacterium* strain not stated;
*** –authors did not provide enough results in the publication that would confirm exactly the fact of obtaining transgenic plants.

Thus, at the moment, transgenic amaranth plants have been already obtained with selective genes, marker genes and genes of interest. Research into the transformation of amaranth continues. In the near future, transgenic amaranths may appear that have an improved biochemical composition and new useful properties.

4. CONCLUSION

Amaranth is unique plant. Its nutritional value and optimal amino acid composition have already been evaluated in many countries around the world. In Western Europe, the plant has already gained popularity and it is possible find products with amaranth on the shelves of supermarkets. In Ukraine, we also have a small range of products that include amaranth.

At the moment, plant regenerants have been obtained for 9 species of amaranth (*A.cruentus* L., *A. hybridus* L., *A.hypohondriacus* L., *A. caudatus* L., *A. paniculatus* L., *A. edulis* L., *A. spinosus* L., *A. tricolor* L., *A. gargenticus* L.), transformed plants for 6 species (*A.hypohondriacus* L., *A. tricolor* L., *A. spinosus* L., *A. retroflexus* L., *A. viridis* L., *A. cruentus* L.), transformed organs and tissues for 4 species (*A. spinosus* L., *A. trisis* L., *A. caudatus* L., *A. tricolor* L.).

As can be understood from our previous experimental work and the work of other authors, there are difficulties in achieving regeneration for many species of amaranths. If regenerants are obtained, the percentage of regeneration does not exceed 30 percent, which is clearly not enough for further obtaining transformed plants after agrobacterial transformation.

Therefore, other transformation techniques are being developed, for which it is not necessary to obtain regenerated plants. The alternative transformation method is called “floral-dip’. According to published studies, transformed plants have been obtained using this method.

At present, mainly transgenic amaranth plants have been obtained, which were transformed by agrobacteria that carried vectors containing selective genes. Only one group of authors obtained transgenic plants with not only selective genes, but also genes of interest.

In the near future, a greater number of amaranth species will be obtained, which will present additional useful features, such as, for example, protein synthesis, which can be used in medicine. The authors hope, that in the near future, amaranth will achieve the position of a recognized niche of the food and medicine industries.

Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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