International Journal of Secondary Metabolite

e-ISSN: 2148-6905

IJSM
International Journal of
Secondary Metabolite

http://dergipark.org.tr/en/pub/ijsm

IJSM

Volume: 7
Number: 2
2020
June

International Journal of Secondary Metabolite (IJSM)
is a peer-reviewed online journal

e-ISSN: 2148-6905

IJSM
International Journal of
Secondary Metabolite

Volume 7, Issue 2, June 2020

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Publisher : İzzet KARA
Frequency : 4 issues per year (March, June, September, December)
Online ISSN : 2148-6905
Website : https://dergipark.org.tr/en/pub/ijsm

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International Journal of Secondary Metabolite

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 2, 63–76

https://doi.org/10.21448/ijsm.669451

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Phytochemical Profile and *in vitro* and *in vivo* Anticonvulsant and Antioxidant Activities of *Epilobium hirsutum*

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Abstract: This study presents the phytochemical profile and *in vitro* and *in vivo* anticonvulsant and antioxidant activities of *Epilobium hirsutum*, which has been traditionally used in the treatment of epilepsy by local people of Turkey. *In vitro* studies revealed that the extract contained a pronounced amount of phenolics (206.3±0.9 mg Gallic acid Eq/g extract) and exhibited significant levels of antioxidant (FRAP; 6226 µmol Fe^{2+}/g extract, ORAC; 6593 µmol Trolox Eq/g extract, DPPH; IC_{50}:33.8 ug/mL and metal chelation; IC_{50}:114 ug/mL) and anticonvulsant (AChE; IC_{50}:71.2 ug/mL, BChE; IC_{50}:92.5 ug/mL, GABA-T; IC_{50}:94.7 ug/mL) activities. *In vivo* studies shown that the extract exhibited high anticonvulsant activities. In addition, the extracts regulated the behaviour, locomotion and mental activities of the mice tested. Biochemical evaluation of the brain tissue revealed that the extract inhibited the production of MDA and stimulated the increasing of antioxidant enzyme levels, which suggest the possible antioxidative role of the extract that worked as neuroprotective agents by scarfing the free radicals produced through PTZ seizure inducer and attenuate convulsions. Moreover the extract regulated serum biochemical parameters, total antioxidant, total oxidant, and ischemia-modified albumin levels. Chromatographic studies were revealed that gallic acid principally might be the major contributor of anticonvulsant and antioxidant activities with the additive contributions of fatty acids and mineral compounds. Findings obtained from this study partially justified the traditional use of *Epilobium hirsutum* in the treatment of epilepsy and suggest potential use of the extract as industrial or pharmaceutical agent.

ARTICLE HISTORY

Received: January 04, 2020
Revised: February 25, 2020
Accepted: April 07, 2020

KEYWORDS

Epilobium hirsutum,
Antioxidant,
Anticonvulsant,
Gallic acid

1. INTRODUCTION

Epilobium species which are commonly known as fireweed, wickopy, niviaqsiaq and most commonly as willowherb belong to Onagraceae, comprises of 200 taxa of herbaceous plants distributed across the world. These species have been traditionally used in the treatment

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ISSN-e: 2148-6905 /© IJSM 2020

of a wide range of disorders such as prostatis, urinary tract, bladder, diarrhoea and gastrointestinal diseases, irritation, inflammation, skin and mucosa diseases and epilepsy. Nowadays extracts prepared from willowerbs are widely used by patients due to their health attributing properties [1]. According to the analytical studies performed on *Epilobium* taxa, phenolic compounds were found as the main bioactive compounds, which were suggested as the physiologically active ingredient [2-4]. Apart from polyphenols, some lipophilic compounds such as triterpenoids and fatty acids were also reported [5-6]. Numerous scientific papers concerning *in vitro* and *in vivo* studies of *Epilobium* taxa focused on various pharmacological activities including antiproliferative [7], analgesic [8], and antioxidant [6, 9-10] activities. However, there is no or limited research studies focused on anticonvulsant effects of *Epilobium* species in the scientific literature. Epilepsy still one of the most complicated electro-hyperactivity neurological disease, in which hyperphosphory mechanism led to brain dysfunction disorders that affect the normal brain performance such as movement, awareness, sensation, consciousness, and behaviour which origin is unclear with the suggestion of genetic mutation, head injury, and hypoglycaemia. It has been estimated that the amount of epilepsy disorder will be reached to 50 million in the forthcoming years [11]. The most common treatment method of epilepsy is performed through anti-seizure drugs. However due to the side effects and drug resistance, alternative treatment methods such as surgically intervention, food regime balance (ketogenic diet), or using traditional herbal medicines have been commonly utilized worldwide [12]. Local people have traditionally used *Epilobium hirsutum* L. (Onagraceae) known as karapil or yakı otu in the treatment of epilepsy in Turkey for a long time. However, clinical and analytical studies regard to *Epilobium hirsutum* are limited in the scientific literature. Therefore, this study aimed to (i) assess the anticonvulsant and antioxidant activities of the hydrophilic extract obtained from *Epilobium hirsutum in vitro* and *in vivo* (ii) determine the effect of the extract on lipid peroxidation and antioxidant enzymes and serum biochemical parameters levels, (iii) identify the individual phenolic composition using HPLC-MS/MS, volatile composition via GC-MS and mineral composition through AAS, ICP-MS and ICP-OS.

2. MATERIAL and METHODS

2.1. Plant Material

Leaf samples of *Epilobium hirsutum* was collected from Van/Turkey (Kurubaş village,) ((38°21'52.54"N, 43°23'25.16"E, 2066 m) in 27 July 2017. Scientific identities of the plants were confirmed at Van Pharmaceutical Herbarium (VPH), Faculty of Pharmacy, Van Yuzuncu Yil University, Turkey and the voucher specimens were stored at VPH (Herbarium code: VPH-340, Collector Code: AD-766). Plant materials were properly cleaned minimizing the loss of chemical components and were left at room temperature in the dark until dry. Subsequently, the samples were ground for a fine powder and stored at -20 °C until analysed.

2.2. Reagents

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Istanbul, Turkey) and were of analytical or HPLC grade.

2.3. Preparation of Lyophilized Extract

The ethanol-based lyophilized extracts were prepared as described previously [13]. Briefly, the ground plant material was mixed with a 20-fold volume of acidified ethanol (80% ethanol, 19% H_2O and 1% of 0.1% trifluoroacetic acid, v/v/v), shaken for 2 h at room temperature (22°C) and centrifuged for 20 min at 15320g (10000 rpm) at 4°C with the supernatant collected. The extraction was repeated one more time. The supernatants from the consecutive extractions were combined and the solvent evaporated under reduced pressure at

37°C using a rotary evaporator (Rotavapor R-205; Buchi, Switzerland). The derived fraction was dissolved in purified water and freeze-dried under a vacuum at -51°C to obtain a fine lyophilized powder.

2.4. *In vitro* Analysis

2.4.1. Antioxidant Capacity

Total phenolics content (Folin-Ciocalteu Reducing-FCR), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacities (ORAC) of the extracts were determined as described previously [13]. DPPH radical scavenging and metal chelating activities were measured according to the method described by Aktumsek and co-authors [14].

2.4.2. Anticonvulsant Activity

Cholinesterase (ChE) inhibitory activities were measured using Ellman's method, as previously reported [14]. GABA-T activity was determined through the method reported by Awad and co-authors [15].

2.5. *In vivo* Analysis

2.5.1. Animals

Swiss albino male mice were chosen according to the approval of the Institutional Animal Ethics Committee (IAEC) of Van Yuzuncu Yil University, Pharmacy Faculty (Approval Number: 5873), which based on minimizing animal suffering and to reduce the number of animals' consumption. Mice were kept in the Institute of Animal House and were fed according to appropriate food facility to gain significant weight of (25-35 g). At the beginning of the study, 40 mice were housed in hygienic of transparent Plexiglas cages (polypropylene cage) of 40×30×20 cm (maximum 8 mice/cage), under standard laboratory conditions. All experiments were performed between 8.00 and 10.00 a.m. in a silent room and constant temperature of 22 - 28 °C, and relative humidity of 50 - 70% with 12/12 h light/dark cycle and free access to standard pellet food and tap water as reported previously [16,17].

2.5.2. Animals

Acute toxicity test was performed according to Vitali and co-authors [18]. Twelve mice were randomly grouped into three groups. Mice were doused with 50, 100, 150 and 200 mg/kg of the extract orally via gastric gavage in each group. The animals were given food and water ad libitum. No signs of toxicity and mortality were observed over a period of 72 h. Consequently, two doses of the extract (100 mg\kg and 200 mg\kg) were selected for anticonvulsant and biochemical evaluation.

2.5.3. Experimental Procedures

The experimental procedures were performed according to Bhosle [16]. Briefly, animals divided randomly into 5 groups, each cage 8 mice as detailed below:

Group 1. Control group: NS + NS (Physiological Normal saline 0.9% NaCl 0.5 mL; i.p). The control group was given normal saline solution NaCl, 0.9% 0.5 mL; i.p for 7 days, which was prepared daily as fresh.

Group 2. PTZ Kindling group: NS +PTZ (65 mg/kg; i.p). Normal saline solution (NaCl 0.9% of 0.5 mL; i.p) was given daily for 7 days to the PTZ group, followed by PTZ (65 mg/kg dissolved in saline solution; i.p) application.

Group 3. Valproate (positive control) group: 45 min incubation after application of Valproate (100 mg/kg; i.p) followed by application of PTZ (65 mg/kg/ i.p). Normal saline solution (NaCl 0.9% of 0.5 mL; i.p) was given daily for 7 days to the Valproate group, followed by PTZ (65 mg/kg dissolved in saline solution; i.p) application.

Group 4. PTZ + Plant Extract Dose 1 (100 mg /kg; i.p) 45 min incubation followed by PTZ application (65 mg/kg/ i.p). *Epilobium hirsutum* lyophilized extract (100 mg/kg, 0.5 mL; i.p) was given daily for 7 days to group 4, followed by PTZ (65 mg/kg dissolved in saline solution; i.p) application and the behaviours of the animals were properly monitored for 30 min as reported previously [16-18].

Group 5. PTZ + Plant Extract Dose 1 (200 mg /kg; i.p) 45 min incubation followed by PTZ application (65 mg/kg/ i.p). *Epilobium hirsutum* lyophilized extract (100 mg/kg, 0.5 mL; i.p) was given daily for 7 days to group 4, followed by PTZ (65 mg/kg dissolved in saline solution; i.p) application and the behaviours of the animals were properly monitored for 30 min as reported previously [16-18].

2.5.4. Induction of Kindled Seizures and Seizure Observation Procedures

In order to determine the potential anticonvulsant effect of *Epilobium hirsutum*, the animals were observed for their response to the PTZ seizure inducer and their attenuation of seizure standing on 5 phases of convulsion scale after the administration of PTZ for 30 min. Thus, immediately after PTZ administration (as previously explained) the mice were observed for, (1) duration of convulsion (number of mice showing convulsions), (2) onset of convulsions (elapsed time from PTZ injection until convulsion occurred) and (3) mortality for 30 min according to Pahuja and co-authors [17]. Stage 0: No respond; Stage 1: Ear and facial twitching; Stage 2: Head nodding, head clonus, and myoclonic jerks; Stage 3: Unilateral forelimb clonus; Stage 4: Rearing with bilateral forelimb clonus and Stage 5: Generalized Tonic–Clonic seizure, with a loss of righting reflex. The animals were accepted as kindled if they exhibited stage 4 or 5 of the consecutive trials; however, if no convulsion occurred within the limited time, the animals were accepted as protected by the extract. Following application of the PTZ, the mice were immediately placed for open field and then Rota rod tests in order to evaluate neurological deformities disorders.

2.5.5. Open Field Test

Open field test was used in order to measure the mental activity, anxiety, exploration, depression, and locomotion as well as seizures psychotic emotion using open-field apparatus or locomotors activity device which made of acrylic / plexiglas (transparent walls so the mice could be visible) with black floor, of the large size 72 x 72 cm divided into 8 squares of equal area. The lines of the apparatus was divided the floor into sixteen 18 x 18 cm squares and the central square (18 x 18 cm) was drawn in the middle of cage (The middle square is used to test the mouse locomotors activity and their behaviour crossing the lines of the trailing chamber many times during a test session). The increase in the count of mice movement was regarded to central nervous stimulation while a decrease in the number of mice movement was regarded to central nervous depressant activity [19].

2.5.6. Rota-rod Test

Rota-rod test was used to test skeleton muscles relaxation as it assesses motor coordination, motor learning, intoxication, sedation, stamina, motor memory (long-term skill or procedural memory) and balances of the mice. The test was conducted by testing the ability of mice to remain on a rotating rod during the 300 seconds. However, the mice failed off the rod rotating at different speeds or under continuous acceleration [20].

2.5.7. Sample Preparation and Biochemical Evaluation of the Animals

Sample preparation of animal materials were conducted as reported previously [21]. Briefly, the animals were sacrificed by removing the brain samples quickly and subsequently washed with cold saline solution twice and stored at -20 °C until analysed. The brains were cut into smaller pieces using scissors, and were homogenized using 5 mL ice-cold, Tris-HCL buffer

(1 mmol/L EDTA, 0.32 mol/L sucrose and 10 nmol/L Tris-HCl, pH 7.4) using a homogenizer and a glass of porcelain homogenizer (20 kHz frequency ultrasonic, Bandelin Sonupuls) for 8 min to break down the cell continents and get a proper solution. Subsequently the homogenate solution was centrifuged at 9500 rpm for 30 min to separate the debris. The Clear upper supernatant fluid was extracted to determine antioxidant enzyme superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activity. All preparation procedures were performed at +4 ^{o}C.

2.5.8. Determination of Malondialdehyde (MDA) Content

MDA level was determined according to the method described previously [16, 21]. The content of malonaldehyde (MDA), expressed as n moles formed per milligram of protein in the tissue, and the following formula was used for calculation: Concentration = A x (V/E) x P. Where, A is the volume of solution, E is extinction Coefficient (1.56 x 105m - 1 cm - 1) and P is the protein content of tissue calculated as milligram of protein per gram of tissue.

2.5.9. Antioxidant Enzyme Activities Determination

Antioxidant enzymes activities were determines as described previously [22]. The amount of glutathione in tissue expressed in μmol/g of tissue μmol/ mg wet tissue: [A/13600] x dilution factor x 1000. Superoxide dismutase (SOD) activity test is based on the principle of the method based on the inhibition of nitroblue tetrazolium (NBT) reduction of the xanthine-xanthine oxidase system as a superoxide generator. It was expressed in Units per milligram protein. Catalase activity (CAT) determination was measured at 230 nm and the results were expressed as μM/min/μg protein. Reduced brain glutathione (GSH) was measured at 412 nm and the amount of glutathione in tissue expressed as μmol/g of tissue.

The blood samples were taken using a cardiac puncture and were put immediately into silicon disposable glass biochemical tubes. Subsequently, the samples were centrifuged at 4000 g for 15 min at 4°C in order to obtaining serum samples for the measurement of biochemical parameters. Serum biochemical parameters including AST, ALT, ALK-P, TRIG, HDL, TBARS, Carbonyl protein, Nox content, SOD, Catalase, Sulfhydryl protein, Ca, K, Cl, Na, Total antioxidant and total oxidant levels were measured by an auto analyser (ARCHITECT 16200, Abbott Park, IL 60064, USA) using the Abbott biochemistry kits (USA).

2.6. Analysis of Phenolic Compounds

Identification and quantification of phenolic compounds by high liquid chromatography–diode array–mass spectrometry (LC-DAD-MS/MS) analysis were conducted as described previously [13].

2.7. Analysis of Volatile and Fatty Acid Compounds

Volatile compounds and fatty acids present in extracts were analysed by gas chromatography mass spectrometry (GC/MS) using a head space solid phase micro extraction and identified by the fragment ions and relative retention indices of their peaks with those of the MS library standards as described previously [23].

2.8. Analysis of Mineral Compounds

Mineral composition of the extracts was evaluated by using AAS, ICP-MS and ICP-OS. The analysis solution was prepared by dissolving the extract in HNO_3. Subsequently, the solution was subjected to microwave assisted extraction procedure. The identity of mineral compounds was confirmed by comparison of authentic standards.

2.9. Data Analysis

The mean of results was calculated based on at least three independent evaluations (n=3) and the standard deviations (SD) were calculated. IC_{50} values were calculated from the

corresponding dose inhibition curve according to their best-fit shapes based on at least four reaction points using Microsoft Excel. Statistical correlation analyses were performed using Graphpad Prism 5 (Graphpad Software, CA, USA), which were considered statistically significant when the $p<0.05$.

3. RESULTS and DISCUSSION

3.1. *In vitro* Analysis

The extract was primarily evaluated for potential antioxidant and anticonvulsant activities through complementary reagent-based *in vitro* methods. As shown in Table 1 the extract contained a pronounced amount of total phenolics (206.3±0.9 mg Gallic acid Eq./g extract) which is equal to 20% of the compounds present in the extract. Antioxidant capacities findings determined through FRAP, ORAC, DPPH and metal chelation activities showed that, the extract had enormous capabilities of regulation of oxidant compounds through different mechanisms. Specifically, the extract exhibited pronounced levels of antioxidant capacities in terms of hydrogen atom (ORAC; 6593 µmol Trolox Eq./g extract) and electron transfer mechanisms (FRAP; 6226 µmol Fe^{2+}/g extract) that indicate a dual antioxidant potential capability. These values were higher than commercial antioxidant agents, which are commonly used in food and pharmaceutical industries. For instance, Ascorbic acid had 5800 µmol Fe^{2+}/g for FRAP and BHA had 6215 µmol Trolox Eq./g for ORAC activities (data not shown in Table 1). With regards to radical scavenging and metal chelation activities, the extract had very low IC_{50} values (33.8 and 114 µg/mL, respectively) which were close to that of the commercial antioxidant agents such as Trolox (4.5 µg/mL) and EDTA (12.3 µg/mL).

Table 1. Extraction yield, *in vitro* antioxidant and anticonvulsant activities of *Epilobium hirsutum* extract

Antioxidant capacity	Extraction yield (%)	22,87
	Folin-Ciocalteu Reducing (FCR; mg Gallic acid/g extract)	206.3±0.9
	Ferric reducing antioxidant power (FRAP; µmol Fe^{2+}/g extract)	6226.1±26.2
	Oxygen radical absorbance capacity (ORAC; µmol Trolox Eq. /g extract)	6593.3±89.8
	DPPH radical scavenging activity (IC_{50}; ug/mL)[1]	33.8±0.2
	Metal chelation activity (IC_{50}; ug/mL)[1]	114.0±10.8
Enzyme inhibition	AChE (IC_{50}; ug/mL)[1]	71.2±8.9
	BChE (IC_{50}; ug/mL)[1]	92.5±7.2
	GABA-T (IC_{50}; ug/mL)[1]	94.7±5.4

The *in vitro* inhibitory activities of the extract against isolated enzymes including AChE, BChE and GABA-T were presented in Table 1. The IC_{50} values, which were lower than 100 µg/mL, showed that the extract had effective suppressive capacities of those enzymes, which are key elements of neurological disorders including epilepsy. Such a treatment method might be useful in the management and/or treatment of the convulsions and epilepsy disorder.

3.2. *In vivo* Analysis

Anticonvulsant activity of the extract through Pentylenetetrazole (PTZ) method was presented in Table 2. According to the acute toxicity test, two doses (100 mg and 200 mg) of the extract were selected. The administration of the Valproate-the positive control successively impeded the onset of the convulsion against effect of PTZ-induced convulsions (3.40 min). However, the extracts that used in a dose dependent manner, particularly 200 mg dose, had pronounced effect against onset of PTZ-induced convulsions (Table 2). On the other hand, 200

mg dose significantly reduced the duration of convulsions from 4.33 min to 1.80 min. PTZ (65 mg\kg) group showed to develop a standard pattern of limbic 5 phases motor seizures of twitch, short-lasting clinic phase, then wild running, falling of animal and finally tonic phase. Moreover, PTZ group showed a significant decrease in the onset of convulsion and a significant increase in duration compared to (200 mg\kg + PTZ) group. With regards to mortality rate, there is no statistically difference between PTZ and extract dose 1 (100 mg) groups, while extract dose 2 (200 mg) and Valproate groups hindered the mortality among mice.

Table 2. Effect of *Epilobium hirsutum* extract on the Pentylenetetrazole (PTZ)-induced convulsion in mice

Groups	Onset of clonic convulsion (min.) (± SEM)	Duration of convulsion (sec.) (± SEM)	Mortality rate (%)
Control	0.0±0.00	0.0±0.0	0
PTZ	2.2± 5.3^{c}	4.3± 0.3^{a,b}	14
100 mg extract+PTZ	2.8± 8.7	2.0± 0.0^{b,c}	14
200 mg extract+PTZ	4.5± 6.7^{c,c1}	1.8 ± 0.2^{a,b1}	0
PTZ + VPA	3.4± 1.5^{c1}	3.6± 0.3^{c,b1}	0

a: $p<0.001$, **b,b1**: $p<0.01$, **c,c1**: $p<0.05$ (Similar letter fields show significance at that letter level)

The open field routinely used to test gross animal behaviour, the nerve excitability, anxiety, mental activity, exploration, and locomotion [19]. However, the epileptic patient has shown to develop different psychotic impairments like anxiety and showed to be less mental activity comparing them with normal individuals. In our current study, mice tested for their gross behaviour and exploration ability by releasing them to the centre of the open field, which were shown in Table 3. No movement achievement was detected after seizure provoked during the given time (5 min). The control group showed almost normally movement by crossing some squares (19.0±5.2) during the given 5 min; however, the number of the centre cross was rarely by 2.75±0.25 times. The mice who expressed seizure provoke with the challenging dose of PTZ (65 mg\kg), did not show any significant movement and almost frizzed for more than 5 min.

Table 3. Comparison parameters of Open-field test

Groups	The number of squre cross (± SEM)	The number of center cross (± SEM)
Control	19.0±5.2	2.8 ± 0.3^{c}
PTZ	-	-
100 mg extract+PTZ	4.33±2.3	1.7 ± 0.3^{c}
200 mg extract+PTZ	15.4± 4.5	-
PTZ + VPA	8.0±4.0	-

c: $p<0.05$

Similar results were obtained for the positive group (valproate 100 mg\kg + PTZ 65 mg\kg), which showed some mice crossing some squares after seizure provoked but most of them did not even cross the centre. However, the extract group (100 mg\kg) showed a significant decrease in exploration behaviour comparing to the control group as they have attenuated seizure provoke. The 200 mg\kg extract group had a high number of the square cross which was close to the control group, but had no influence and significant difference in the number of the centre cross. The rota rod studies used to test the motor coordination of the

experimental mice. No significant results were obtained among groups tested. Almost all the groups had the same result, falling twice within the 5 seconds.

Table 4 presents the effect of assayed groups on lipid peroxidation (MDA) and antioxidant enzymes levels of brain tissue. MDA is a significant indicator of injury caused by reactive oxygen species. The application of the PTZ significantly increased the level of MDA according to the control group. Administration of Valproate and extracts relatively reduced the level of MDA. With regards to antioxidant enzymes such as SOD, GSH-Px, CAT and GSH, administration of PTZ significantly reduced their levels, and subsequently application of both the extracts and Valproate considerably regulated their levels according to the control group.

The effect of the extract on 18 serum biochemical parameters were presented in Table 5. There is a negative tendency of the PTZ application and a positive tendency of the positive control-Valproate in serum biochemical parameters evaluation. The results showed that the extract (specifically 100 mg) regulated the serum biochemical parameters such as AST, ALT, ALK-P, TRIG, TBARS, SOD and mineral levels, total antioxidant, total oxidant, and ischemia-modified albumin levels. The results indicate that the dose of 100 mg was slightly effective than that of the 200 mg.

Table 4. Effect of *Epilobium hirsutum* extract on lipid peroxidation (MDA) and antioxidant enzymes levels on brain tissue

Groups	MDA (nmol/mg pt.) (± SEM)	SOD (IU/mg pt) (± SEM)	GSH-Px (IU/mg t.) (± SEM)	CAT (IU/mg pt.) (± SEM)	GSH (µmol/g pt.) (± SEM)
Control	1.29 ± 0.038^{b}	282.12±4.08^{a,b,c}	0.40 ± 0.021^{b,c}	1.66 ± 0.074	21.11 ± 0.36^{b,c}
PTZ	1.51 ± 0.027^{b,c,c1}	235.11± 4.09^{a,c1}	0.27 ± 0.015^{b}	1.49 ± 0.060	17.10 ± 0.49^{b,b1}
100 mg extract+PTZ	1.33 ± 0.034^{c}	259.48 ± 7.37^{c1}	0.32 ± 0.014	1.55 ± 0.134	19.12 ± 0.63
200 mg extract+PTZ	1.43 ± 0.040	247.81 ± 4.43^{b}	0.29 ± 0.027^{c}	1.53 ± 0.078	18.42 ± 0.83^{c}
PTZ + VPA	1.32 ± 0.045^{c1}	253.37 ± 5.26^{c}	0.32 ± 0.021	1.58 ± 0.114	20.84± 0.60^{b1}

a p<0.001, b. b1 p<0.01, c. c1 p<0.05 (Similar letter fields show significance at that letter level). MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; CAT: Catalase; GSH: Reduced glutathione; PTZ: Pentylenetetrazole; VPA: Valproate

3.3. Phytochemical composition

In order to reveal the major contributor of anticonvulsant and antioxidant activity of the extract chromatographic studies were conducted, which showed that the extract was rich in mainly phenolics and accompanied by volatiles and mineral compounds (Table 6).

Phenolic composition of the extract was presented in Table 6 and Figure 1. MS/MS data showed that four major individual phenolic compounds present in the extract, which comprised mainly of phenolic acids. The compounds were tentatively characterized based on molecular weight, neutral loss, fragment ions, spectrum properties and co-chromatography studies. Compound 1 was tentatively identified as gallic acid (Table 6, Figure 1) which gave a negatively charged molecular ion ([M–1]-) at m/z 169 and displayed a MS/MS fragment of 125 m/z. Based on molecular weight, fragmentation pattern and absorbance spectrum, this compound was tentatively identified as gallic acid. Compound 2 showed a negatively charged molecular ion ([M–1]-) at m/z 353 and produced MS/MS fragment ions of 191 m/z respectively.

Table 5. Effect of *Epilobium hirsutum* extract on serum biochemical parameters

	Control	PTZ	Valproate	100 mg extract+PTZ	200 mg extract+PTZ
AST (U/L)	66,5±6.5	81,0±7.0	73,7±6.0	68,0±7.9	74,7±12.2
ALT (U/L)	52,0±3.9	64,8±.6.0	57,7±6.9	55,0±8.7	57,2±5.6b
ALK-P (U/L)	154,2±7.7	169,7±10.3	161,7±14.0	152,2±11.3	163,0±13.3
Trig (mg/dl)	28,0±2.8	30,3±1.9	31,0±2.3	26,8±2.6	26,2±2.9
HDL (mg/dl)	37,8±4.3	38,8±3.3	41,0±3.2	41,2±2.9	41,2±2.8
TBARS (nmol MDA/mg protein)	0,19±0.0	0,30±0.1	0,25±0.1	0,24±0.0	0,21±0.0
Carbonyl protein (nmolDNPH/mg protein)	1,12±0.2	1,74±0.3	1,08±0.3	1,16±0.5	1,01±0.3
Nox content (mg/mL sodium nitroprusside/mg protein)	0,40±0.1	0,57±0.1	0,43±0.1	0,45±0.1	0,46±0.1
SOD (U SOD/mg protein)	0,62±0.1	0,18±0.0	0,51±0.1	0,46±0.1	0,59±0.1
Catalase (mmol H2O2/mg protein)	0,14±0.0	0,06±0.0	0,13±0.1	0,14±0.0	0,15±0.0
Sulfhydryl protein nmol DTNB/mg protein	0,14±0.0	0,06±0.0	0,13±0.1	0,14±0.0	0,15±0.0
Ca (mg/dl)	9,0±0.1	9,0±0.6	9,1±0.2	8,8±0.3	8,6±0.4
K (mEq/L)	4,2±0.5	4,2±0.6	4,2±0.5	4,7±0.3	4,3±0.4
Cl (mEq/L)	105,3±1.4	104,0±0.8	104,4±1.3	106,7±1.3	107,5±1.4
Na (mEq/L)	143,2±3.2	144,5±2.5	143,4±3.3	141,4±1.6	140,5±1.5
Total antioxidant level	3,1±0.2	2,5±0.4	3,0±0.4	3,1±0.2	3,0±0.1
Total oxidant level	6,3±0.3	7,2±0.6	6,1±0.4	6,3±0.6	6,2±0.7
Ischemia-modified albumin	0.645±0.003	0.675±0.005	0.657±0.01	0.653±0.006	0.647±0.010

No neutral loss fragment ions were detected. On the basis of molecular weight, fragmentation pattern and absorbance spectrum, these compounds were tentatively characterized as 5-O-Caffeoylquinic acid. The third compound was tentatively identified as caffeic acid by the presence of negatively charged molecular ions ([M – 1]-) at m/z 179 and MS/MS fragments -/135 m/z. No fragment ion was detected through the neutral loss studies. Compound 4 had a loss 162 amu, which indicate the presence of hexoside moiety. It had a negatively charged molecular ions ([M –1]-) at m/z 463 and produced MS/MS fragment ions of 301. According to molecular weights, fragmentation patterns and absorbance spectrums, these compounds were tentatively identified as Quercetin-3-O-glucoside (Table 6, Figure 1). The findings obtained through gas chromatography studies were presented in Table 6, which showed that the volatile composition of the extract was consisted of fatty acids compounds. The major fatty acid was determined as palmitic acid followed by linoleic and linolenic acids through co-chromatography studies, molecular weights and fragmentation patterns. Additionally, an accumulation of a benzothiazole derivative was also identified in the extract (Table 6). With regards to the mineral compound accumulation of the extract, high levels of K, Mg, Ca and Si were detected. The levels of heavy metals such as Ag, As, Cd, Cr and Pb were found as trace levels (Table 6). A safe and reliable treatment that able to fully control the seizures has not been found until now and due to the unwanted and side effects of modern synthetic anticonvulsant medicines, biological activity evaluation researches have been focused on local medicines used in the treatment of seizures in order to find alternative new medicines with minimum side effects or less toxicity [24]. Reactive oxygen species are endogenous sources of mitochondrial homogeneous system subversion or enzymatic autooxidation reactions that form imbalance between (ROS) and cellular antioxidant defense system.

Table 6. Bioactive composition of *Epilobium hirsutum* extract

	Phenolic composition Compound	MS/MS $[M+1]^+$/ $[M-1]^-$	Fragments (m/z) (+/-)	Concentration (mg/g extract) (±STD)
1	Gallic acid	-/169	-/125	79.2±2.4
2	5-*O*-Caffeoylquinic acid	-/353	-/191	18.4±0.8
3	Caffeic acid	-/179	-/135	21.0±0.6
4	Quercetin-3-O-glucoside	-/463	-/301	8.4±0.8

Fatty acid composition

	Compound	Retention time	Fragmant ions	Contribution (%)
1	Palmitic acid	36.8	60, 73, 83, 97, 129, 143 185, 213, 227, 239, 256	22.49
2	Benzothiazole derivative	38.5	54, 63, 69, 82, 91, 108, 135	22.29
3	Stearic acid	40.1	55, 57, 60, 69, 71, 73, 83, 87, 129, 185, 241, 284	13.19
4	Linoleic acid	42.2	67, 81, 95, 150, 220, 263, 294	16.30
5	Linolenic acid	43.6	55, 67, 79, 93, 108, 121, 135, 149, 177, 191, 209, 222, 235, 249, 264, 278	7.86

Mineral composition

Compound	Level (µg/g extract)	Mineral compound	Level (µg/g extract)
Ag	T	Mn	174.2±10.0
As	T	Mo	T
B	18.2±1.4	Pb	T
Cd	T	Se	2.7±0.1
Co	3.2±0.0	Si	1560.2±15.4
Cr	T	Zn	37.4±2.8
Cu	196.3±1.2	Na	420.1±8.2
K	108940.4±34.5	Fe	305.6±2.4
Mg	15353.4±18.1	Ca	11148.7±6.3

T: traces; ND: not detected.

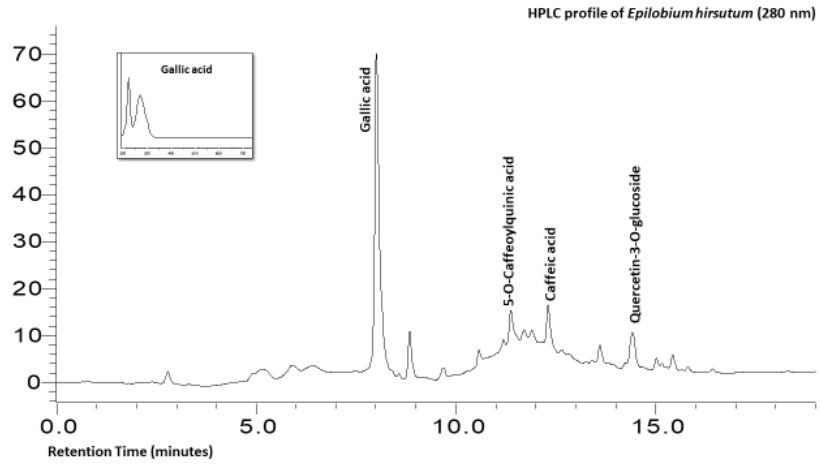

Figure 1. Phenolic composition of *Epilobium hirsutum*

Most of these oxidation products produced during seizure provoke that lead to tissue injury in epilepsy. Since most of the antioxidants defend enzymes in the brain are in small quantity, they lose their function against the potent of (ROS). Chronic disease like Alzheimer's, Parkinson's, anoxia and ischemia of brain and heart as well as arteriosclerosis, rheumatism, and cancer, showed almost the same deduction [21]. Within this study only the control and extract applied (particularly 100 mg/kg) groups showed proper immobility behaviour and normal interaction with the environment around after seizure provoke. PTZ kindling model, which is a

chemical kindling seizure inducer, has been routinely used in rodent model in order to examine the anticonvulsant effect of modern or folk medicines. It blocks the chloride channel of GABA A receptor, cause local injury of brain tissue, increase the hyperactivity of glutamic receptors (NMDA) and cause calcium ions entering into the nerve cells and liberation of free radicals [25]. Biochemically, PTZ influence the glutamate receptors (NMDA) by opening its channel and induce calcium ions entering into the nerve cells. One of the main mechanism of action of anticonvulsant activities of medicinal plants was associated to the decrease in MDA levels and increases in antioxidant enzymes levels in brain [16-17]. In the present study, the administration of the plant extract significantly decreased the levels of MDA and increased the levels of antioxidant enzymes (SOD, GSH-Px, CAT and GSH) and therefore it can be suggested that *Epilobium hirsutum* extract caused an increasing of brain antioxidant enzymes that worked as neuroprotective agents by scarfing the free radicals produced by PTZ seizure inducer and attenuate convulsion.

The extract contained a rich composition of phytochemicals, particularly phenolic compounds including gallic acid, 5-caffeoylquinic acid, caffeic acid and quercetin-3-O-glucoside. Apart from phenolic compounds some lipophilic compounds such as fatty acids and mineral compounds have also detected within this study. It was reported that caffeic acid can reduce the levels of free radicals and DNA damage in the epilepsy model induced by PTZ [26]. Chlorogenic acid able to ameliorate the decrease the MDA and ROS levels after H2O2-induced oxidative stress [27]. The ameliorative effect of gallic acid in the intracerebroventricular streptozotocin-induced oxidative damage was evaluated previously and it was reported that there was a significant link between the normalization of thiobarbituric acid, reactive substances and thiol contents, as well as antioxidant enzyme levels and oxidative stress deactivation [28]. Huang and co-authors reported that gallic acid can reduce the maximal seizure classes, predominant behavioral seizure patterns, and lipid peroxidation *in vivo* epilepticus status. The *in vitro* protective mechanism study showed that gallic acid decreased Ca^{2+} release, ROS, and lipid peroxidation from kainic acid-stressed PC12 cells. According to the neuroprotective effects of gallic acid against excitotoxins, it was suggested for a significant clinical application in epilepsy [29]. Addition to the phenolic compounds, it was suggested that polyunsaturated fatty acids including linoleic and linolenic acids, may have neuroprotective and anticonvulsant effects which provides a potential use in the treatment of epilepsy [30]. Mineral compounds are necessary elements for proper development and functioning of the central nervous system (CNS). Among them manganese, magnesium, zinc and copper are key mineral compounds in the treatment or management of CNS disorders [31]. For instance, it has been reported that manganese is required for activity of glutamine synthetase, which converts glutamate to glutamine. The deficiency of manganese might cause accumulation of glutamate and consequently leads to generation of seizures [32]. The magnesium level of epilepsy patients was found lower than that of people without epilepsy and the lower magnesium concentration associated with seizures [33]. Though the correlation of zinc-copper ratio has not been fully understood for epilepsy until now, it is proposed to intake adequate levels of zinc-copper in order to minimize the seizure occurrences [34]. Granica and co-authors reported that plant materials belong to *Epilobium* genus including *Epilobium hirsutum* are considered as nontoxic on various organs such as brain, liver, kidneys, spleen and thymus, which was confirmed by the experimental data using different animal models [1], which is in agreement with our findings. The main objectives of the present study were to evaluate phytochemical composition and protective effect of *Epilobium hirsutum*, which has been traditionally used in the treatment of epilepsy by local people in Turkey, on brain from the elevation of the oxidation reaction, and prevent or minimize the convulsions. Our findings showed that gallic acid dominated phenolic composition and other phytochemical compounds (linoleic acid, linolenic acid, manganese,

magnesium, zinc and copper) of *Epilobium hirsutum* had anticonvulsant and antioxidant activities in vitro and *in vivo*.

4. CONCLUSION

This is the first report of *in vitro* and *in vivo* anticonvulsant and antioxidant activity and chemical profile of *Epilobium hirsutum*. The extract contained a pronounced level of total phenolics and exhibited high *in vitro* antioxidant (serum biochemical parametres total antioxidant-oxidant levels) and anticonvulsant activities (through detaining the onset of the convulsions and shortening the duration of the convulsions). The extract was found as a rich source of phytochemical compounds such as phenolics (specifically gallic acid), fatty acids and minerals. Findings obtained from this study partially justified the traditional use of *Epilobium hirsutum* in the treatment of epilepsy.

Acknowledgements

None of the authors has a commercial interest, financial interest, and/or other relationship with manufacturers of pharmaceuticals, laboratory supplies and/or medical devices or with commercial providers of medical services. The authors are grateful to the Van Yuzuncu Yil University Scientific Research Projects Council for providing financial assistance of the research (Project number: TYL-2017-5873).

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 author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 2, 77–87

https://doi.org/10.21448/ijsm.674930

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Investigation of the Antioxidant Activity and Phenolic Compounds of *Andricus quercustozae* Gall and Host Plant (*Quercus infectoria*)

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Abstract: *Andricus quercustozae* (Bosc, 1792) is a cynipid gall wasp, which induces gall on oaks (*Quercus* spp.). It is known that both cynipid galls and oaks are used in traditional medicine. In this study, some biological characteristics of various extracts (acetone and ethanol) of *A. quercustozae* asexual gall and its host plant, *Quercus infectoria* Olivier, were investigated. The antioxidant capacities of the extracts were evaluated using radical scavenging activity (ABTS and DPPH assays), the β-carotene-linoleic acid method, the phosphomolybdenum method, and the reducing power (CUPRAC method). Total phenolics, flavonoid and tannin contents were measured in the gall and the oak leaf extracts. Moreover, ethanol extracts of the gall and the host plant were evaluated using HPLC for the composition of phenolics. Generally, the gall extracts (acetone and ethanol, respectively) exhibited the strongest radical scavenging (DPPH, IC_{50} value of acetone extract: 11.00 µg/mL and IC_{50} value of ethanol extract: 8.67 µg/mL; ABTS, 52.27 µg/mL and 44.97 µg/mL) and antioxidant activities with the highest level of phenolics. The antioxidant activity of the gall extracts was in the range of 80.74 to 87.49 % for β-carotene-linoleic acid method, while and it was ranged from 75.68 to 78.20 mgAEs/g for phosphomolybdenum method. In the results of some antioxidant methods (ABTS and β-carotene-linoleic acid), it is observed that the host plant extract has values close or high to the gall extract. In this context, our results suggested that the cynipid gall extracts could be used as a natural agent in food, medicinal and pharmaceutical applications.

ARTICLE HISTORY

Received: January 14, 2020
Revised: March 24, 2020
Accepted: April 01, 2020

KEYWORDS

Cynipidae,
Gall,
Antioxidant,
Phenolic,
Tannin

1. INTRODUCTION

Quercus infectoria belonging to the Fagaceae family is a small tree or a shrub widely grown in Turkey (Anatolia), Syria, Iran, and Greece. The oak is known as one of the medicinal plants, which has been traditionally used in oriental folks [1, 2]. The gall wasps or cynipids (Cynipidae), which are known as the gall inducers, is a large group with roughly 1400 species

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ISSN-e: 2148-6905 /© IJSM 2020

worldwide [3], and 151 cynipid species are also known from Turkey [4-6]. All cynipid species induce their galls on their host plant species, which are mostly the oaks (Fagaceae), Rosaceae, Lamiaceae, Asteraceae and Papaveraceae. The galls are structures formed by plants entirely for the benefit of the gall inducer. The gall provides nourishment, shelter, and protect the cynipids [3]. Besides, the gall has been used in folk medicine for having various therapeutic properties since ancient times [7]. In the ancient Sumerian (5000 BC) and Babylonian periods, the galls of *Andricus infectorius* (Hartig) (known as *Turkish Gall*) were presented in the prescriptions for typhoid cure. Furthermore, it was used in the cure of bleeding and respiratory diseases in ancient Syria [8, 9]. In Traditional Uyghur Medicine, it is seen that the gall (*A. infectorius*) was widely used in the cure of intestinal dysmotility, dysentery, functional enteritis, hemorrhagic sores, alopecia areata, dental caries, periodontitis, halitosis, pharyngolaryngitis and tympanitis [10, 11]. According to last two decades pharmacological researches, the various biological activities of the oak galls such as antibacterial, antiviral, antifungal, antioxidant, astringent, antidiabetic, antiparkinsonian, antitumor, antidiabetic, local anaesthetic, antipyretic, anti-inflammatory and many more [10, 12-16] have been revealed. In addition, there are many types of research on antioxidant activities, total phenolic and flavonoid compounds of *A. infectorius* galls. The gall extracts possess a strong antioxidant activity against free radicals because of the greater amount of phenolic and flavonoid compounds [17-23].

Previous phytochemical investigations have shown that the oak galls contain high amounts of tannin (50-70%) [24-28]. Gallic acid, ellagic acid and some sugars are the main constituents found in the cynipid galls in addition to tannin [29-31]. The amount of tannin is more concentrated in gall tissues than leaf tissues. The tissues with lower tannins are used as nutrients by cynipid larvae [32-34]. There is a highly significant positive correlation between the tannin level in oak and gall wasp diversity or abundance. The cynipids, along with their defence development against oak tannins, used them for their benefit. Tannins protect the cynipid larvae to fungal pathogens, hyper-parasites, leaf herbivores and some pathogenic factors [35].

The study aimed to investigate the amounts of the total secondary metabolites of the gall and host plant extracts. Besides, it reveals some biological characteristics (antioxidant activities) and phenolic components of all extracts for future investigations. In this context, some biological characteristics of the oak gall and the host plant are considered to be an accessible and important data source for antioxidant activity. In short, to determine in which areas (like the food industry, medicinal and pharmaceutical) the benefits of the cynipid gall used in traditional medicine, can be used.

2. MATERIAL and METHODS

2.1. Collection and Preparation of Gall and Host Plant Extracts

Andricus quercustozae (Bosc, 1792) asexual gall and the leaves of the host oak (non-galled leaves), *Q. infectoria*, were collected from Denizli, Turkey in May 2015. The species identified by the Entomology Laboratory in Pamukkale University. The galls and leaves of the host oak were dried in the shadow, broken into small pieces with an electric blender. And then gall and leaves were extracted with acetone and ethanol using the previous method [36] and stored in Secondary Metabolite Laboratory, Pamukkale University, Turkey.

2.2. Determination of Total Bioactive Components

2.2.1. Quantification of Total Phenolic Content

The Folin–Ciocalteu method [37] with slight modification was used to determine the total phenolic contents of each extract. The sample solution (1 mg/mL) was mixed with diluted Folin–Ciocalteu reagent (1 mL) and dH_2O (46 mL). After 3 min, sodium carbonate solution (3

mL, 2%, Na_2CO_3) was added. The absorbance of the mixture was measured at 760 nm after the incubation (in the dark, 2 hours, room temperature). The total phenolic content was expressed as equivalents of gallic acid (mgGAEs/g).

2.2.2. Quantification of Total Flavonoid Content

The total flavonoid contents of each extract were analysed according to the method [38]. Briefly, aluminium trichloride (1 mL, 2% $AlCl_3$) was mixed with the same volume of extract solution (2 mg/mL). The absorbance was measured at 415 nm after the incubation (10 min, room temperature). The total flavonoid content was expressed as equivalents of quercetin (mgQEs/g).

2.2.3. Quantification of Total Tannin Content

The vanillin method [39] with slight modification was used for analysing the total tannin content. The solution (0.5 mL) was mixed with vanillin reagent (1.5 mL, 1% in 7 M H_2SO_4) in an ice bath. The solution absorbance was measured at 500 nm after the incubation (15 min, room temperature). The total tannin content was expressed as equivalents of (+)-catechin (mgCEs/g).

2.3. Radical Scavenging Activity

2.3.1. Determination of DPPH Radical-Scavenging Activity

The radical scavenging activity of the extracts was determined using the method [40]. Different concentration (5 to 25 µg/mL) of the extracts (1 mL) was mixed with 4 mL of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical methanolic solution. The absorbance was measured at 517 nm after 30 min. Synthetic antioxidant BHT (butylated hydroxytoluene) was used as a positive control. The results were expressed as IC_{50} values.

2.3.2. Determination of ABTS Radical-Scavenging Activity

The method [41] with slight modification was used to determine the radical scavenging activity of the extracts. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) cation was produced by reacting ABTS solution (7 mM) with potassium persulfate (2.45 mM) and then the mixture to stand in dark (12-16 hours, at the room temperature). The mixture was diluted with ethanol to give an absorbance of 0.700 ± 0.02 units at 734 nm for the study. The extract solution (1 mL) and ABTS solution (2 mL) were mixed. The mixture absorbance was read at 734 nm after the incubation period (15 min, at room temperature). The results were expressed as IC_{50} values.

2.4. Total Antioxidant Activity

2.4.1. β–Carotene–Linoleic Acid Method

The total antioxidant activity of the extracts was analyzed using the β–carotene–linoleic acid method [42] with slight modifications. β-Carotene (0.2 mg) was dissolved in chloroform (1 mL) and added linoleic acid (20 µL) and Tween–20 (200 mg). The chloroform was evaporated using a rotary evaporator. The mixture was diluted with dH_2O (100 mL). As soon as the emulsion (4.8 mL) and 1 mL extracts (1 mg/mL) were placed into test tubes, initial absorbance was measured at 470 nm. The measurement was carried out at 30 min intervals for 2 hr. BHA and BHT were used as standards. The antioxidant activity was calculated using the equation below:

$$AA = \left[1 - \left(\frac{A_0 - A_t}{A^\circ_0 - A^\circ_t}\right)\right] x\ 100$$

Where A_0 and A^o_0 are the absorbance values measured at the initial incubation time for samples and control, respectively. While A_t and A^o_t are the absorbance values measured in the samples or standards and control at 2 hr.

2.4.2. Phosphomolybdenum Method

The phosphomolybdenum method [43] with slight modification was used to determine the antioxidant activity of the extracts. Extract solution (0.3 mL) was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture absorbance was read at 695 nm after the incubation period (90 min, at 95 °C). The total antioxidant capacity was expressed as equivalents of ascorbic acid (mgAEs/g).

2.5. Reducing Power

2.5.1. Activity Cupric Ion Reducing (CUPRAC) Method

The method [44] was used to determine the cupric ion reducing activity (CUPRAC). Extract solution (0.5 mL) was added to premixed reaction mixture containing $CuCl_2$ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM) and NH_4Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank was prepared by adding sample solution (0.5 mL) to the premixed reaction mixture (3 mL) without $CuCl_2$. The mixture and blank absorbances were measured at 450 nm after the incubation period (30 min, at room temperature). The absorbance of the blank was subtracted from that of the sample. CUPRAC activity was expressed as equivalents of Trolox (mgTEs/g).

2.6. Quantification of the Phenolic Compounds by HPLC

Phenolic compounds were analyzed by high performance liquid chromatography (HPLC) according to the method [45] with some modification. Detection and quantification were performed with a diode array detector (SPD–M20A), a LC–20AT pump, a CTO–10ASVp column heater, SIL–20ACHT auto sampler, SCL–10Avp system controller and DGU–14A degasser. The mobile phases were A: 3.0% formic acid in distilled water and B: methanol. Methanol was used to dissolve samples, and then 20 µL of this solution was injected into the column. Gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, chlorogenic acid, vanillic acid, epicatechin, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, quercetin, and cinnamic acid were used as standards. The differentiation and quantitative analysis were made by comparing the standards. The quantity of each phenolic compound was expressed as µg per gram of the extract.

2.7. Statistical Analysis

The SPSS Statistical Package program were used to analyze the results. The results were presented as mean ± std. Differentiations between the extracted groups were tested using Analysis of Variance and Tukey method were performed ($p < 0.05$).

3. RESULTS and DISCUSSION

3.1. Determination of Total Bioactive Components

The calibration curve generated from the analysis of the standard (gallic acid) was linear with $y=0.0016x+0.0407$; $r^2=0.983$. The *A. quercustozae* gall extracts showed the highest phenolic content, acetone (479.56 ± 45.36 mgGAE/g) and ethanol (437.27 ± 3.14 mgGAE/g), while *Q. infectoria* extracts showed the lowest contents. Among the studied extracts, total phenolic content ranged from 79.35 to 479.56 mgGAE/g (Table 1). Phenolic contents of the gall extracts were found to be more than the extracts of host oak (*Q. infectoria*). In addition, there were no differences between the acetone and ethanol extracts for each species ($p>0.05$), but the gall extracts and host plant extracts were found to be statistically different from each other ($p<0.05$).

In the present study, total flavonoid contents were analysed with the spectrophotometric method [38], and the flavonoid contents ranged from 33.19 to 110.28 mgQE/g (Table 1). Statistical differences among each group were found ($p<0.05$).

Table 1. Total Phenolic, Flavonoid and Tannin Contents (mean±SD) of the Extracts.

Species	Solvent	Total Phenolic Content (mgGAEs/g)	Total Flavonoid Content (mgQEs/g)	Total Tannin Content (mgCEs/g)
A. quercustozae asexual gall	Acetone	479.56±45.36^{b}	47.84±3.06^{b}	23.10±1.22^{b}
	Ethanol	437.27±3.14^{b}	33.19±1.91^{a}	7.03±0.02^{a}
Q. infectoria leaf	Acetone	87.90±8.97^{a}	110.28±3.47^{d}	34.04±0.75^{c}
	Ethanol	79.35±6.13^{a}	75.89±0.71^{c}	32.81±1.62^{c}

GAE = Gallic Acid Equivalents; QE = Quercetin Equivalents; CE = Catechin Equivalents; Data were given as the mean of the measurements ± std. The letters after the mean values in each column refers to statistically different than the others ($p<0.05$).

Tannin content was analysed using the vanillin method and these results were evaluated as catechin equivalents. The total tannin content of the extracts varies from 7.03 to 34.04 mgCE/g (Table 1). There were no differences ($p>0.05$) between the acetone and ethanol extracts of the host plant, but the gall extracts and host plant extracts were found to be statistically different from each other ($p<0.05$). Unlike phenolic compounds, *Q. infectoria* extracts showed the highest flavonoid and tannin content while the gall extracts showed the lowest contents. These results suggested that the phenolic, flavonoid and tannin contents were best extracted with acetone for both the gall and the host plant (Table 1).

Secondary metabolite constituents are known as phenolic, flavonoid and tannin etc. that serve as powerful antioxidants [46]. Moreover, there are important biological properties as antiallergenic, antimicrobial, antiartherogenic, antithrombotic, antiinflammatory, vasodilatory and cardioprotective effects [47-50]. Similar to the previous studies [21, 51-53], this study also shows that the cynipid gall and the host plant contain high amounts of phenolic, flavonoid and tannin.

3.2. Radical Scavenging Activity (DPPH and ABTS Assays)

The free radical scavenging activities of the samples of *A. quercustozae* gall and *Q. infectoria* leaves were tested by DPPH and ABTS assays. The DPPH and ABTS are known as radicals, they can be readily undergone scavenging by an antioxidant [54, 55]. The lower IC_{50} reflected a higher antioxidant activity in both assays (Table 2). In DPPH assay, the gall extracts more than the leaf extracts, have stronger scavenging activity. Among all the extracts, only ethanolic gall extract exhibited the highest radical scavenging capacity with IC_{50}: 8.67 µg/mL, followed by acetonic gall extract. The lowest scavenging activity was observed in ethanolic leaf extract with a very high IC_{50} value of 54.37 µg/mL. The ABTS scavenging capacity of the extracts was determined and IC_{50} values are given in Table 2.

The extracts showed scavenging activities in the range of 19.75 to 52.27 µg/mL. The acetonic leaf extract showed significantly stronger ABTS scavenging capacity (IC_{50}: 19.75 ± 0.92 µg/mL) than that of all other extracts ($p<0.05$). In compared extracts, *A. quercustozae* gall extracts had higher antioxidant capacity than *Q. infectoria* leaf extracts. The results of the strong radical scavenging capacity of the gall extracts are related to the high concentration of phenolic compounds in the gall extracts (Tables 1, 2).

3.3. Total Antioxidant Activity (β–carotene–linoleic Acid and Phosphomolybdenum Methods)

The total antioxidant capacities of the extracts were evaluated by using β–carotene–linoleic acid assay. The extracts of both samples exhibited generally high antioxidant activities (Table 2). However, the leaf extracts had strong antioxidant activity (91.05 ± 1.29% and 89.45 ± 1.72%) more than the gall extracts (80.74 ± 7.39% and 87.49 ± 1.27%). When compared with the inhibition values of all extracts, the acetone extract of the leaf (91.05 ± 1.29%) had a higher

value than others, but lower than synthetic antioxidants (BHA and BHT). In phosphomolybdenum assay, the extracts obtained from the gall had a higher antioxidant capacity than leaf extracts (p<0.05). Furthermore, the acetone extracts showed the highest antioxidant activity while the ethanol extracts showed the lowest activity (Table 2). Therefore, both the gall and the leaf extracts can be considered as natural inhibitors in the food industry. It must be the first study to compare the antioxidant capacities of the gall and the host plant.

Table 2. Antioxidant Properties of the Extracts.

Species	Solvent	ABTS (IC_{50}) (µg/mL)	DPPH (IC_{50}) (µg/mL)	β–carotene /linoleic acid method (%)	Phosphomolybdenum method (mgAEs/g)	CUPRAC assay (mgTEs/g)
A. quercustozae asexual gall	Acetone	52.27±4.49^{c}	11.00±0.39^{a}	80.74±7.39^{a}	78.20±1.63^{c}	237.74±5.55^{c}
	Ethanol	44.97±2.56^{bc}	8.67±0.58^{a}	87.49±1.27^{ab}	75.68±0.44^{c}	245.82±1.06^{c}
Q. infectoria leaf	Acetone	19.75±0.92^{a}	46.16±4.60^{b}	91.05±1.29^{b}	57.83±3.55^{b}	146.73±1.77^{a}
	Ethanol	40.71±2.19^{b}	54.37±3.61^{c}	89.45±1.72^{ab}	37.76±2.52^{a}	155.62±2.29^{b}
BHA	-	nt	nt	100.00±0.00	nt	nt
BHT	-	12.05±0.44	18.00±0.30	92.89±0.52	nt	nt

AE: Ascorbic Acid Equivalents; TE: Trolox Equivalents; nt: not tested; Data were given as the mean of the measurements ± std. The letters after the mean values in each column refers to statistically different than the others (p<0.05).

Table 3. Phenolic Compounds in the Ethanolic Extracts (µg/g extract).

No.	Phenolic Component	RT (min)	*A. quercustozae* asexual gall	*Q. infectoria* leaf
1	Gallic Acid	6.8	113.384	46.337
2	3,4-dihydroxybenzoic acid	10.7	210.835	84.87
3	4-hydroxybenzoic acid	15.7	383.299	100.285
4	2,5-dihydroxybenzoic acid	17.2	6002.886	2181.12
5	Chlorogenic acid	18.2	132.297	40.314
6	Vanillic acid	19.2	1002.53	360.975
7	Epicatechin	21.3	27286.511	3679.685
8	Caffeic acid	22.7	31979.335	8982.126
9	*p*-Coumaric acid	26.1	2.203	89.31
10	Ferulic acid	30.1	1.311	90.41
11	Rutin	45.6	< LOD	< LOD
12	Ellagic acid	47.7	2854.833	2635.375
13	Naringin	49.7	29.915	911.861
14	Quercetin	70.4	< LOD	285.237
15	Cinnamic acid	71.1	6.316	97.219

LOD = Limit of Detection; RT: Retention Time.

3.4. Reducing Power (CUPRAC Method)

Cu^{2+} reduction is used to determine electron donation activity which is known an important mechanism of antioxidant. Therefore, in order to analyse extracts' electron-donating power, their ability to reduce Cu (II) was tested. The high values of TEs reflected a high reducing activity. The reducing power activities of the extracts are presented in Table 2. When compared to the cupric reducing ability of all extracts, the ethanol extracts had a high cupric reduction potential for both samples. Moreover, the gall extracts exhibited higher values than the leaf extracts (p<0.05). The high reducing power of the gall extracts might relate to the high phenolic compounds that act as electron donors.

There are investigations on the antioxidant activities of both the host plant [51] and the cynipid gall [17-20, 22, 56, 57]. For the first time, a different species (*A. quercustozae*) and its host plant (*Q. infectoria*) were compared with this study. This and similar studies should reveal the important biological characteristics of many cynipid galls and host plant, which has been used since ancient times against many diseases, in terms of human health.

3.5. Phenolic Composition (HPLC)

In the study, the phenolic components of the ethanolic extracts of *A. quercustozae* asexual gall and *Q. infectoria* leaf were determined using the HPLC method (Table 3). For both species' extracts, caffeic acid has the highest concentration followed by epicatechin. Caffeic acid and epicatechin are abundant in medicinal plants and possess many biological effects such as antioxidant, anti-aging [58, 59]. Moreover, other phenolic compounds found in the extracts such as gallic acid also possess beneficial effects on human health. Radical scavenging activity can thus be explained by the presence of epicatechin and caffeic acid.

4. CONCLUSION

The results reported in this study revealed that tested total phenolic compounds were significantly found more in the gall extracts. However, the gall extracts were strong radical scavenging because of the highest level of phenolics. The non-galled leaves extracts have the highest flavonoid and tannin contents while the gall extracts showed the lowest contents. These results suggested that the phenolic, flavonoid and tannin contents were best extracted with acetone for both the gall and the host plant. Due to the high antioxidant activities were observed, it is suggested that they can be used as a natural agent in food, medicinal and pharmaceutical applications. This study clearly indicates that the gall derived remedies may have distinct therapeutic effect as compared with analogues produced from other parts of the host plant. The galls can also be used for the prevention and treatment of various diseases. Although cynipid diversity is rich in worldwide, only gall extracts of a few species have been studied so far. Further studies are necessary to determine some biological characteristics of other cynipid galls extract for the food industry and medicine.

Acknowledgements

We would like to thank crew in Secondary Metabolite Laboratory, Pamukkale University, which helped during the study.

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.

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 2, 88-98

https://doi.org/10.21448/ijsm.646558

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Some Quality Criteria of Valerian (*Valeriana dioscoridis* Sm.) Growing in Different Environments

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Abstract: Valerian (*Valeriana dioscoridis* Sm.) is a perennial herb of the Caprifoliaceae family. The genus *Valeriana* L. is represented by 14 species (15 taxa) in Turkey. This plant contains flavone glycosides, iridoids and lignans. Among these components, the medically important active ingredient is valerianic acid. Essential oils from valerian roots and rhizomes are used for the treatment of various diseases, including insomnia, mental illness, anxiety, menstrual cramps and physical stress conditions. In this study, evaluations were made of the root and rhizome of valerian plants grown in a natural environment and in greenhouse condition. The macro and micro nutrient contents of the powdered plant samples, and the antioxidant and antimicrobial activity values of the extracts were reported. Except for some macro and micro elements, it was determined that antioxidant and antimicrobial activities of plants cultivated and grown in nature were not different. The major components of both the natural and cultivated forms were determined to be 9-Borabicyclo [3.3.1] nonane, 9-[3-(dimethylamino) propyl]- (17.55% and 22.65%, respectively). The heavy metales such as Fe (415.21±47.8 mg/kg), Cu (50.9±0.2 mg/kg) and Mn (274.6±9.5 mg/kg), were obtained above limit values in grown plants of natural environment conditions.

ARTICLE HISTORY

Received: December 24, 2019
Revised: May 14, 2020
Accepted: May 22, 2020

KEYWORDS

Antioxidant,
Antimicrobial Activity,
Valerian,
Macro-Micro Elements

1. INTRODUCTION

Valeriana L. of the Caprifoliaceae family, has approximately two hundred species throughout the world [1, 2]. In Turkey, there are 14 species (15 taxa) [2-5] *Valeriana dioscoridis* Sm. is a perennial herbaceous plant with rhizomes, pink flowers and is commonly known as

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ISSN-e: 2148-6905 /© IJSM 2020

Kediotu or Çobanzurnası in Turkey. The flowering time is February-May. It grows on rocky slopes and scrubland, at up to 1500 meters above sea level [2, 3].

The dried roots and rhizomes are known to be cause soothing and sleep. It is also used in wound treatment [6] and is known to calm the nerves and relieve spasms [7]. In the roots of *V. dioscoridis*, there are lipophilic compounds known as valtrates at the rate of 0.5%. Another species, *Valeriana officinalis*, is reported to have been used as a poisonous plant and in witchcraft at the time of Cervantes [8]. Essential oils obtained from *V. dioscoridis* rhizomes have been found to have an antifungal effect [9] and the aqueous extracts of *V. dioscoridis* have an antioxidant effect [10].

Although the use of this plant is so common, there are limited data on its reliability [11]. Most of the medicinal and aromatic plants are collected from natural areas and are offered to trade. For drugs that are mostly obtained from naturally growing plants, it can be difficult to provide a sufficient amount of medicinal plants or these plants are not of the necessary quantity and quality due to mistakes made during harvesting and drying. When this is the purpose of medicinal and aromatic plants, such factors reduce the utility values of plants and even adversely affect them.

Different relaxation methods are used to eliminate the problems caused by the stressful conditions of modern life. Anxiety, which is caused by stress, has become an important problem affecting the quality of life of current society. Although various drug treatments are available, because of the side-effects of these drugs, many people have turned to alternative medicine and as a result there is an increase in the use of medicinal and aromatic plants. There is a constantly increasing demand for valerian plants, which are medicinal and aromatic plants with potential effects on the significant modern-day disorder of anxiety. In order to meet this demand, the roots and rhizomes of the valerian plant are collected from areas of natural growth. Seed production can be a problem of valerian plants as there is a low percentage of seed production and germination [12]. At the same time, irregular collection of this plant throughout the world is endangering populations of the species [13], resulting in insufficient numbers of plants, and the risk of extinction. Production or cultivation of these plants is one of the factors protecting natural flora. Therefore, cultivation and germplasm studies of this plant are important [13]. Furthermore, sometimes the wrong or various chemical strains of the plant can be collected, and there are specific periods when the effective substances in medicinal plants are highest, so they must be harvested at that time. However, it is not easy to determine when and how plants are collected, since it is not possible to control the collectors. Therefore, often plants are not of the desired quality, with regulated cultivation, the quality and productive varieties of these plants can be improved.

The aim of this study was to determine and evaluate some quality criteria of samples taken from the subsoil organs of plants grown in greenhouse conditions and in plants collected from natural areas. There are ongoing studies to eliminate the danger of extinction of the valerian plant. These studies can be considered of value as the cultivation of these plants to specific standards, which are in high demand for drugs, will contribute to the economy of Turkey.

2. MATERIAL and METHODS

The research was carried out between 2016 and 2019 in The Greenhouse of Crop and Animal Production Department, Sivas Vocational School, Cumhuriyet University, Department of Plant and Animal Production and Cumhuriyet University Advanced Technology Research Center laboratories. *Valeriana dioscoridis* Sm. plant were used as materials.

2.1. Supply of Plant Materials

The valerian (*Valeriana dioscoridis* Sm.) plants used in this research were collected from Ahmetler village, in the Manavgat district of Antalya (Turkey. C3 Antalya: Manavgat, Ahmetler village, near a stream, in a red pine forest and damp places, 36 S 0383784-UTM 4076925, 639 m, 19.II.2016, Çinbilgel s.n.).

Later, some of these plant roots were grown in pots in a greenhouse environment. Samples were collected from the root and rhizome of plants grown in both the natural environment and in the greenhouse culture conditions. The obtained plant samples were dried in the shade and grinded to the appropriate size for extraction with a laboratory grinder.

2.2. Obtaining Extracts

The powdered plant materials were macerated with 80% ethanol. After one day of agitation in the shaker, the plant particles were filtered, and dried in an oven to obtain the extracts [14].

2.3. Gas Chromatography-Mass Spectrometry (GC/MS) and GC Analysis of Extracts

Gas Chromatography / Mass Spectrometer was used to identify the components of the extracts and Gas Chromatography was used to determine the relative percentages [15]. GC–MS analyses were worked with mass spectrometer detector. Helium gas was used as a carrier gas at a constant flow rate of 1.5 mL in minutes, and 1 µL injection volume using splitless mode was programmed among 80-300 at rate of 5 in minutes. Post run was set at 300 °C for 2 min. Total run time was 60 minutes [16].

2.4. Determination of Macro-Micro Element Contents

First, the samples were ground for further analysis. The N content was determined using the modified Kjeldahl method [17]. For the P, K, Fe, Mn, Zn and Cu contents, 0.200 g plant samples were weighed in a porcelain crucible then dried in the oven at 550 ºC for 5 hours to obtain ash as contents. After removal from the oven, 1/3 HCl and distilled water were added to the extracted samples. Using a P 880 nm UV-spectrophotometer [18], the levels of K, Fe, Mn, Zn and Cu were determined with a Atomic Absorption Spectrophotometer (AAS) [19].

2.5. *In vitro* Antioxidant Activity

2.5.1. DPPH Radical Method

The DPPH radical scavenging activity of the extracts was evaluated according to the Blois method [20]. Briefly, 1mL of 1.5×10^{-4} M DPPH solution in methanol was mixed with 3mL sample solution in ethanol at different concentrations and incubated for 30 min in the dark. Absorbance was measured at 520 nm. Gallic acid was used as a positive control. The percent of DPPH radical scavenging activity was calculated according to the following equation:

$$\% \text{ DPPH radical scavenging activity} = (Ac - As)/ Ac) \times 100$$

Where Ac was the absorbance of the control without the sample and As was the absorbance in the presence of the extract.

2.5.2. Linoleic Acid/Thiocyanate Method

To determine the antioxidant activity of the extracts, the ferric thiocyanate method was used. In this method, linoleic acid oxidation is formed in vitro, and during oxidation Fe^{+2} ions are oxidized to Fe^{+3} ions. Specifically, the formation of peroxides is monitored by spectrophotometric measurement of a sample of the mixture in the incubation period. A high absorbance value indicates a high peroxide concentration. The sample solution (10 mL) and standard solution (Vitamine A and BHT) at concentrations of 100-1000 µg/ml were mixed with

10 mL of linoleic acid (2.52 %), 20 mL of phosfate buffer (0.02 M, pH 7.0) and 9.74 mL of distilled water. After vortexing, the mixture was incubated for 53 h at 37 ºC. The negative control was prepared without linoleic acid. Thereafter, at 0, 5, 8, 24, 27, 32, 48, and 53 hours, 9.6 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate were added to the 0.2 mL mixture. After 3 min, 0.1 mL of 20 Mm ferrous chloride in 3.5 % HCI was added to the mixture. The absorbance was calculated at 500 nm after 5 min incubation [21].

2.5.3. Thiobarbutric Acid Method

In this method, 2 mL of sample solution as prepared in the FTC method was mixed with 2 mL of 20% trichloroacetic acid (TCA) and 2 mL of 0.67% thiobarbituric acid (TBA), then incubated for 10 min in a water bath. After cooling, it was centrifuged for 10 min at 3000rpm/min. The absorbance of supernatant was measured at 532 nm [22].

2.5.4. Ferric Reducing Antioxidant Power Assay

The ferric reducing power for plant extracts was evaluated according to the Oyaizu method [23]. For the experiment protocol, 1 mL of plant extract (50-1000 µg/mL) and standard was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% $K_3Fe(CN)_6$. The mixture was kept in the dark for 20 min at 50 ºC. Then, 5 mL of 10% trichloro-acetic acid was added and centrifuged at 2500 rpm for 10 min. 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ solution were added to the 2.5 mL of supernatants. The absorbance of the mixture was measured at 700 nm.

2.6. Antimicrobial Activities of *Valeriana* Extracts

The microdilution Broth method [24] was used to determine the Minimum Inhibition Concentration (MIC) of *Valeriana* extracts against microorganisms of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus cereus, Candida albicans* and *Candida tropicalis*. Extracts were dissolved in dimethylsulphoxide (DMSO) to prepare stock solutions (50 mg/mL). The Mueller Hinton Broth (Accumix® AM1072) and Saboraud Dekstroz Broth (Himedia ME033) were used to grow and dilute bacteria and yeast, respectively. 10 µL extract was added to the first line of the microtiter plate, which was diluted with 90 µL broth. Next, 50 µL sample was added to the second line of the microtiter plate, which was serially diluted two-fold with broth. Concentration of the extracts in the wells ranged from 2.5 to 0.004 mg/mL. In the 11th well, 100 µL broth was added to be used as the sterilizitation control. The last well (12th) containing broth and inocula without extracts was used as the growth control. Final inoculum size was 5 $x10^5$ CFU/mL of bacteria and 0.5-2.5 $x10^3$ CFU/mL of Candida in every well [25, 26]. The bacteria and yeast suspension (50 µL) was added to the prepared samples. Samples with added bacteria were incubated at 37 (±1) °C and the samples to which *Candida* was added were incubated at 35 (±1) °C for 16-24 hours. The lowest concentration of extract that was capable of inhibiting visible growth of the microorganism was accepted as the MIC value.

3. RESULTS and DISCUSSION

Plants of the same species, obtained from two different growing environments; the cultivated form (grown in greenhouse conditions) and the natural form (collected from nature) were used in the scope of this experiment. The different growth media the effect of on the essential oil content of plants and the quality criteria of plant extracts were evaluated.

3.1. GC/MS Analysis of the Obtained Extracts

A very small amount of volatile oil could be obtained from the plants and the amount obtained remained at the hydrolysate level. Therefore, the plant extracts were used tto determine the content. Gas Chromatography-Mass Spectrometry (GC/MS) was used for the analysis and the results are shown in Table 1. When the 80% ethanol extracts of the plants collected from

the natural areas and the greenhouses were compared, they were seen to have different components.

The major components of both the natural and cultivated forms were determined to be 9-Borabicyclo[3.3.1]nonane,9-[3-(dimethylamino)propyl]-(17.55% and 22.65%, respectively). A high level of valerianic acid (pentanoic acid) is desired as this gives the plants medical properties. However, this component remained at the rate of 0.30% in the plants grown in the natural environment, and could not be determined in the plants cultivated in greenhouses. As the environmental temperature sometimes reaches very high levels during GC-MS analysis, this could cause a rupture of the bonds between chemical components of some plants and may have prevented the determination of valerianic acid (pentanoic acid). In addition, as the plants were grown in the controlled conditions of a greenhouse, this may have caused secondary metabolites to be expressed at a low rate as they are usually expressed by the plant to protect itself. As varying environmental conditions are usually produced to protect the plant, there may be differences in metabolite amounts and content. Lopes et al. [27] reported that *Valeriana* roots have highly isovaleric acid. Bogacz et al. [11] and Dimpfel [28] stated that the roots of valerian carry chemical components, especially valerianic acid, and the part of valerian used as herbal medicine is the roots.

Table 1. Main compounds identified in the methanolic extract of *Valeriana dioscoridis* Sm. by GC-MS

No	RT (Retention Time)	Components	Relative Percentage (%)	
			Culture form	Natural form
1	7.550	Pentanoic acid	-	0.30
2	9.055	1,2-Cyclopentanedione	-	1.29
3	28.275	1-Dodecanol	-	1.51
4	28.281	1-Tetradecanol (CAS)	1.54	-
5	29.133	o-Diethyl benzene	1.53	3.29
6	29.391	Phenol, 2,4-bis(1,1-dimethylethyl)- (CAS)	0.73	0.88
7	30.856	4,5-dimethyl-11-methylenetricyclo [7.2.1.0 (4.9)]dodecane	3.20	-
8	33.247	Valeranone, (+)-	0.20	-
9	33.471	Acrylic acid dodecanyl ester	1.47	1.46
10	34.752	1,3-Dimethylthieno[3,4-d]thiepin	6.12	-
11	36.572	1,3-Butadienylidene)cyclohexane	12.34	7.89
12	36.910	1-(3'-Hydroxypropyl)-2,5-dimethoxy -3,4,6-trimethylbenzene	9.50	1.89
13	37.739	Hexadecanoic acid, methyl ester	1.87	4.24
14	38.397	(+-)-3-(3,4-Dimethoxyphenyl)pyrrolidine	2.37	-
15	38.414	1,3-Dimethyl-3-hydroxy-5-methoxyox indole	4.06	-
16	38.861	Hexadecanoic acid, ethyl ester (CAS)	-	2.36
17	39.782	9-Borabicyclo[3.3.1]nonane, 9-[3-(dimethylamino)propyl]-	22.65	17.55
18	40.457	Methyl linoleate	-	1.88
19	41.544	9-Octadecenoic acid, ethyl ester	-	2.71
20	43.261	n-Nonadecanol-1	-	1.31
21	44.388	Oleic acid amide	3.31	8.22
22	49.549	Isophthalic acid, 2,6-dimethoxyphenyl ethyl ester	1.68	-
Total			72.57	56.78

3.2. Macro-Micro Nutrient Element Concentrations

The macro and micro nutrient content of *Valeriana dioscoridis* plants grown in different growth media are presented in Table 2.

Table 2. The macro and micro nutrient content values of *Valeriana dioscoridis* plants collected as the natural form and cultivated form grown in a greenhouse.

Growing Area	Mn (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	Ca (%)	Mg (%)	N (%)	P (%)	K (%)
Natural Form	274.6±9.5	50.9±0.2	35.9±0.4	4152.1±47.8	3.4±0.02	0.5±0.0	1.29±0.006	0.88±0.01	3.22±0.2
Cultivated Form	133.4±3.7	11.85±0.3	31.99±0.0	3761.1±5.4	1.8±0.01	0.8±0.01	1.86±0.005	1.02±0.01	3.17±0.1

The results of this study showed that the amount of potassium was obtained as 3.22 K % and 3.17 K %, respectively in natural form and cultivated form. Both values were found close to each other. While the N concentration of the natural form was determined as 1.29 N %, the nitrogen concentration of the plant grown in cultivated form was determined as 1.86 N %. At the same time, phosphor concentration was obtained in the nearly same proportions in plants grown in both environments (natural and cultivated form contain 0.88 P % and 1.02 P % , respectively)

The limit value of the micronutrients such as Zn, Mn and Cu are in the range of 23.2-39.4, 55-104.3 and 4.8-13.5 µg/g, respectively [29]. The Zn contents and range were similar in plants obtained from both growth environments. The Mn content exceeded the limit values in plants grown in both environments, with higher Mn content determined in plants grown in natural conditions. Similarly, Petenatti et al. [29] determined high Mn value in Valeriana officinalis. The Cu content was within the limit values under greenhouse conditions and exceeded the limits in plants grown in natural conditions. The Ca content was remained proportionally low in plants grown under greenhouse conditions. The amount of Fe was found to be higher in the plants grown in the natural environment than other condition. Petenatti et al. [29] reported that the content of Fe obtained as 0.97 mg g^{-1} in *Valeriana officinalis*. The high values of micro elements, some of which are heavy metals, suggested that these plants collected from fields may have been obtained from high traffic areas. When the plant is evaluated in terms of nutrients, it is necessary to cultivate the plants avoiding heavy metal pollution, so cultivation should be in areas with low traffic density.

3.3. Antioxidant Activity

3.3.1. DPPH Radical Scavenging Activity

The percentage DPPH radical scavenging capability of cultured and natural *V. dioscoridis* extracts are illustrated in Figure 1. The scavenging effect of the extract on DPPH radical increased in a linear manner with increasing concentration from 0.1 to 2.0 mg/mL, although at a lower level than the standard gallic acid. Duaheh et al. [30] reported that *V. officinalis* species showed high DPPH radical scavenging activity (IC_{50} = 38 mg/mL). Sudati et al. [31] and Malva et al. [32] reported that *V. officinalis* had an antioxidant effect and this property, made medically valuable to the *Valeriana* plant.

3.3.2. Linoleic Acid/Thiocyanate Method

This method is based on the measurement of the amount of lipid peroxide formed by incubation of an unsaturated fatty acid linoleic acid with oxygen at 40 ºC in an emulsion medium formed by phosphate buffer. A higher absorbance value shows lower antioxidant activity. The extracts obtained from the valerian plants grown in nature and collected in the

culture medium and the results of total antioxidant activity of Vitamin E are given in the graph below (Figure 2). According to the linoleic acid/ferric thiocyanate (FTC) method, the results are quite low compared to the reference. When evaluated in terms of both cultivation conditions, there were no significant differences in antioxidant capacity.

3.3.3. Thiobarbutric Acid Method (TBA)

The TBA method represents the inhibition of degradation of peroxides in the final stage in the production of carbonyl compounds. The TBA test is used to measure secondary peroxide oxidation products such as aldehyde and ketone. The antioxidant activity values of extracts obtained from valerian plants grown in the two different growing environments, the natural habitat and culture conditions, according to the thiobarbituric acid method, are shown in Figure 3. When the data were examined, it was observed that the results of the antioxidant activity of the extracts were quite low compared to the reference. No significant differences were determined between the two growth environments in respect of antioxidant capacity.

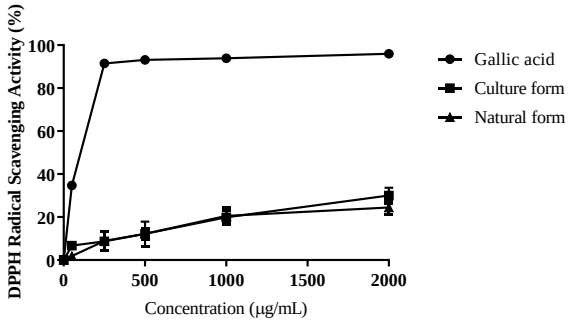

Figure 1. DPPH radical scavenging activity of 80% ethanol extracts of cultured and natural *Valeriana dioscoridis*

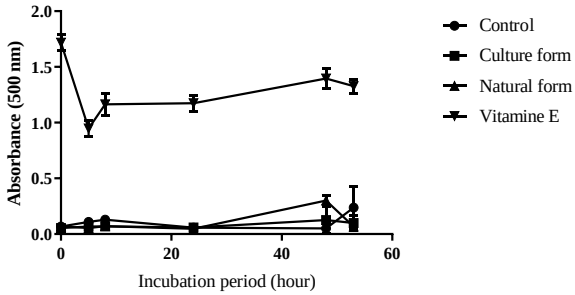

Figure 2. Antioxidant capacity of 80% ethanol extracts of cultured and natural *Valeriana dioscoridis* using the FTC method

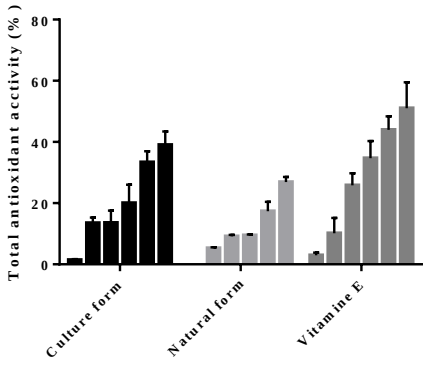

Figure 3. Antioxidant capacity of 80% ethanol extracts of cultivated and natural *Valeriana dioscoridis* using the TBA method

3.3.3. Thiobarbutric Acid Method (TBA)

The reduction power method is based on the principle that potassium ferrocyanide (Fe^{2+}) is formed by reacting the substances with the potential for reduction with potassium ferricyanide (Fe^{3+}), then reacting with ferric chloride to give maximum absorbance at 700 nm. Figure 4 below shows the comparative results of plant extracts collected from nature and grown in culture conditions and vitamin E used as reference for reducing power. According to the obtained data, the antioxidant activity values of the extracts did not show good results compared to the reference compound and there were no significant differences between the two growth conditions.

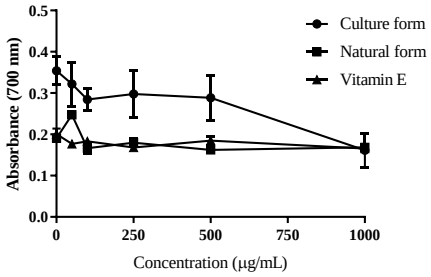

Figure 4. Ferric reducing power of 80% ethanol extracts obtained from cultivated and natural *Valeriana dioscoridis*

3.4. Antimicrobial Activities

The antimicrobial activity results of the *Valeriana* extracts are shown in Table 3. It has been reported that antimicrobial activity of plant extracts to be significant if the MIC value is 0.1 mg/mL or less, moderate if the MIC value is in the range of $0.1 < MIC \leq 0.625$ mg/mL and weak if the MIC value is bigger than 0.625 mg/mL [33, 34]. There is little difference in terms of antimicrobial activity in the comparisons of the extracts. Among the tested microorganisms, *Bacillus cereus* was more susceptible to some extracts, with MIC values ranging between 0.312 and 2.5 mg/mL. According to Düzgüner and Erbil [35] reported that *Valeriana* plant extracts have low antibacterial effect.

Table 3. Antimicrobial capacity of 80% ethanol extracts of cultivated and natural *Valeriana dioscoridis*

Growing area	*E. coli* ATCC 25922	*S. aureus* ATCC 29213	*P. aeruginosa* ATCC 27853	*B. cereus* ATCC11778	*C. albicans* ATCC10231	*C. tropicalis* DSM11953
Culture form	>2.5	>2.5	>2.5	1.25	>2.5	>2.5
Natural form	>2.5	>2.5	>2.5	1.25	>2.5	>2.5

4. CONCLUSION

Phenolic compounds that one of the most important substances with antioxidant activity, can prevent oxidative cell damage in living organism. The generally, medicinal and aromatical plants have antioxidant compounds. In this context, these plants are in high demand and consumed. If plants are harvested only from nature, their generation may face the danger of extinction. Some application mistakes are made during the collection from nature. This can also affect the plant's quality criteria. In this study, it was evaluated whether there is a decrease or increase in nutrient content, antioxidant and antimicrobial activity values of plants grown in nature and cultivated. It was observed that there was no difference in the quality criteria among plants grown in nature and cultivated. In fact, it is thought that quality criteria can be increased as a result of some plant cultivation processes.

Acknowledgements

The authors are grateful to the the Coordinatorship of Scientific Research Projects of Cumhuriyet University for their kind financial support (Project No: SMYO19).

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 author(s).

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IJSM International Journal of Secondary Metabolite

International Journal of Secondary Metabolite
2020, Vol. 7, No. 2, 99–108

https://doi.org/10.21448/ijsm.677102

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Enhancement of Plant Regeneration in Lemon Balm (*Melissa officinalis* L.) with Different Magnetic Field Applications

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Abstract: *Melissa officinalis* L. (lemon balm) is a valuable medicinal and aromatic plant in the Lamiaceae family. Two independent experiments were performed to improve the productivity of the plant regeneration. Firstly, the most efficient *in vitro* culture system of *M. officinalis* was determined using 7 different explant types (leaf, petiole, stem, root, axillary buds, shoot buds and cotyledon buds) on medium containing Murashige and Skoog minimal organics (MSMO) medium with different concentrations and combinations of plant growth regulators (PGRs). Micropropagation was obtained only with explants containing meristematic cells (axillary buds, shoot tip buds and cotyledon buds). Lemon balm had a very low regeneration capacity and in the second part of the experiment, enhancement of regeneration was aimed with the applications of different magnetic fields (MFs). Two different MFs (50 and 100 mT) were generated using neodymium block magnets. There was no MF exposure with control treatment. Three different explants (axillary, shoot tip and cotyledon buds) were cultured on media including BA in combination with indole-3-acetic acid (IAA) or naphthalene acetic acid (NAA) with the application of two different MFs at 1 hour duration. As a result, it was determined that MF applications enhanced the regeneration capacity of *M. officinalis* and the best shoot formation was observed with axillary bud explant cultured in 1.5 mg/L BA at 100 mT MF application for 1 hour duration.

ARTICLE HISTORY

Received: January 19, 2020
Revised: February 19, 2020
Accepted: March 31, 2020

KEYWORDS

In vitro culture,
Lemon balm,
Magnetic field,
Melissa officinalis,
Micropropagation

1. INTRODUCTION

Melissa officinalis L. (lemon balm) is an aromatic perennial plant belonging to Lamiaceae family. It has been used in the treatment of dyspepsia, irritability, insect bites, melancholy, insomnia, hysteria, depression and heart failure in folk medicine [1,2]. Strong medicinal properties of lemon balm are ascribed to the phenolic compounds such as rosmarinic acid, tannins and flavonoids [3,4]. Magnetic field (MF) applications in agriculture can be used to enhance the quality and quantity of the product [5]. Some studies reported the positive effects of various static MF intensities on seed germination, growth, regeneration and content in some plant species [6-17]. MF affects cell metabolism of meristem cells and has a crucial influence on mitosis affecting G1 phase of cell cycle [18]. MF applications increase antioxidant enzyme

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ISSN-e: 2148-6905 /

activities [superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) and ascorbate peroxidase (APX)] and raised antioxidant activity also makes the plant more resistant to other abiotic stress factors [18].

Lemon balm is a valuable medicinal herb and according to previous studies [19-24] regeneration capacity of this plant is very low (≤5.5 shoot). The first objective of this study was to establish an *in vitro* culture protocol of *M. officinalis* and secondly to improve the regeneration capacity of this plant with the application of two different MF intensities (50 mT and 100 mT).

2. MATERIAL and METHODS

2.1. *In vitro* culture studies of *M. officinalis*

Seeds of *M. officinalis* were collected from Bolu, Turkey. Identification of the plant was made by using "Flora of Turkey and the East Aegean Islands" [25]. Seeds were washed with an anti-bacterial soap, rinsed with distilled water and surface disinfested with 0.1 % mercuric chloride ($HgCl_2$) for 15 min and 70 % ethanol for 2 min, and then finally rinsed with sterile water for three times. Seeds were placed in aseptic, disposable petri dishes containing Murashige and Skoog's minimal organics (MSMO) medium (4.43 g/L MSMO, Sigma Chemical Co., St. Louis, MO, USA) [26] with 30 g/L sucrose, 8 g/L Difco Bacto-agar (pH 5.7, autoclaved for 20 minutes at 121°C and 105 kPa).

For adventitious shoot formation, after six weeks incubation on germination medium, four different explants (leaf, petiole, stem and root) were excised from sterile seedlings and were cultured on MSMO medium supplemented with different combinations and concentrations of cytokinins [benzyladenine (BA), kinetin (KIN) and thidiazuron (TDZ)] and auxins [indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA)]; BA (0.2, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/L) + IAA (0, 0.2, 0.5, 1.0, 1.5, 2 and 3.0 mg/L) or NAA (0, 0.2, 0.5, 1.0, 1.5, 2 and 3.0 mg/L); BA (0.1, 0.5, 1 and 3mg/L) + IAA (0, 0.5 and 1 mg/L) + gibberellic acid (GA_3; 0.5 mg/L); BA (0.5 and 1 mg/L) + NAA (0, 0.5 and 1 mg/L) + GA_3(0.5 mg/L); BA (0.5 and 1 mg/L) + IBA (0, 0.5 and 1 mg/L) + GA_3 (0.5 mg/L); BA (0.1, 0.5, 1 and 3 mg/L) + IAA (0, 0.1, 0.5 and 1 mg/L) + GA_3 (0.5 mg/L) + Ascorbic acid (AA; 100 mg/L); 1.5 mg/L BA + Chitosan (30 and 60 mg/L); BA (0.1, 0.5, 1 and 3 mg/L) + NAA (0, 0.1, 0.5 ve 1 mg/L)+ polyvinylpyrrolidone (PVP; 0.5 mg/L) + GA_3 (0.5 mg/L); KIN (0.5, 1 and 3 mg/L) + NAA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L); KIN (0.5, 1 and 3 mg/L) + NAA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L) + AA (100 mg/L); KIN (0.5, 1 and 3 mg/L) + IAA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L); KIN (0.5, 1 and 3 mg/L) + IAA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L) + AA (100 mg/L); KIN (0.5, 1 and 3 mg/L) + IBA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L); KIN (0.5, 1 and 3 mg/L) + IBA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L) + AA (100 mg/L); KIN (1, 3 and 5 mg/L) + 2,4D (1 mg/L); Kinetin (0.1, 0.5, 1 and 3 mg/L) + NAA (0, 0.1, 0.5 and 1 mg/L)+ PVP (0.5 mg/L) + GA_3 (0.5 mg/L); TDZ (0.1 and 0.5 mg/L) + IAA (0, 0.5 and 1 mg/L); TDZ (0.05, 0.1, 0.5 and 1 mg/L) + IAA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L); TDZ (0.1 and 0.5 mg/L) + NAA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L); TDZ (0.1 and 0.5 mg/L) + 2,4D (0, 0.1, 0.5 and 1 mg/L) + GA_3 (0.5 mg/L); TDZ (0.1 and 0.5 mg/L) + IBA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L); TDZ (0.1 and 0.5 mg/L) + IAA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L); TDZ (0.1, 0.5 and 1 mg/L) + IAA (0, 0.5, 1 and 3 mg/L) + GA_3 (0.5 mg/L) + AA (100 mg/L); TDZ (0.5 and 1 mg/L) + NAA (0, 0.5 and 1 mg/L) + silver nitrate ($AgNO_3$; 0.25 mg /L); TDZ (0.5 and 1 mg/L) + IAA (0, 0.5 and 1 mg/L) + $AgNO_3$ (0.25 mg /L); TDZ (0.5 and 1 mg/L) + NAA (0, 0.5 and 1 mg/L) + $AgNO_3$ (0.25mg /L) + PVP (0.5 mg/L); TDZ (0.5 and 1 mg/L) + IAA (0, 0.5 and 1 mg/L) + $AgNO_3$ (0.25mg /L) + PVP (0.5 mg/L); TDZ (0.1, 0.5, 1 and 3 mg/L) + NAA (0, 0.1, 0.5 and 1 mg/L)+ PVP (0.5 mg/L) + GA_3 (0.5 mg/L); TDZ (0.1 and 0.5 mg/L) + IAA (0, 0.5 and 1 mg/L)

+ Chitosan (30 and 60 mg/L-after autoclaving); TDZ (0.1 and 0.5 mg/L) + IAA (0, 0.5 and 1 mg/L) + Chitosan (30 and 60 mg/L-before autoclaving).

Half strength sucrose (15 gr/L) and agar (4 gr/L) were also used with above combinations. For shoot formation from preexisting meristems, three different explants (axillary buds, shoot buds and cotyledon buds) were placed in MSMO medium including BA (0.2, 0.5, 1, 1.5, 2.0 and 3 mg/L) + IAA (0.2, 0.5, 1.0, 1.5, 2 and 3.0 mg/L) or NAA (0.2, 0.5, 1.0, 1.5, 2 and 3.0 mg/L).

For rooting, after four weeks, all regenerated shoots were transferred to Magenta containers (GA-7 Vessel, Sigma-Aldrich® Chemical Co., St. Louis, MO, USA) containing the MS medium for an additional four weeks for shoot elongation. On the 6th week of culture, shoot number per shooted explants and percentage of explants producing shoots were recorded. After 6 weeks, shoots were then separated individually and placed in rooting medium including MS and different concentrations of auxins (IAA; 0.5, 1, 3 and 5 mg/L, IBA; 0.5, 1, 3 and 5 mg/L, 2,4-D; 0.05, 0.5 and 1 mg/L and NAA; 0.5 and 1 mg/L). Each auxin concentration had 10 replications and each experiment was repeated for three times. After 6 weeks, the number of roots and percentage of explants producing roots were recorded. After cleaning the roots of regenerated shoots with sterile distilled water, they were transferred to plastic pots containing potting soil.

2.2. Magnetic field experiments

Neodymium block magnets (100x50x5 mm) mounted side by side were placed in an aluminum stand. The magnetic fields (50 ± 5 mT and 100 ± 5 mT) were adjusted with magnets and a magnetic channel was formed between two magnet set to fit a 90 mm diameter petri plate (Figure 1). Magnetic field intensity was adjusted using Teslameter.

For magnetic field experiment, three different explants (axillary buds, shoot buds and cotyledon buds) were placed in petri plates containing 1.5 mg/L BA exposing 2 different magnetic fields (50 mT and 100 mT) at 1 hour duration and there was no MF exposure with control treatment. Explants were lined up in binary rows (3-6 explants in each row) in the middle of petri plate to obtain the uniform magnetic field intensity (Figure 1 and Figure 2a).

All cultures were incubated at 22 °C under a 16-h photoperiod (cool-white fluorescent lights, 22-28 µmol $m^{-2}s^{-1}$). Experiment was repeated for three times for each treatment.

2.3. Statistical analysis

All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc, Chicago, IL, USA).

3. RESULTS and DISCUSSION

After many tested different concentration and combinations of PGR, it can be concluded that *M. officinalis* was very recalcitrant species for adventitious shoot formation using leaf, petiole, stem and root explants. Media supplemented with different PGR concentrations and combinations with full and half strength of MSMO and agar were not effective for adventitious shoot regeneration. Also, medium including silver nitrate, chitosan, ascorbic acid, polyvinylpyrrolidone and activated charcoal were not effective for adventitious shoot regeneration.

Table 1. Regeneration of axillary buds in magnetic fields (50 mT and 100 mT) and without magnetic field exposure (control) in media containing different PGR combinations.

	Axillary bud					
	Mean number of shoots (±SE)			% explants forming shoots		
PGRs (mg/L)	50 mT	100 mT	Control	50 mT	100 mT	Control
PGR free	0.6±0.5[d]	0.6±1.1[c]	0.3±0.6[c]	33	33	33
BA						
0.2	2.75±2.2[d]	-	-	55	-	-
0.5	4.25±3.3[c]	-	-	20	-	-
1.0	1.75±1.0[d]	-	-	35	-	-
1.5	9.5±1.3[a]	12.5±2.1[a]	8.3±2.5[a]	70	75	65
2.0	-	-	-	-	-	-
3.0	-	-	-	-	-	-
BA + NAA						
0.2 + 0.2	-	-	-	-	-	-
0.5 + 0.5	-	-	3.5±1.8[b]	-	-	35
1.0 + 1.0	-	-	-	-	-	-
1.5 + 1.5	-	-	-	-	-	-
2.0 + 2.0	-	-	-	-	-	-
3.0 + 3.0	-	9.5±1.3[b]	-	-	60	-
BA + IAA						
0.2 + 0.2	6.3±2.6[a]	-	2.3±1.3[b]	100	-	50
0.5 + 0.5	-	-	-	-	-	-
1.0 + 1.0	-	-	-	-	-	-
1.5 + 1.5	-	-	-	-	-	-
2.0 + 2.0	-	-	-	-	-	-
3.0 + 3.0	-	-	-	-	-	-

[*]Data presented as mean number of shoots per explant ± standard error (SE). [a,b,c,d] Mean values with the same letters within vertical columns are not significantly different (p>0.05) (i.e., Comparison of PGR combinations for each explant and MF applications).

Only explants having preexisting meristem were successful for shoot regeneration. Shoot multiplication via meristem proliferation was obtained with explants containing meristematic cells (axillary buds, shoot buds and cotyledon buds). Among control treatments (no MF applications), best shoot proliferation was obtained with axillary bud at 1.5 mg/L BA (8.3±2.5 shoots per explant at 65% shoot frequency). Hypocotyl bud was also efficient for shoot regeneration at 0.5 BA + 0.5 NAA combination (6.8±2.2 shoots per explant at 75% shoot frequency) (Table 1, Table 2 and Table 3). Low regeneration efficiency of *M. officinalis* was reported with previous studies via meristem proliferation [19-24]. Tavares *et al*. [19] reported multiple shoot formation from cotyledonary node explants of 10-day-old *M. officinalis*. The highest average number of shoots was obtained with 2 mg/L of BA (4 shoots). Meszaros *et al*. [20] developed a micropropagation technique which is applicable for field cultivated plants for *M. officinalis* using shoot tips. The combination of 1 mg/L IAA and 1.5 mg/L BA resulted in the best multiplication (5.5 shoots). Tantos *et al*. [21] investigated the effect of triacontanol supplemented with 1 mg/L BA and 0.5 mg/L IAA on micropropagation of lemon balm using shoot tips. They obtained less than 3 shoots with triacontanol free medium with 1 mg/L BA and 0.5 mg/L IAA. Addition of triacontanol was not effective to increase the shoot number (<5.5 shoots). Silva *et al*. [22] reported that nodal segments of *M. officinalis* cultured on 2 mg/L BA

produced 2.57 shoots. Meftahizade *et al.* [23] investigated the optimization of micropropagation in *M. officinalis*. They tried different concentrations of alone BA or BA with combinations of IAA, IBA or NAA. But shoot number was less than 5 shoots with all of the treatment. Statistical analysis of their results showed that 3 mg/L BA in combination with 1 mg/L NAA had the highest regeneration in shoot tips explants (5 shoots).

Table 2. Regeneration of shoot tip buds in magnetic fields (50 mT and 100 mT) and without magnetic field exposure (control) in media containing different PGR combinations.

	Shoot tip bud					
	Mean number of shoots (±SE)			% explants forming shoots		
PGRs (mg/L)	50 mT	100 mT	Control	50 mT	100 mT	Control
PGR free	1.0±1.0^{d}	0.6±0.5^{e}	0.3±0.6^{c}	33	33	66
BA						
0.2	-	7.25±3.2^{ab}	-	-	25	-
0.5	-	6.3±1.3^{b}	-	-	40	-
1.0	-	-	-	-	-	-
1.5	3.5±1.7^{b}	8.5±2.1^{a}	3.3±1.5^{b}	50	80	66
2.0	1.8±1.5^{d}	6.0±2.2^{b}	-	33	33	-
3.0	-	-	-	-	-	-
BA + NAA						
0.2 + 0.2	2.8±1.5^{c}	7.3±2.6^{ab}	-	50	60	-
0.5 + 0.5	1.5±1.3^{d}	4.8±2.2^{c}	3.8±1.9^{b}	50	55	45
1.0 + 1.0	-	-	-	-	-	-
1.5 + 1.5	-	-	-	-	-	-
2.0 + 2.0	-	-	-	-	-	-
3.0 + 3.0	-	3.3±1.7^{d}	-	-	30	-
BA + IAA						
0.2 + 0.2	5.3±1.0^{b}	-	6.3±1.5^{a}	60	-	30
0.5 + 0.5	-	-	-	-	-	-
1.0 + 1.0	-	-	-	-	-	-
1.5 + 1.5	-	-	-	-	-	-
2.0 + 2.0	-	-	-	-	-	-
3.0 + 3.0	-	7.25±3.3^{ab}	-	-	70	-

*Data presented as mean number of shoots per explant ± standard error (SE). ^{a,b,c,d} Mean values with the same letters within vertical columns are not significantly different (p>0.05) (i.e., Comparison of PGR combinations for each explant and MF applications).

Mohebalipour *et al.* [24] evaluated the regeneration capacity of different landraces of nodal segments of *M. officinalis* on MS medium containing 2 mg/L BA alone or in combination with 2 mg/L IAA. Maximum average shoot number (4.97 shoots) was observed with 2 mg/L BA. Regenerated shoots were cultured on shoot elongation medium containing MSMO for additional 4 weeks. After 2 weeks, regenerated shoots were separated individually and cultured on MSMO medium including IAA, IBA, 2.4-D or NAA. Regenerated shoots were rooted in basal MS medium (control) (Figure 2c). The best root formation was obtained with basal medium or 0.5 mg/L IAA regarding the root number and root formation frequency (Table 4). Although 0.5 or 1 mg/L IAA caused root formation, increasing concentrations of IAA (3 mg/L) seriously inhibited root development. IBA and NAA concentrations were not productive for root formation, and root induction was not occurred with all tested concentrations of 2,4-D. The rooted plants were transferred to plastic pots containing sterile soil and kept in growth room

conditions at 22 °C under a 16-h photoperiod (Figure 2d). The rooted plants had 80% survival rate through the hardening off process. Similar to our result, Tavares *et al*. [19] showed that roots were developed in MS medium alone or supplemented with IBA or NAA. Meszaros *et al*. [20] also observed root development with PGR free medium. Meftahizade *et al*. [23] obtained the best root formation (3.2 roots) with 1 mg/L NAA. On the other hand, 7.75 roots were obtained with 1 mg/L NAA in our study (Table 4).

Figure 1. Magnetic field setup-50 mT (above), 100 mT (below left) and control (MF free) (below right).

In the second part of the experiment, explants having preexisting meristem (axillary buds, shoot buds and cotyledon buds) were used for MF applications. Explants were cultured on MSMO medium including BA in combination with IAA or NAA at 50 mT or 100 mT for 1 hour duration (Table 1, Table 2 and Table 3). No MF was also applied as control and regeneration efficiency of 3 explants were compared [50 mT, 100 mT and control]. One hour MF exposure was chosen for this experiment in regarding to the result of our previous study [17]. Ulgen *et al*. [17] reported that exposure time of MF was a significant factor for lemon balm seed germination and low duration (1 hour) gave the best germination rate. Explant was also cultured on media including no PGR was also tested for shoot regeneration capacity exposing 50 mT or 100 mT MF applications. Only MF applications without PGR were not effective for shoot regeneration. Very low shoot regeneration capacity was observed without PGRs (0.3 to 1 shoots) (Table 1, Table 2 and Table 3).

Regeneration capacity of lemon balm was improved with two different MF applications (50 mT and 100 mT) at 1 hour duration. The best shoot development was obtained with axillary bud cultured on MSMO medium suplemented with 1.5 mg/L BA exposed to 100 mT MF (12.5±2.1 shoots per explant at 75% shoot frequency) (Table 1, Table 2 and Table 3) (Figure 2a and b). Shoot regeneration showed 50.6% increase with 100 mT MF application (from 8.3 shoots to 12.5 shoots per explant) (Table 1). Explants (shoot tip and hypocotyl buds) exposed to100 mT MF also showed increased number of shoot regeneration (from 3.3 to 8.5 shoots for shoot tip bud explant and from 2.8 to 9.3 shoots for hypocotyl bud explant). Shoot frequency (% explants forming shoots) was also improved with 100 mT MF application for axillary bud and shoot tip explants (from 65% to 75% for axillary bud and from 66% to 80% for shoot tip bud explants) (Table 1, Table 2 and Table 3).

Several studies showed that MF aplications considerably affect the cell metabolism and mitosis in plant meristematic cells [27]. Many studies with different plant species reported the

positive effect of low MF applications on plant growh, regeneration rate, shoot and root number, plant fresh and dry weight, leaf number and stem length [5,18,28]. Similar to our findings, MF application improved the regeneration capability of *Paulownia tomentosa* (Thunb.) Steud. and *P. fortunei* (Seem.) Hemsl. node explants cultured on MS medium supplemented with 1 mg/L BA+0.1 mg/L NAA [27]. Regeneration capacity of soybean shoot tip explants cultured on Gamborg's medium and vitamins supplemented with 40 mg/L adenine sulfate+0.1 g/L glutamine+0.1 mg/L 2,4-D was also improved with MF applications [29].

Table 3. Regeneration of hypocotyls buds in magnetic fields (50 mT and 100 mT) and without magnetic field exposure (control) in media containing different PGR combinations.

	Hypocotyl bud					
	Mean number of shoots (±SE)			% explants forming shoots		
PGRs (mg/L)	50 mT	100 mT	Control	50 mT	100 mT	Control
PGR free	1.0±0.6^{d}	0.3±0.5^{c}	-	66	33	-
BA						
0.2	-	-	-	-	-	-
0.5	2.0±0.8^{d}	-	-	25	-	-
1.0	5.3±0.9^{b}	-	-	40	-	-
1.5	8.0±0.8^{a}	9.3±1.5^{a}	2.8±0.5^{c}	80	50	75
2.0	-	-	-	-	-	-
3.0	3.8±1.7^{c}	-	-	25	-	-
BA + NAA						
0.2 + 0.2	-	-	-	-	-	-
0.5 + 0.5	-	-	6.8±2.2^{a}	-	-	75
1.0 + 1.0	-	-	-	-	-	-
1.5 + 1.5	-	-	-	-	-	-
2.0 + 2.0	-	-	-	-	-	-
3.0 + 3.0	3.3±1.5^{c}	4.3±0.9^{b}	-	50	40	-
BA + IAA						
0.2 + 0.2	4.8±2.2^{bc}	-	5.5±2.4^{b}	65	-	50
0.5 + 0.5	-	-	-	-	-	-
1.0 + 1.0	-	-	-	-	-	-
1.5 + 1.5	-	-	-	-	-	-
2.0 + 2.0	-	-	-	-	-	-
3.0 + 3.0	-	-	-	-	-	-

*Data presented as mean number of shoots per explant ± standard error (SE). ^{a,b,c,d} Mean values with the same letters within vertical columns are not significantly different (p>0.05) (i.e., Comparison of PGR combinations for each explant and MF applications).

Racuciu *et al.* [29] demonstrated an increase in chlorophyll, carotenoid and nucleic acid levels in *Zea mays* L. with low static MF application (50 mT). Reina and Pascual [7] showed that lettuce seeds exposed to a stationary magnetic field increased water uptake. Applied MF altered in intracellular levels of Ca^{2+} and other ionic current density across cellular membrane. This shift caused changes in osmotic pressure and the capacity of cellular tissues to absorb water. Saktheeswari and Subrahmanyam [30] investigated the effects of pulsed magnetic field on *Oryza sativa* L. and inferred that the ion-cyclotron resonance might have interfered with the Ca^{2+} ion sequestering and thereby allowed a rise in free Ca^{2+} concentration in the system that

might be a signal to the cell to enter into early mitotic cycle. Enhanced regeneration capability of *M. officinalis* explant exposed to MFs may be related to increased uptake of Ca^{2+} ions.

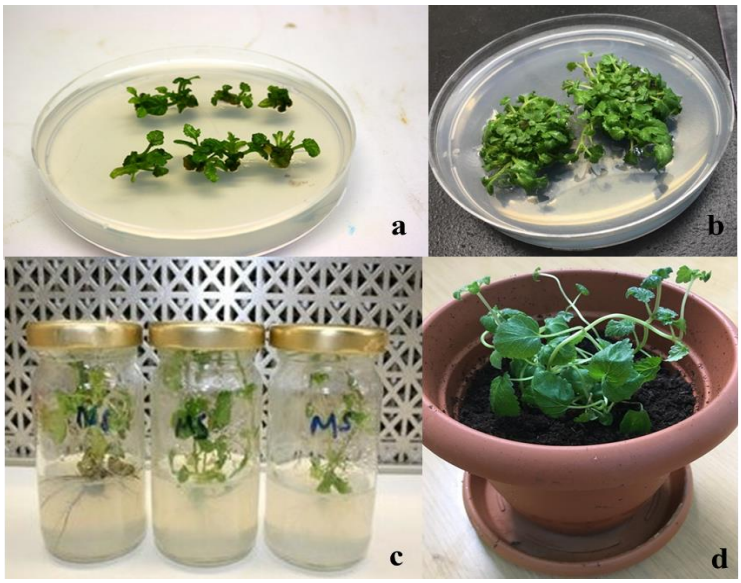

Figure 2. *In vitro* regeneration of *M. officinalis*: a) Axillary bud explants lined up in binary rows for MF application, b) Shoot regeneration from axillary bud explants cultured on MSMO medium containing 1.5 mg/L BA, with 100 mT MF application,c) Rooting of the regenerated shoots on MS basal medium, d) Regenerated plant under growth room conditions

Table 4. Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots.

Auxins (mg/L)	Mean number of roots (±SE)	% explants forming roots
Control	12.25±1.3[a]	100
IAA		
0.5	11±2.5[a]	100
1.0	10±2.1[ab]	100
3.0	-	-
IBA		
0.5	6.5±2.4[bc]	75
1.0	8±2.0[b]	75
3.0	4.25±2.5[c]	50
NAA		
0.5	-	-
1.0	7.75±2.6[b]	75
3.0	-	-
2,4-D		
0.1	-	-
0.5	-	-
1.0	-	-

*Data presented as mean number of roots per explant ± standard error (SE). Means with the same letter within columns are not significantly different at $p>0.05$. Control means no auxin added to the media. [a,b,c] Mean values with the same letters within vertical columns are not significantly different ($p>0.05$)

4. CONCLUSION

An efficient *in vitro* regeneration system was developed for *M. officinalis* via explants containing preexisting meristems (axillary buds, shoot tip buds and cotyledon buds) and then regeneration efficiency was enhanced with 2 different MF applications (50 mT and 100 mT). Especially axillary bud explant exposed to 100 mT MF for 1 hour improved the regeneration capacity of *M. officinalis*. Medicinal value of *M. officinalis* propagated with different magnetic field applications should be investigated making comparison in terms of phenolic content (especially rosmarinic acid), antioxidant potential and antioxidant enzymes in future studies.

Acknowledgements

This study was supported by the Abant Izzet Baysal University Research Foundation (BAP 2016.03.01.1027). We are grateful to Dr. Asaf Tolga Ülgen for his technical supports.

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 author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 2, 109–118

https://doi.org/10.21448/ijsm.699292

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

The Effect of 24-Epibrassinolide Treatments at Different Concentrations on Some Growth Parameters and Crocin Level in Saffron (*Crocus sativus* L.)

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Abstract: Brassinosteroids (BRs) are steroid phyotohormones that play roles in plant growth, secondary metabolite accumulation, stress response, and adaptation. Saffron (*Crocus sativus* L.) is an important plant with significant pharmacological effects due to its rich phytochemical content. Crocin, the main pigment of the saffron stigma, is a natural food colorant and has anti-cancer activity. In this study, the effects of 24-Epibrassinolide (EBL) at different concentrations (10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M) on corm (mother corm) and aerial part related parameters as well as the level of crocin in saffron were investigated. The experiment was conducted in a plant growth cabinet as randomized plots design with 3 replications. 24-Epibrassinolide treatments did not affect the rates of shoot emergence from saffron corms, while 10^{-8} and 10^{-9} M EBL treatments increased the number of active nodium and cormlet (daughter corm) as compared to control. All the applied EBL concentrations significantly increased the root and shoot lengths of saffron plants. The maximum number of flowers per plant was observed as a result of the 10^{-8} M EBL treatment. Exogenous treatment of 10^{-9} M EBL enhanced the crocin level by 96.0% compared to the control. This is the first study to evaluate the effects of BRs in saffron.

ARTICLE HISTORY

Received: March 05, 2020
Revised: April 02, 2020
Accepted: April 23, 2020

KEYWORDS

Saffron *(Crocus sativus)*,
24-Epibrassinolide,
Growth,
Crocin.

1. INTRODUCTION

Saffron (*Crocus sativus* L.) is a perennial, sterile and triploid plant that belongs to the Iridaceae family. Both plant itself and spice obtained from dried stigmas are called saffron. Besides being widely used in different industrial areas like food, dye, and cosmetics, saffron is an important plant with pharmacological properties grown since ancient times [1]. Corms of saffron survive for only one season and reproduce only vegetatively by cormlet formation [2]. The flowering time of saffron corms planted in August is known as autumn. *C. sativus* is grown in Italy, Morocco, Egypt, Greece, Spain, and Turkey and is also cultivated in Azerbaijan, China, India, Pakistan, and Ireland [3]. Saffron is one of the most expensive spices in the world and this is due to the labor in its cultivation process and harvest. A stigma of a saffron is about 2

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ISSN-e: 2148-6905 /

mg and stigmas of around 110.000-170.000 flowers should be collected by hand to obtain one kg of dry saffron [4].

Chemical analyses demonstrate that numerous seconder metabolites are found in the stigma of saffron containing sugars, minerals, oils, and vitamins as well as terpenes, flavonoids, anthocyanins, and carotenoids [5]. Crocin ($C_{44}H_{64}O_{24}$), which gives color to the saffron, picrocrocin ($C_{16}H_{26}O_7$) which is responsible for taste, and safranal ($C_{10}H_{14}O$) that gives flavor, are the most bioactive compounds [6]. Crocin, which constitutes 6-16% of dry stigma, is a diester and a water-soluble carotenoid [7]. It has been shown in various studies that crocin and crocetin showed inhibitory effects due to their apoptosis-stimulant effect at the beginning and progression stages of various cancer types [8] such as leukemia [9], brain cancer [10], lung cancer [11], and prostate cancer [12].

In vivo and *in vitro* studies in different plants have revealed that secondary metabolite production is promoted by plant growth regulators (PGRs) [13]. Brassinosteroids (BRs), are a class of polyhydroxysteroids considered as "sixth class plant hormones" [14]. Studies carried out with BR mutants of *Arabidopsis thaliana* (L.) Heynh: [15] and *Pisum sativum* L. [16] provided strong proof that BRs are essential for plant growth and development. Brassinosteroids play pivotal roles in a wide range of physiological processes including such as cell division and expansion, gene regulation, protein, and nucleic acid synthesis, photosynthesis, vascular differentiation, seed germination, flowering, and fruit formation as well as increase tolerance against various stress factors [17, 18]. In addition, it has been determined that BRs promote the production of secondary metabolites in different plants [19, 20], however, there are few studies on this subject [21].

The market value of saffron is quite high and demand is increasing for various purposes. On the other hand, saffron production is limited and is gradually decreasing [22]. Therefore, a great interest has emerged in understanding and increasing the biosynthesis and production of saffron carotenoids [23]. In this study, the effects of the EBL hormone on corm and aerial part related parameters as well as the level of crocin in saffron were examined.

2. MATERIAL and METHODS

2.1. Plant Material and 24-Epibrassinolide Treatments

Saffron (*Crocus sativus* L.) corms used in this study were obtained from Safranbolu district of Karabük (Turkey) province. The experiment was carried out for three months in the plant growth cabinet in 16 days (23 ±1°C) and 8 hours night (10±1°C) period conditions with 50±2% relative humidity. In the study, a total of 120 healthy and uniform saffron corms (with an average of 2.5 cm in diameter) were used. 24-Epibrassinolide (Sigma, Germany) hormone was prepared in accordance with the method of Fariduddin *et al*. [24] and EBL concentrations used in the study were prepared by the dilution of stock solution. Saffron corms planted at a depth of 1.5-2 cm in pots (9 x 8 cm) containing peat were given 150 mL of water on the first day and water application of 150 mL containing EBL hormone in concentrations of 10^{-6}, 10^{-7}, 10^{-8} or 10^{-9} M at intervals of 5 days was carried out. Control samples were given 150 mL of water every 5 days. A total of eighteen applications were made in three-months. In the experiment carried out in 3 replications in accordance with a randomized block pattern, one corm was planted in each pot and 24 saffron corms were used for each application group.

2.2. Determination of Growth Parameters

Shoot emergence (%) was calculated by recording numbers of shoots spring from saffron corms during the experiment (n=24). Cormlets (1 cm or more in diameter) and the number of active nodiums on corms were determined at the end of the application period. Root and shoot lengths (cm) of saffron were determined by means of a ruler (n=15). During the flowering

period, the number of flowers in control and experiment groups was determined by daily count (n=15).

2.3. Determination of Crocin Level by High Performance Liquid Chromatography (HPLC)

2.3.1. Sample Preparation

Flowers were collected daily and their stigmas were separated by hand and left to dry in the shade [25]. The extraction of saffron stigmas was carried out in accordance with Zeng *et al*. [26] method. After stigmas were dried at 40ºC for 8 hours, they were powdered in a mortar. Then, 4 mL of methanol was added to stigma samples (10 mg) and the mixture was shaken for 4 hours in dark. The final volume was completed to 5 mL by methanol after the extraction. Samples were then passed through a 0.45 µm pore size syringe filters.

2.3.2. Chromatographic System

A Shimadzu HPLC system (LC 20A Prominence, Germany) was used equipped with a degasser (DGU-20A3), pump (LC-20AD), autosampler (SIL-20AC HT), column oven (CTO-10AS VP), detector (DAD, SPD-M20A), and data communication module (CBM-20AC).

2.3.3. Chromatographic Conditions

Quantitation of crocin in stigmas was carried out by UV detection at 440 nm wavelength using the C18 reverse-phase Inertsil ODS 3 (250 mm × 4.6 mm; 5 µm) column (GL Sciences, Japan). Acetonitrile and ultra-pure water as the mobile phase with gradient elution and mobile phase flow rate was 0.8 mL min^{-1}. Injection volume was set at 50 µL and column temperature at 25°C. The retention time of crocin was determined as 5.6 min in these conditions. Crocin used in the study was obtained from Sigma-Aldrich (Tokyo, Japan) and solvents (HPLC grade) from Merck (Darmstadt, Germany). Crocin concentrations (mg L^{-1}) were given as the mean (± SE) of three replications.

2.4. Statistical Analysis

Parametric assumptions (normal distribution and homogeneity of variances) for each data set were tested using Shapiro-Wilk and Bartlett's tests. Data obtained from control and EBL hormone treatments were subjected to one-way analysis of variance (ANOVA) and then, multiple comparisons of means were made using Tukey's HSD test. Results are given as mean (±SE). The significance level was determined as 0.05 in all analyses.

3. RESULTS and DISCUSSION

Biotechnological methods provide large amounts of reproducive material for saffron as well as offering opportunities to produce commercially important chemical substances such as crocin, picrocrocin and safranal [1]. However, micropropagation protocols take a long time, labor-intensive, complex, and not repeatable. Genetic improvement of saffron by means of molecular plant breeding is difficult because it is a triploid (2n=3x=24) plant that does not produce seeds [27]. Quality of saffron depends on the content and composition of metabolites, responsible for red color, taste and flavor, which are affected by environmental conditions to which corms are exposed [28]. Modern agriculture is facing several difficulties due to reasons such as a global reduction in soil fertility as well as increasing consumer demands such as pure and high-quality ecological products. For these reasons, alternatives that protect the ecological balance of nature are needed. Plant growth regulators, a product of modern biotechnology, are significant in this respect [29]. But so far, there are very few studies investigating the possible effects of PGRs on saffron [30-32]. To our best knowledge, there is no investigation on the effects of BRs on plant growth and seconder metabolites of saffron.

In the present study, exogenous EBL treatments did not affect shoot emergence compared to the control, while they caused statistically significant differences in active nodium and cormlet numbers (Table 1). It has been found that the number of active sodium and cormlet numbers varies depending on the EBL concentration. Active nodium number per corm increased by 35% as a result of the 10^{-9} M EBL treatment. Treatment with 10^{-8} and 10^{-9} M EBL caused an increase in cormlet numbers by 92% and 70%, respectively. In the field study carried out by Aytekin and Açıkgöz [29], hormone (polystimulin), biohumus, effective microorganisms (EM) or biohumus + EM were applied to saffron corms and an increase in the number of cormlets (an average of 2.47) was obtained in all treatments. In another study about saffron, effects of seven different bacteria isolates as well as indol-3-acetic acid (IAA) and gibberellic acid (GA_3) on cormlet formation and plant growth parameters were tested. Hormone applications did not result in a significant difference in the parameters about shoot emergence and cormlet (cormlet number, diameter, length and weight) compared to control [30]. Yıldırım *et al*. [31] reported that dormancy was broken and numerous cormlets were formed in different sizes of saffron corms which were pre-treated with 5 ng μL^{-1} BAP and 5 ng μL^{-1} BAP + 150 ng μL^{-1} GA_3 for 150 minutes. While picloram, plays a delaying role in parameters related to nodium activation and corm in saffron among the substances picloram, paclobutrazol or zeatin applied in greenhouse conditions exogenously, the best corm yield was obtained from paclobutrazol (10 mg L^{-1}) application compared to the other treatment groups. Zeatin application (3 mg L^{-1}) caused led to the formation of a high number of active corms (2.81 active nodium/corm) [32].

Table 1. Effects of different concentrations of 24-Epibrassinolide (EBL) hormone treatments on shoot emergence (%), active nodium (number/corms) and cormlet number (number/corms) in saffron (*C. sativus*)

Treatments	Shoot emergence (%)	Active nodium number (number/corm)	Cormlet number (number/corm)
Control	79.20 ± 4.20^{a}	3.76 ± 0.12 ^{bc}	1.52 ± 0.25 ^{b}
10^{-6} M EBL	70.80 ± 4.20^{a}	3.20 ± 0.15 ^{c}	1.05 ± 0.50 ^{b}
10^{-7} M EBL	75.00 ± 7.20^{a}	3.13 ± 0.20 ^{c}	1.27 ± 0.16 ^{b}
10^{-8} M EBL	87.50 ± 7.20^{a}	4.03 ± 0.25 ^{b}	2.93 ± 0.28 ^{a}
10^{-9} M EBL	83.30 ± 4.20^{a}	5.10 ± 0.14 ^{a}	2.59 ± 0.22 ^{a}

Values in the table are mean (±SE) data. Different letters in the same column indicate significant differences according to Tukey's HSD test ($p < 0.05$).

As all EBL concentrations applied in the present study revealed statistically significant increases in root and shoot length compared to control, maximum values were determined in both parameters consequent to 10^{-8} M EBL treatment (Table 2). Ali *et al*. [33] recorded that applications of 10^{-9} M EBL and 28-homobrassinolide (HBL, BR analogue) increased root and shoot length in *Vigna radiata* (L.) R.Wilczek. Similarly, HBL applications at different concentrations (10^{-7}, 10^{-9} and 10^{-11} M) to *Zea mays* L. for 7 days led to an increase in root and shoot length compared to control [34]. Brassinosteroids are involved in the cell expansion process with their effects on gene expression and enzyme activity [35]. Besides, this hormone group stimulates cell elongation and cell division which is underlined by physiological ways such as control of modification of cell wall, carbohydrate assimilation and aquaporin activities [36]. On the other hand, EBL treatments have been determined to demonstrate inhibitory effects on root formations in mung bean, wheat, and corn plants [37]. Müssig *et al*. [38] reported that applications with lower concentrations promote root growth as high concentrations cause an inhibitory effect. Additionally, it shows that roots need less BRs than shoots as BR amount was found to be high in tomato shoots while they were very low in roots [39]. In the present study,

EBL treatments (10^{-8} and 10^{-9} M) caused an increase in the number of flowers (45.0% and 41.0%, respectively) (Table 2). It was determined in the study conducted by Ghanbari *et al*. [27] for 3 years that single or combined applications of different types of fertilizers and *Glomus mossae*, a mycorrhizal fungus, affect flower number in saffron and that organic fertilizer (inoculated with *Glomus mossae*) is the application which increases the number of flowers most compared to other applications. While EBL hormone (10^{-6} M) applied to *Capsicum annuum* L. by spraying method on day 15, 45 and/or 75 did not change the number of flowers compared to control [40], 10^{-8} M EBL treatments performed after 15th and 30th days to *Tagetes erecta* L. days from planting with the same method led to an increase in the number of flowers [41]. Brassinosteroids affect flower formation by modulating metabolic pathways as well as interacting with other signaling pathways and phytohormones [42, 43].

Table 2. Effects of different concentrations of 24-Epibrassinolide (EBL) hormone treatments on shoot length (cm), root length (cm) and the number of flowers (per plant) in saffron (*C. sativus*)

Treatments	Shoot length (cm)	Root length (cm)	Number of flowers (per plant)
Control	10.87 ± 0.52 ^{d}	2.52 ± 0.25 ^{c}	1.73 ± 0.04 ^{b}
10^{-6} M EBL	16.73 ± 0.75 ^{c}	4.72 ± 0.16 ^{b}	1.46 ± 0.12 ^{b}
10^{-7} M EBL	24.59 ± 1.59 ^{b}	7.69 ± 0.28 ^{a}	1.69 ± 0.13 ^{b}
10^{-8} M EBL	31.62 ± 2.06 ^{a}	8.86 ± 0.57 ^{a}	2.50 ± 0.21 ^{a}
10^{-9} M EBL	24.11 ± 1.21 ^{b}	7.70 ± 0.17 ^{a}	2.44 ± 0.06 ^{a}

Values in the table are mean (±SE) data. Different letters in the same column indicate significant differences according to Tukey's HSD test ($p < 0.05$).

Besides their dynamic roles in the adaptation of plants to their environments, secondary metabolites are important to drug sources [44]. It has been revealed that crocin and its analogues (α- crocin, crocin-2, crocin-3, crocin-4 and crocin-5) with many biological activities such as anti-tumor, anti-cancer, anti-microbial, anti-inflammatory and antioxidant were more efficient than the other secondary compounds in the therapeutic use of saffron [45]. According to the results of HPLC analysis results in the present study, 10^{-8} and 10^{-9} M EBL treatments increased crocin, a natural carotenoid, amount compared to control by 21.0% and 96.0% (Figure 1 and Figure 2). Based on the obtained results, we can interpret that the use of EBL in the cultivation of saffron may be one of the important approach for increasing the level of crocin. In this study, it was determined that low-concentration EBL treatments gave more effective results within the investigated parameters. Similarly, studies conducted in different plants have shown that BRs applied at low doses increase product yield and tolerance to different stress factors [46].

In a study in which effects of different culture conditions, carbon sources and PGR on development of callus and crocin level in saffron, it has been revealed that crocin level is increased in mediums containing 30 g L^{-1} sucrose and 20 g L^{-1} starch hydrolyzate as well as IAA (4 mg L^{-1}), GA_3 (2 mg L^{-1}) or uniconazole (1.25 mg L^{-1}) [47]. Chen *et al*. [2] obtained three times higher amount of crocin in calluses obtained from the nutrient medium containing 0.5 g L^{-1} 6-benzyl adenine (BA) and 2 mg L^{-1} IAA hormones using two-stage *in vitro* culture system. Zeng *et al*. [26] stimulated the formation of stigma-like structures as a result of the application of sodium acetate *in vitro* culture, but also caused an increase in the amount of crocin. In addition, it was reported that polyvinylpyrrolidone (PVP) application increases the crocin level in stigma-like structures, as well. In another *in vitro* study, effects of sodium azide and/or NaCl applications in two different saffron ecotypes on pharmaceutical content of saffron were investigated. While application with 0.09 mg L^{-1} sodium azide increased safranal, picrocrocin and crocin amount in both ecotypes, NaCl applications caused a decrease in the amount of secondary metabolites investigated [48].

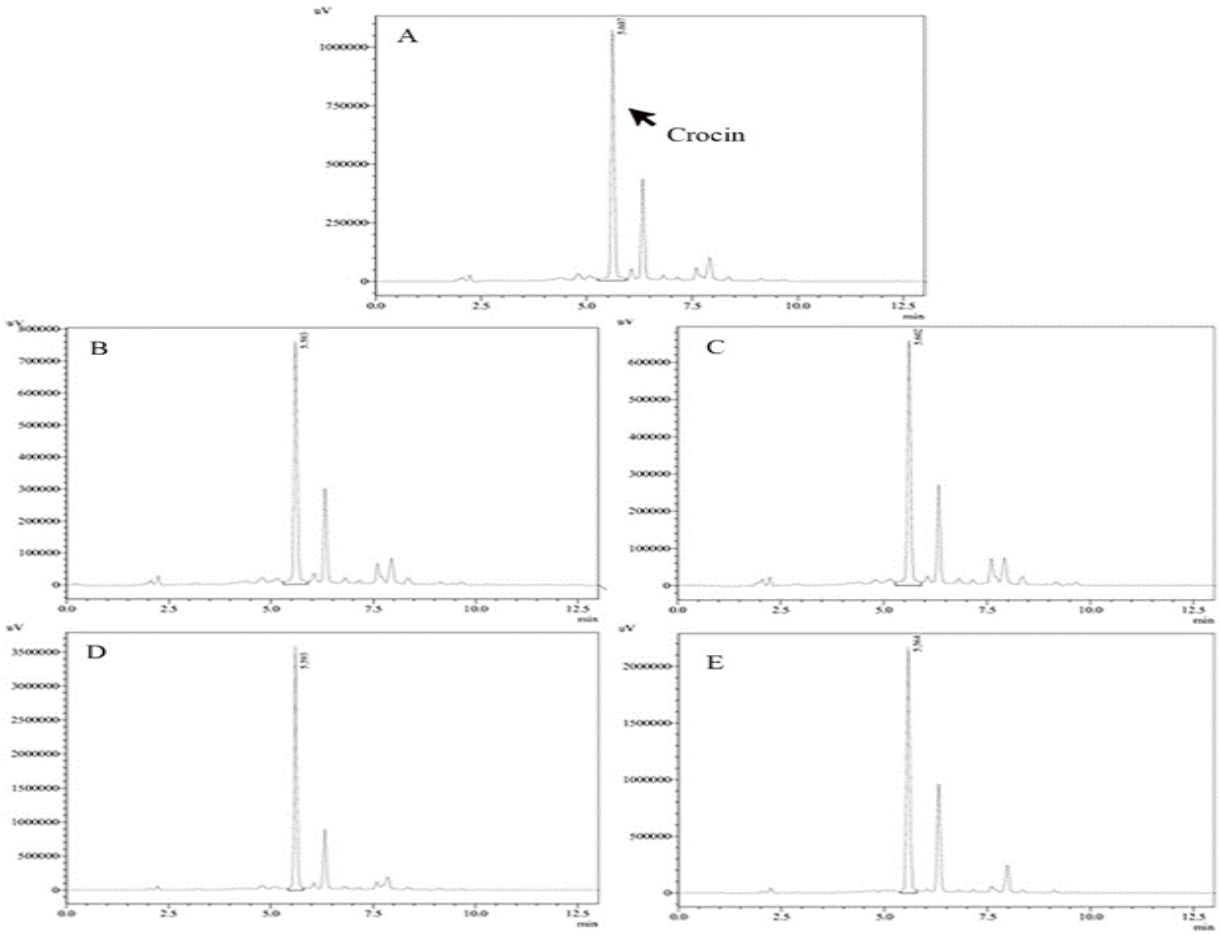

Figure 1. HPLC chromatograms of crocin extracts in saffron (*C. sativus*) stigmas. A) Control, B) 10^{-6} M EBL, C) 10^{-7} M EBL, D) 10^{-8} M EBL and E) 10^{-9} M EBL treatments.

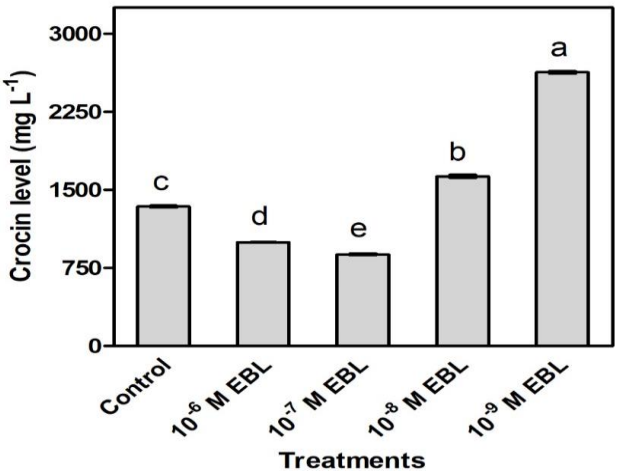

Figure 2. Effect of different concentrations of EBL hormone treatments on the level of crocin in saffron (*C. sativus*) stigmas. Data given are mean (± SE) values (n = 3). Different letters on the bars indicate significant differences according to Tukey's HSD test ($p < 0.05$).

In the study investigating effects of EBL applications of different concentrations (0.75, 1.50, and 2.25 mg L^{-1}) on plant growth and secondary metabolite production in *Lavandula angustifolia* Mill. (munstead), it has been shown that 0.75 and 1.50 mg L^{-1} hormone applications increased parameters related to yield as well as total phenolic content and essential oil yield per

plant [49]. Liu *et al*. [21] demonstrated that Brassinazole Ressistant 1 (*BZR1*), which plays a role in BR signalling [50], increases the carotenoid level by regulation carotenoid biosynthesis genes.

4. CONCLUSION

When the economic value of saffron is considered, different alternatives are necessary in order to increase its production and secondary metabolite content. In the present study, low concentration EBL treatments in *Crocus sativus* have led to an increase in active nodium, cormlet, and flower numbers as well as in root and shoot lengths. Another significant finding obtained in the study is the determination of an increase in crocin levels depending on the EBL concentration applied, which requires further investigations. We believe that this study, which is the first one to demonstrate the effects of 24-Epibrassinolide in saffron, will constitute the basis for detailed and comprehensive studies in the future.

Acknowledgements

We appreciate Senior Biologist Işıl Kesbiç and Dr. Tülden İnanan for their assistance in HPLC analyses.

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 author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 2, 119–125

https://doi.org/10.21448/ijsm.693816

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Investigation of Some Corticosteroids as Glutathione Reductase Inhibitor

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Abstract: The detection of glutathione reductase inhibitors (GRIs) has recently become very popular due to their use as malarial and cancer drugs. Today, steroidal compounds have several clinical roles due to their potent immunomodulating and anti-inflammatory properties. In this study, GR inhibitory capacity of some corticosteroids (dexamethasone, prednisolone and methylprednisolone) has been reported. Amongst these steroidal molecules dexamethasone showed the weakest inhibitory effect on GR enzyme. IC_{50} values were determined by drawing % activity-[I] graphs for these corticosteroids showing inhibition effects. These corticosteroids have inhibition ranging micromolar for GR with IC_{50} values. These corticosteroids exhibit very potent inhibitory activity against GR enzyme at low micromolar concentrations when compared to well-known GRIs.

ARTICLE HISTORY

Received: February 25, 2020
Revised: April 02, 2020
Accepted: May 09, 2020

KEYWORDS

Steroid,
Glutathione reductase,
Inhibition,
Oxidative stress,
Cancer

1. INTRODUCTION

Glutathione reductase (EC 1.6.4.2, GR) is a homodimeric enzyme with flavin adenine dinucleotide (FAD) as a prosthetic group in its active site. This enzyme catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) using nicotinamide adenine dinucleotide phosphate (NADPH) molecules. GR is involved in balancing the intracellular redox system. For the GR enzyme, both GSSG and NADPH, analogs of their substrates, and complexes containing many different ligands have been investigated in detail by the enzyme's high-resolution crystal structures [1-3]. It has been reported that the glutathione reductase enzyme from Baker's yeast (*Saccharomyces cerevisiae*) shows 50% similarity to *Escherichia coli* (*E. coli*) and human GRs and all of them exhibit well-preserved secondary structural elements [3,4]. Inhibition of the GR enzyme has been studied using many different compounds. As a result of these trials, it was observed that there was a decrease in GSH / GSSG ratio and an increase in NAD(P)H / $NAD(P)^+$ ratio [4-6]. In some studies, it has been determined that the inhibition of the GR enzyme does not affect the production of free radicals or the expression of other enzymes involved in GSH biosynthesis [6-8]. However, the strong activity of the GR enzyme in cancer cells helps to provide resistance to various chemotherapeutic drugs. High intracellular GSH levels provide an advantage for tumor cells to survive, especially in lung,

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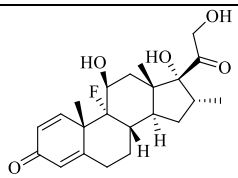
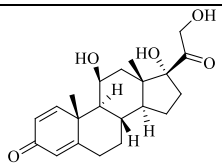
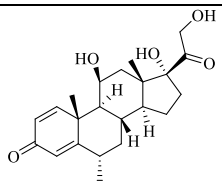
ISSN-e: 2148-6905

breast, larynx, colon and bone marrow cancers [7,8]. Considering this situation, it can be seen that substances that inhibit the GR enzyme can be used for anticancer treatment.

The term steroid is used for a wide variety of molecules with different physiological effects. More specifically, corticosteroids are a class of chemical that are both synthesized in the lab and have naturally produced hormonal effects. Glucocorticoids usually regulate metabolism and inflammation. Mineralocorticoids regulate sodium and water levels. Corticosteroids are in a spectrum ranging from glucocorticoid effects to only mineracocorticoid effects, and steroid compounds are selected based on their suitability to a particular treatment [9]. Many clinical roles of steroid molecules have been associated with potent anti-inflammatory and immunomodulatory properties [10]. Dexamethasone, prednisolone and methylprednisolone are synthetic corticoids. All three are used as medicines for many diseases, especially anti-inflammatory therapy (Table 1).

Table 1. Substances tested, medical usage and molecular structures.

Compound	Medical usage	Molecular structure
Dexamethasone	Inflammation, Rheumatoid arthritis	
Prednisolone	Ulcerative colitis, Asthma, Crohn's disease, Rheumatoid arthritis	
Methylprednisolone	Arthritis, Bronchidal inflammation	

In this study, the GR enzyme inhibitory potential of some corticosteroids (dexamethasone, prednisolone and methylprednisolone) was tried to be determined. For this purpose, both *in silico* (docking) and *in vitro* experiments were carried out.

2. MATERIAL and METHODS

2.1. Measurement of Glutathione Reductase Activity

GR enzyme activity was performed according to the Beutler method [11]. In order to determine the activity of GR enzyme from baker's yeast, the decrease of NADPH in the presence of the substrate GSSG (oxidized glutathione) was done by spectrophotometrically at 340 nm under the assay condition at 25°C, 100 mM Tris-HCl buffer pH 8.0 [5].

2.2. Inhibition Studies

First, 1mg of each corticosteroid was dissolved in 1mL DMSO and then diluted to various concentrations with pure water. To determine GR inhibition activity, five serial dilutions of the inhibitors were measured. The reaction system was composed of 5-30 µL inhibitor sample, 200 µL buffer (0.1M, pH 8.0: Tris-HCl), 660-695 µL pure water, 50 µL GSSG, 50 µL NADPH and 10 µL enzyme (0.3 units/mL). The reaction was initiated upon addition of the enzyme. The reaction system was prepared at room temperature in a quartz cuvette. The blank reading was composed of all chemicals except the inhibitor. The absorbance of the reaction mixture was

measured at 340 nm within 3 minutes from the start of the reaction on a Thermo Scientific Evolution 200 Series UV-VIS spectrophotometer. The absorbance for each reaction mixture was measured three times within 3 minutes of adding the enzyme, and the results were reported as mean ± standard deviation. The inhibition properties are reported as IC_{50} values that were determined graphically from inhibition curves of log inhibitor concentration versus percent of inhibition. IC_{50} values represent the concentration of inhibitor required for 50% inhibition of the enzyme.

2.3. *In silico* Studies

In silico placement studies were conducted to investigate interactions between ligand (corticosteroid substance) and amino acid residues around the active site of the GR enzyme (PDB: 1DNC). *In silico* docking figures and values are taken from Swissdock (Figure 1 and Table 2).

3. RESULTS and DISCUSSION

GR, which is an antioxidant enzyme, performs a very important task by performing the regulation of GSH in the redox metabolism in the cells of many living things. It is involved in the formation of deoxyribonucleotides in GSH redox hemostasis. The viability of fast-proliferating cells and cells that are resistant to oxidative stress is relatively dependent on the regeneration of GSH. Therefore, potential GR inhibitors play an important role in the development of antitumor and antiparasitic drugs [2,12,13]. In this study, we demonstrated the *in vitro* inhibitory effects of prednisolone, methylprednisolone and dexamethasone corticosteroid drugs on the GR enzyme. These corticosteroid drugs were found to be highly potent inhibitors of GR enzyme at submicromolar level as shown in the Table 1. The IC_{50} (µM) values of the compounds was determined as 0.0116, 0.0210 and 0.0352 for prednisolone, methylprednisolone and dexamethasone, respectively. For carmustine (N, N-bis (2-chloroethyl) -N-nitro) known as a strong and specific GR inhibitor, the IC_{50} value for the same enzyme was obtained as 647 µM [14]. When the IC_{50} values we obtained for the corticosteroids we used in our study were compared with carmustine; Approximately 55.77 times prednisolone, methylprednisolone 30.8 times and dexamethasone 18.38 times lower show the same effect. In experiments performed on GR enzyme by Grellier *et al.*, [15] it was determined that nitro aromatic substances containing quinoline ring affect GR enzyme. Therefore, it has been reported that it can be used as antistimulants and anticancer drugs [15]. In studies conducted by different research groups on GR enzymes isolated from different sources, some drugs and chemicals containing different functional groups were found to have an inhibition effect [14-19]. The aim of these studies in the literature and our study is the same, to identify new drug molecules in which GR inhibitors can be used in treatment (primarily diseases such as cancer and malaria). For this purpose, there are two major advantages of trying substances previously used as drugs. The first is that there is no need to conduct very costly and time consuming human cytotoxicity experiments. The second is that the production system already exists.

We also made *in silico* studies of these three corticosteroid drugs, which we determined to be effective GR enzyme inhibitors. Prediction binding models between these three molecules and GR enzyme we obtained from PDB through Swisdock program were created. For this purpose, the fully flexible placement methodology of these ligands and the estimated ΔG values and binding models were determined by Swissdock on the simulation of protein residues of the GR enzyme. Prednisolone was placed in the binding site of methylprednisolone and dexamethasone GR enzyme. Estimated ΔG scores and corresponding binding interactions of fixed inhibitors in GR targets are shown in Table 2. In addition, docking models for three corticosteroids with GR enzyme are shown in Figure 1. In addition to these data, the graphs we

use to determine IC_{50} values experimentally for three corticosteroids with GR enzyme are shown in Figure 2.

Table 2. Inhibition values and docking scores for the enzyme GR used in the research.

Inhibitor	GR IC_{50} (µM)*	Estimated ΔG, kcal/mol
Prednisolone	0.0116 ± 0.0002	-7.42
Methylprednisolone	0.0210 ± 0.0005	-7.23
Dexamethasone	0.0352 ± 0.0017	-7.02
Carmustine[a]	647.00	-

*Mean from at least three determinations. [a]Ref [19].

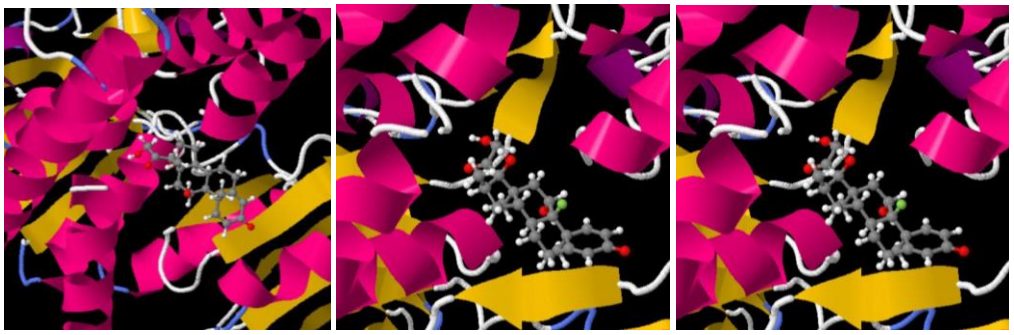


Figure 1. 3D docking binding models of prednisolone, methylprednisolone and dexamethasone with GR enzyme, respectively.

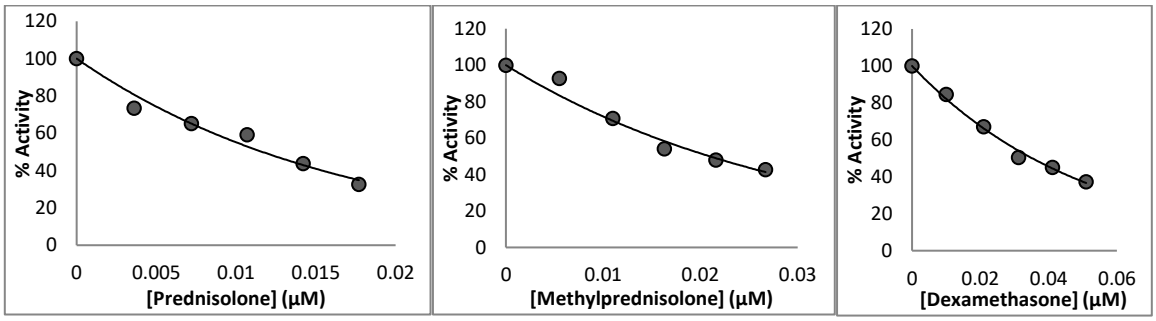


Figure 2. IC_{50} inhibition graph of GR enzyme with prednisolone, methylprednisolone and dexamethasone, respectively.

Medicines are molecules used to treat or prevent diseases. In recent years, both pharmaceutical companies and scientists have been extensively investigating whether drug molecules are used outside of the diseases in which they are used for treatment. This is done because the time to register a new molecule as a medicine is very long and expensive. A standardized production process in a molecule used as a drug and the completion of all phase studies provide an important advantage. In this reported study, the inhibition effects of three different corticosteroid drugs on GR enzyme were tried and also docking studies with human GR enzyme were done and shown in Figure 1, Figure 2 and Table 2. When the values obtained in Table 2 are examined, it is seen that both experimental and theoretical data are compatible for GR enzyme. In our study, it was found that the most effective GR inhibitor from the corticosteroids tested was prednisolone and the placement score supported this. When these findings were compared with literature [15-18], it was determined that these three corticosteroids we tried were very effective GR inhibitors. In the literature, both known as a strong GR inhibitor (IC_{50}: 647 µM) and used as a cancer drug, carmustine has high toxicity and causes inhibition of human DNA synthesis, causing various side effects [19]. The GR enzyme

plays a critical role in thiol homeostasis, maintaining a high GSH / GSSG ratio [20]. Although physiologically essential, the human cellular GSH pool can also increase tumor formation and resistance to anticancer drugs. It is also seen as the target enzyme in the treatment of viral infections such as malaria and HIV [21]. Here we report these three corticosteroids that we use in our study as potent GR inhibitors. The IC_{50} values of these corticosteroids were found to be 18.38 to 55.77 times lower than the carmustine we used as positive control, so these compounds were considered to be potent GR enzyme inhibitors.

4. CONCLUSION

Resistance in the use of cancer drugs is the most important reason for failure in the treatment of cancer. Many cancer drugs induce the production of intracellular reactive oxygen species (ROS) as part of their mechanism of action. Resistance to such drugs develops by increasing the level of intracellular GSH of the cancer cell. Increasing intracellular oxidative stress increases the sensitivity of cancer cells to anticancer drugs. GR inhibition is a new approach that can be used in cancer treatment. GR enzyme inhibitors are similarly seen as the target enzyme in the treatment of viral infections such as malaria and HIV by increasing the intracellular oxidative stress level of the host cell. Here we report these three corticosteroid drugs (prednisolone, methylprednisolone and dexamethasone) that we found to be potent GR inhibitors in our study. Strong enzyme inhibitors can have inhibitory effects even at very low concentrations. Therefore, it is thought that their toxicity and, therefore, their side effects will be less. It showed a very strong inhibitory effect against GR for corticosteroid drugs used in this study. Blockade of GR occurs as an atherapic method to treat various oncological diseases or some viral infections. These results indicate that these corticosteroids have the potential to be used in the treatment of cancer or some viral infections, but should be confirmed by *in vivo* and/or clinical trials for more precise results.

Acknowledgements

This work study was presented as a summary in the 3rd International Conference on Advances in Natural and Applied Sciences (ICANAS 2018), 2018, Antalya, Turkey. Authors gratefully thanks to Swissdock for *in silico* data. The authors gratefully acknowledge the financial support from the AİÇÜ Scientific Research Council (ECZF.19.001).

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.

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 2, 126–138

https://doi.org/10.21448/ijsm.711952

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Pharmacognostical Studies on *Acer campestre* L. subsp. *campestre*

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Abstract: This study on *Acer campestre* L. subsp. *campestre*, used traditionally in treatments of various inflammation disorders, especially hemorrhoids and rheumatism, aimed to determine the total phenolic contents, the antioxidant and antimicrobial potentials, and the chemical composition profile of the extracts and subextracts from maple syrup and *Acer campestre* L. subsp. *campestre* leaves and twigs. The antioxidant activities of maple syrup extracts and subextracts were examined by 2,2-diphenyl-1-picryl-hydrazyl (DPPH), and the total phenolic contents of all samples were determined using the Folin-Ciocalteu reagent (FCR) method. Also, they were tested for their antimicrobial activities by microbroth dilution technique. Catechic tannin, saponins and flavonoids are determined in its leaves and twigs by preliminary qualitative phytochemical analysis. The alcoholic extracts obtained by 48-hour maceration (91.2%), ethyl acetate subextract of alcohol extracts obtained by 24-hour maceration (91.1%) and alcohol extracts obtained by 24-hour maceration (88.8%) exhibited higher radical scavenging activity than other samples, while the decoction and infusion had moderate activities. Beside of this, it was found that the syrup and its subextracts have more total phenolic contents than other extracts. The alcohol extracts obtained by 24-hour maceration and by 48-hour maceration exhibited higher activity against *Candida albicans* (78 µg/mL and 156 µg/mL, respectively). Only the alcohol extract obtained by 48-hour maceration and ethyl acetate subextract of syrup showed an activity against *Escherichia coli*, while all samples except butanol subextract of syrup have an antibacterial activity against *Pseudomonas aeruginosa*.

ARTICLE HISTORY

Received: March 31, 2020
Revised: April 27, 2020
Accepted: May 13, 2020

KEYWORDS

Acer campestre subsp. campestre,
Maple syrup,
Antioxidant,
Total phenolic content,
Antimicrobial

1. INTRODUCTION

Oxygen-derived free radicals have a major role in the development of oxidative stress and may cause various chronic and degenerative diseases such as coronary heart disease, stroke, inflammatory diseases, aging, cancer and diabetes mellitus. In addition to these, the role of these radicals in the inflammatory process was already proved and it was specified that they are

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ISSN-e: 2148-6905 /© IJSM 2020

implicated in the activation of nuclear factor kB, which induces the transcription of inflammatory cytokines and cyclo-oxygenase 2. The antioxidant agents scavenge or increase the effects of these radicals and play therefore an important role to prevent and also to treat the diseases that are caused by oxidative stress. In recent years, natural antioxidant compounds come into prominence in many investigations because of the unfavorable effects of synthetic antioxidants. Many studies on the contribution of phenolic compounds to antioxidant activities of plants exist in literature [1,2]. It is well known that these compounds exhibit also good anti-inflammatory activity [3,4]. This relationship between antioxidant and anti-inflammatory properties are subject to an increasing number of studies. As a matter of fact, it was designated with the recent studies on anti-inflammatory plants that their anti-inflammatory activity is related to the synergistic effects of free radical scavenging activities, effects like corticoid or pro-inflammatory enzyme inhibitors [1,2].

Maple syrup, used in food industry as a sweetener, is prepared from various *Acer* species and is mostly produced in eastern North America, Canada and the United States, with the province of Quebec (in Canada) responsible for the majority of the world's supply (approximately 80%) [5]. Many investigations on maple syrup exist in literature [6-19]. Apart from the content of maple syrup such as amino acids (arginine, threonine, proline, etc.), vitamins (riboflavin, niacin, thiamine, etc.), minerals (K, Ca, Mg, Na, Mn, Al, Zn, Fe, etc.), phytohormones (abscisic acid and phaseic acid and their metabolites) and organic acids (fumaric acid, malic acid, etc.) [6-8], the phenolic compounds (belonging to lignan, phenolic acid, stilbene, coumarin and flavonoid subclasses) are secondary metabolites, mostly isolated from the syrup [9-14]. The anticancer, antioxidant, α-glucosidase enzyme inhibitory, and anti-inflammatory activities were determined in previous *in vitro* studies [15-17] as well as liver-protective effects in animal studies [18] and the potential to reduce plasma glucose levels [19]. Some of the studies on maple syrup and *Acer* species is mostly used in the production of maple syrup (*A. saccharum* Marsh., *A. rubrum* L.) are very interesting and useful. The mechanism of action of the anti-inflammatory effect was studied, and its potency was displayed by comparing resveratrol, curcumin and quercetin, which are known as strong anti-inflammatory compounds [17]. The antioxidant, antiradical and antimutagenic activities of the phenolic compounds in the syrup were also investigated and it was found that all the studied phenolic compounds showed antioxidant and antiradical activity and the glycosylated phenolic compounds were more effective than the aglycons [20].

In a study on cytotoxicity of maplexins A-I from *Acer rubrum* L., it was shown that the two (maplexins C–D) or three (maplexins E–I) galloyl derivatives had antiproliferative effects against HCT-116 and MCF-7 cells, and the cancer cells were up to 2.5-fold more sensitive to the maplexins than the normal cells [21]. In an investigation on the anti-hyperglycemic activity of the methanol extract prepared from *Acer saccharum* Marsh. leaves, the extract exhibited a potent inhibitory effect on α-glucosidase *in vivo* and *in vitro* and acertannin was isolated via assay-guided fractionation of the crude extract [22]. Wu et al. were studied the antibacterial activities of extracts prepared with 50% ethanol from five *Acer* species (*Acer platanoides* L., *Acer campestre* L., *Acer rubrum* L., *Acer saccharum* Marsh. and *Acer truncatum* Bunge) against 24 standard bacteria strains. They showed more inhibitor activity against Gram-positive bacteria and were effective by a different mechanism from levofloxacin. They also inhibited β-ketoacyl-ACP reductase (FabG), which has an important role in bacterial fatty acid synthesis. Additionally, tannic acid and two maple leaf extracts, exhibited better inhibition on bacteria, showed time-dependent irreversible inhibition of FabG [23]. In another study on the antiprotease activity of certain medicinal plants from Slovakia, only the extracts of *Acer platanoides* and *Rhus typhina* L. showed urokinase inhibition activity [24]. The ash of the bark of *Acer campestre* subsp. *campestre*, is used to treat the inflamed wounds in traditional

medicine, in Turkey (Catalca) [25]. The juice from gallnut on its leaves and branches is preferred for the treatment of eczema traditionally in Turkey (Kocaeli) [26].

In this study, the chemical composition profile by preliminary qualitative phytochemical analysis of various extracts obtained from the leaves and twigs of *Acer campestre* subsp. *campestre* (ACC), used traditionally in various inflammatory diseases especially hemorrhoids and rheumatism in Turkey, and the antioxidant activity, total phenolic content and antimicrobial activity of various extracts obtained from the leaves and twigs of ACC and also of the maple syrup and its subextracts were investigated. Additionally, the activities and total phenolic content of ACC extracts were compared with the activities of the maple syrup and its extract, for the aim to make a prediction about the possibility for usage in infectious diseases and about the anti-inflammatory activity of ACC.

2. MATERIAL and METHODS

2.1. Plant Material

The maple syrup was procured from the overseas market. The leaves and twigs of *Acer campestre* L. subsp. *campestre* were collected from Catalca - Istanbul (Turkey), in July 2017 and the voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE 115948).

2.2. Preliminary Qualitative Phytochemical Analysis

The infusions were prepared from 5 g of air-dried and powdered plant part with 100mL boiled water in 30 min. After cooling, the infusions were filtered. Following tests were performed with these filtrates for qualitative detection of phytochemicals e.g. flavonoids, anthracenes, saponins, tannins (catechic tannin and gallic tannin). In addition to these, the dried and powdered plant part was used for the tests alkaloids [27]. The results were expressed in the presence (+) and absence (−) of the phytochemicals.

2.2.1. Test for flavonoids

Over 5mL infusion, 5mL Shibata Reagent (conc. HCl+water+ethanol) and a piece of Magnesium were added and it has been observed whether orange, red or purple color has occurred (Shibata Reaction = Cyanidin Reaction).

2.2.2. Test for Anthracene Compounds

Over 10mL infusion, 5 drops concentrated H_2SO_4 was added. The mixture was warmed in boiling water for 15 min. and extracted with 5 mL toluene after cooling. Over the toluene phase, 3mL NH_3 solution (10%) was added and it has been observed whether rose pink to red color has occurred.

2.2.3. Test for Saponins

10 mL infusion was shaken vigorously in a graduated cylinder for 30 sec. and it has been observed after 15 min. whether minimum 1 cm foam layer has occurred.

2.2.4. Test for Tannins

2.2.4.1. Gelatin Test (for the General Determination of Tannins)

Over 5mL infusion, 2mL Gelatin-salt Reagent (Gelatin solution (1%) saturated with NaCl) was added. It has been observed whether cream-beige precipitate has occurred.

2.2.4.2. Separation of the Type of Tannins

1st step - Ferric Chloride Test: Over 10mL infusion, 3 drops $FeCl_3$ (5%) was added. It has been observed whether blue-black colour (gallic tannin) or dark olive green (catechic tannin) has occurred.

2nd step – Stiasny Reaction (for the separation of tannin types): Over 10mL infusion, 5mL Stiasny Reagent (formol in water (30%) + conc. HCl) was added. The mixture was warmed in water (80°C) for 30 min. When the material contains catechic tannin, precipitates in potions appeared. After cooling of the mixture, it was filtered. 3mL filtrate was saturated with sodium acetate. After adding of 3 drops diluted $FeCl_3$ solution, it has been observed whether blue-black precipitate or colour has occurred.

2.2.5. Test for Alkaloids

1 g of air-dried and powdered plant part was extracted with 10mL H_2SO_4 solution (3%) in hot water. It was cooled and filtered. After adding 5 mL NH_3 solution (10%), it was stirred with 10mL ether. The layers were allowed to separate. The etheric phase was evaporated to dryness. The residue was dissolved in 10mL H_2SO_4 solution (3%). The alkaloid control reactions were made on this solution in 3 portions.

1. After adding of the Mayer Reagent (mercuric chloride + potassium iodide + water), it has been observed whether milk-white precipitate has occurred.
2. After adding of the Bouchardat Reagent (iodine + potassium iodide + water), it has been observed whether dark red precipitate has occurred.
3. After adding of the Dragendorff Reagent (bismuth carbonate + potassium iodide + water), it has been observed whether orange-red precipitate has occurred.

2.3. Preparation of the Plant Material for the Preliminary Analysis

2.3.1. Total Moisture Content (Loss on Drying)

Air-dried and powdered plant part (weight: A) was put into a pre-dried and weighed (WAc1) crucible. The samples were dried in an oven at 100°C for 2 hours. It was cooled in desiccators and reweighed (WAc2). The percent loss of weight of air-dried sample was calculated by equation: % = (WAc2-WAc1) x100 / A

2.3.2. Total Ash Content

Air-dried and powdered plant part (weight: A) was put into a pre-dried and weighed (Wc1) crucible. The sample was ignited gradually in an electrical muffle in 300°C for 15 min and after this 500°C for 15 min. The process was allowed to cool by taking a break for a while. After dropping 1-2 drops of water, the temperature was gradually increased. When the temperature reached 800°C by gradually increasing, it was ignited in 800°C for 30 min. It was cooled in desiccators and reweighed (Wc2). Total ash content was calculated as in equation: % = (Wc2 – Wc1) x 100 / A

2.4. Extraction and Preparation of Samples

After dissolution of syrup in alcohol-water mixture, it was subjected to liquid–liquid partitioning sequentially with ethyl acetate (SEtOAc) and butanol (SBuOH). The dried and powdered plant part was macerated with ethanol, separately with stirring for 24 h (Ac1) and 48 h (Ac2). Only the half of Ac1 was subjected to liquid–liquid partitioning sequentially with ethyl acetate (Ac1EtOAc), but the partition could not continue with butanol because of a phase separation problem. An infusion and a decoction were prepared separately from another portions as in its traditional use. The organic extracts were evaporated to dryness and the aqueous extracts were lyophilized. The extracts were stored at ± 4°C till further used.

2.5. Antioxidant Assay and Determination of Total Phenolic Contents

All samples were applied to the microplates by DPPH method and Folin-Ciocalteu reagent (FCR) method, as shown in Figure 1 and Table 1.

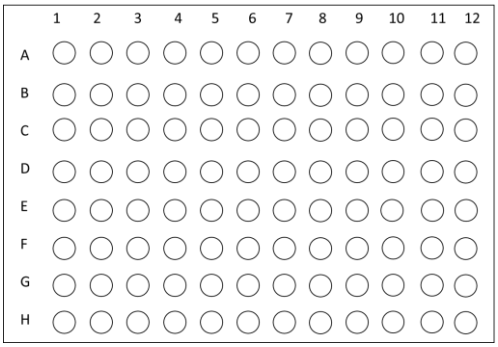

Figure 1. Microplate in antioxidant assay

Table 1. Application order in antioxidant assay by DPPH method

Wells	References and Samples	Wells	References and Samples	Wells	References and Samples
A1-A3	BHT (46.3 µg/mL)	A4-A6	Decoction	A7-A9	SEtOAc
B1-B3	BHT (92.5 µg/mL)	B4-B6	Infusion	B7-B9	SBuOH
C1-C3	BHT (187.5 µg/mL)	C4-C6	Ac1	C7-C9	Syrup
D1-D3	BHT (375 µg/mL)	D4-D6	Ac2		
E1-E3	BHT (750 µg/mL)	E4-E6	Ac1EtOAc		
F1-F3	BHT (1.5 mg/mL)				
G1-G3	BHT (3 mg/mL)				

2.5.1. DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picryl-hydrazil) radical scavenging activity of all samples were measured by the DPPH method described by Bardakci et al. [28]. 25 µL of extracts (5mg/mL) were individually mixed with 200 µL of DPPH solution (0.1 mM) freshly prepared in methanol. Following the incubation period in the dark at room temperature, the absorbance was measured against the reference using a micro plate reader at 517 nm. Butylated hydroxytoluene (BHT) was preferred as reference substance.

DPPH radical-scavenging activity was calculated as follows:

DPPH radical scavenging activity (%)= $[(Abs_{control}-Abs_{sample})/Abs_{control}]*100$

$Abs_{control}$ = the absorbance value of the control group

Abs_{sample} = the absorbance of the samples

2.5.2. Determination of Total Phenolic Contents in Extract

Total phenolic content of plant extracts was measured with slight modifications on previously described method by Singleton and Rossi [29]. 20 µL of the extracts (5mg/mL) were mixed with 100 µL of Folin-Ciocalteu reagent [FCR; diluted with distilled water (9:1)] in basic medium [pH ~10; 300 µL sodium carbonate solution (20%)]. After a 30 min. incubation period at 45 °C, absorbance was read at 765 nm. Gallic acid was used as a standard and a calibration curve was plotted in a concentration range of 50–500 mg/L. All experiments were performed triplicate and total phenolic content was expressed as mg of gallic acid equivalents per g of the extract.

2.6. Antimicrobial Assay

In vitro antibacterial activities of samples were investigated against Gram positive bacteria (*Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228 and

Enterococcus faecalis ATCC 29212); Gram negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352 and *Pseudomonas aeruginosa* ATCC 27853) and also their antifungal activities against *Candida albicans* ATCC 10231. Minimum inhibitory concentrations (MICs) of compounds were determined by microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI) [30,31].

For this purpose, serial two-fold dilutions of extracts ranging from 5000 µg/mL to 9.76 µg/mL were prepared in Mueller-Hinton Broth (MHB) (Difco, Detroid, USA) for bacteria and RPMI-1640 (Sigma) medium for yeast. The inoculums were diluted in broth media to give a final concentration of 5 x 10^5 cfu/mL for bacteria and 0.5×10^3 to 2.5×10^3 cfu/mL for yeast in the test tray. The trays were incubated at 37°C for 18–24 h. The MIC was defined as the lowest concentration of compound giving complete inhibition of visible growth. To verify the standardization of test procedure, Levofloxacin and Fluconazole were used as reference antibiotics for bacteria and yeast, respectively.

3. RESULTS and DISCUSSION

3.1. Antimicrobial Assay

The results of the preliminary qualitative phytochemical analysis are summarized in Table 2. Catechic tannin, saponins and flavonoids are determined in *Acer campestre* L. subsp. *campestre* leaves and twigs.

Table 2. The preliminary qualitative phytochemical analysis results to determine the contents of *Acer campestre* L. subsp. *campestre* leaves and twigs

Secondary metabolites		Plant sample
Alkaloids	Bouchardat Reaction	-
	Dragendorff Reaction	-
	Mayer Reaction	-
Anthracene analogs		-
Flavonoids		+
Saponins		++
Tannins	Gallic tannin	-
	Catechic tannin	++

++ = higher amount of the secondary metabolite; + = presence of the secondary metabolite; – = absence of the secondary metabolite

3.2. Total Moisture (Loss on Drying) and Total Ash Contents

The total moisture and total ash contents of the *Acer campestre* L. subsp. *campestre* leaves and twigs was found 7.82% and 5.31%, respectively.

3.3. Antioxidant Assay and Determination of Total Phenolic Contents

The extracts Ac2 (91.2%), Ac1EtOAc (91.1%) and Ac1 (88.8%) exhibited the higher radical scavenging activity than other samples, while the decoction and infusion had moderate activities. Beside of this, it was found that the syrup and its subextracts had more total phenolic contents than other extracts. The images of the microplates for DPPH method and Folin-Ciocalteu reagent (FCR) method, were shown in Figure 2 and Figure 3, respectively. The results were shown at Table 3.

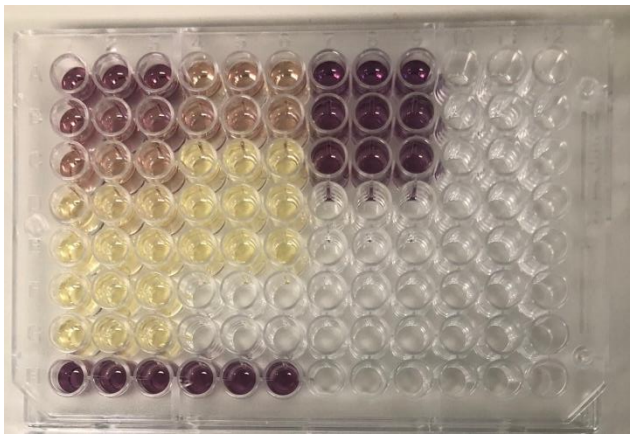

Figure 2. Microplate image in antioxidant assay by DPPH method

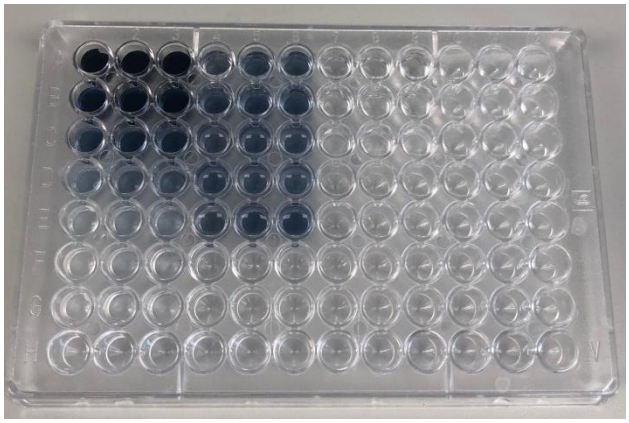

Figure 3. Microplate image for total phenolic contents by Folin-Ciocalteu reagent (FCR) method.

Table 3. The results of DPPH antioxidant activity assays and total phenolic content of the extracts (5mg/mL) *Acer campestre* L. subsp. *campestre* leaves and twigs.

Extracts	DPPH (% scavenging)	Total phenolic content (mg of GAE per g of extract)
Decoction	67.60268	70.19
Infusion	64.39584	78.86
Ac1	88.79661	12.91
Ac1EtOAc	91.08663	6.72
Ac2	91.17296	6.49
Syrup	1.549973	248.7
SETOAc	2.088558	247.25
SBuOH	2.043333	247.37
BHT	88.00	-

These values are the means of three replicates. DPPH: 2,2-diphenyl-1-picrylhydrazyl; GAE: gallic acid equivalents

3.4. Antimicrobial Assay

Among tested samples, all samples except SBuOH had an antibacterial activity against *P. aeruginosa* while only SEtOAc and Ac2 showed an activity against *E. coli*. In addition, all samples except SBuOH and Ac1EtOAc exhibited antifungal activity against *C. albicans*. Especially the extract Ac1 and Ac2 were the most active samples against *C. albicans*. The results of *in vitro* antimicrobial activities of all samples were shown in Table 4.

Table 4. *In vitro* antimicrobial activities of samples

	MIC values (μg/mL)						
Extracts	S.a	S.e	E.f	E.c	K.p	P.a	C.a
Decoction	-	-	-	-	-	1250	625
Infusion	-	-	-	-	-	1250	1250
Ac1	-	-	-	-	-	625	78
Ac1EtOAc	-	-	-	-	-	1250	-
Ac2	-	-	-	1250	-	625	156
SETOAc	-	-	-	1250	-	1250	312.5
SBuOH	-	-	-	-	-	-	-

S.a: *S.aureus* ATCC 29213; S.e: *S.epidermidis* ATCC 12228; E.f: *E.faecalis* 29212; E.c: *E.coli* ATCC 25922; K.p: *K.pneumoniae* ATCC 4352; P.a: *P.aeruginosa* ATCC 27853; C.a: *C.albicans* ATCC 10231, -: no inhibition

Reactive oxygen radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) are known to play a major role in the development of oxidative stress. As a result, diseases such as cardiovascular diseases, inflammation, diabetes, degenerative diseases, cancer, anemia occur. In the literature, the relationship between antioxidant activity and anti-inflammatory activity has been revealed by studies in which plants' antioxidant and anti-inflammatory activities are investigated simultaneously [1-4]. As a result of this study, it was stated that there was a high correlation between these activities, and the plants rich in total phenolic content and flavonoids exhibited anti-inflammatory activity [1]. Oxygenated radicals are known to play a role in inflammation. In another study, antioxidant activities of 20 plants, known with their anti-inflammatory effects, were studied and the relationship between these activities was shown again [2]. There is a research in the literature that there is also a correlation between antioxidant and antimicrobial activities of plants [32]. With all these in mind, the maple syrup, the ethanol extracts of ACC prepared with different extraction method, and their subextracts were tested for their antioxidant and antimicrobial activities and total phenolic contents. All these results were examined comparatively and the samples with anti-inflammatory potential were evaluated. In parallel with the usage in the treatment of inflammatory wounds in traditional medicine and the relationship between antioxidant and anti-inflammatory activities, the expected antioxidant activity from the extracts of the species was determined. In fact, some extracts showed higher activity then the standard BHT, as in previous on some other *Acer* species. In a study on *A. tegmentosum* Maxim., the 70% ethanolic extract of its leaves exhibited more stronger activity than BHT even at the concentration of 0.1 mg/mL [33]. Also, Watanabe and Devkota determined that the antioxidant activity of 70% methanolic extract of *Acer tataricum* subsp. *ginnala* (Maxim.) Wesm. (syn. *A. ginnala* Maxim.var. *aidzuense* Franch.) leaves (EC_{50}: 7.0 μg/mL) had higher activity then the standard Trolox (EC_{50}: 12.2 μg/mL) [34]. Unlike these, the methanol extract elicited equivalent activity (IC_{50}:15 μg/mL) to that of standards (BHA: IC_{50}:14 μg/mL; α-Tocopherol: 12 μg/mL) in a investigation on the biological activities of *A. tataricum* subsp. *ginnala* (Maxim.) Wesm. (syn. *A. ginnala* Maxim.), while the water and hexane subextracts (50.0 μg/mL and 46.3 μg/mL, respectively) were less active than the standards [35]. Also, the alcohol extract of *A. rubrum* (antioxidant capacity = 3325 ± 129) showed higher activity and the alcohol extract of *A. saccharum* (antioxidant capacity = 968 ± 155) exhibited less activity than the commercial standardized French maritime pine bark extract Oligopin® (antioxidant capacity = 1930 ± 101) [36]. In another study, the phenolic contents and antioxidant activities of the methanol extracts prepared from the leaves and branches of *A. campestre* collected in different locations were

evaluated comparatively. Their antioxidant activity were found more than BHT or similar to it (IC50 values of BHT: 27.80 μg/mL; of leaves collected in Guerrouche and Ain El Kebira: 16.67 ± 4.26 μg/mL and 28.33 ± 7.26 μg/mL, respectively) [37]. In this present study, the alcohol extracts showed higher activity than BHT; but the ethyl acetate subextract also exhibited high activity unlike previous studies (DPPH radical scavenging activity (%) at the concentration of 5 mg/mL = Ac1: 88.79661%; Ac1EtOAc: 91.08663%; Ac2: 91.17296%; BHT: 88.00%). It was also noteworthy that despite the high phenolic content, syrup samples showed lower activity compared to alcohol extracts. Alcohol macerates and their subextracts exhibited the highest radical scavenging effect. Beside of this, the syrup and their subextracts were found to have the highest phenolic content. Having parallel results in phenolic content and antioxidant activity tests have become an expected result in the literature. Extracts with high phenolic content also exhibit high antioxidant activity; however, this may not apply to *Acer* species. While the extract of *A. tegmentosum* Maxim. leaves was more active than standard, its total phenolic content was only 116.35±1.4 mg GAE/g [33]. Although alcohol extract of *A. rubrum* was found to be twice times more active than Oligopin®, its total phenolic content was determined 378.6 ± 1.1 mg GAE/g, the total phenolic content of the alcohol extract of *A. saccharum* with less activity was 211.7 ± 9.6 mg GAE/g [36]. In another study, the water extracts of *A. rubrum* bark, exhibited approximately 2-3 times less activity than Oligopin®, had similar total phenolic contents to this standard (extracts: 528.8 and 540.3 mg GAE/g; Oligopin®: 572.9 mg GAE/g); but the *A. saccharum* water extract with the half amount of total phenolic compounds (298.6 mg GAE/g) showed higher effect than standard [38]. In spite of these, a correlation between antioxidant activity and total phenolic content was determined in a study on the *A. campestre* by Atroune et al. [37]. On the other hand, this correlation did not detected in this present study

Considering that the DPPH method gives information about the radical scavenging activity of polar compounds, the fact that the extracts prepared with alcohol show very high antioxidant activity despite their low phenolic content suggests that polar phenolic compounds in these samples may be highly potent antioxidant agents. However, the extracts prepared with water showed less activity than those prepared with alcohol; this means that polar phenolic compounds only in alcohol extracts have higher antioxidant potential. Having high antioxidant activity also brings to mind the high anti-inflammatory potential as mentioned above. However, other antioxidant methods are needed to make a full assessment upon the low antioxidant activity of these samples At the same time, the secondary metabolite group, tannins, saponins and flavonoids, were found to be present in the plant in preliminary qualitative phytochemical analysis. In other studies on *Acer* species, the fact that different tannin compounds are responsible for many different activities [35,39,40]. The presence of such tannin compounds in this species suggested that the tannins content of ACC extracts should be examined in future studies.

Moskalenko investigated 8 *Acer* species [*A. barbinerve* Maxim. ex. Miq., *A. tataricum* subsp. *ginnala* (Maxim.) Wesm. (syn. *A. ginnala* Maxim.), *A. mandshuricum* Maxim., *A. pictum* Thunb. (syn. *A. mono* Maxim.), *A. negundo* L., *A. pseudosieboldianum* (Pax.) Kom., *A. Tegmentosum* Maxim., *A. caudatum* subsp. *ukurundense* (Trautv. & C.A.Mey.) E.Murray (syn. *A. ukurunduense* Trautv. & C.A. Mey)] for their antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Shigella sonnei*, *S. flexneri*, *Bacillus subtilis* and *Mycobacterium smegmatis*. The ethanol (70%) extracts of *A. tataricum* subsp. *ginnala* (syn. *A. ginnala*) had the highest activity against all bacteria among the *Acer* extracts, while the ethanol extract of *A. pictum* (syn. *A. mono*)exhibited no activity [41]. In a study on the antibacterial effect mechanism of *A. truncatum*, the 50% ethanolic extract was found to be effective on *S. aureus*, *S. epidermidis* and *E. coli* (MIC values are 0.5 mg/mL,0.25 mg/mL, 0.5->0.5 mg/mL, respectively), and its ethyl acetate subextract showed an effect against all bacteria (*S. aureus*, *S. epidermidis*, *E. coli* and *Pseudomonas aeruginosa*) with the MIC values of 0.25 mg/mL,

0.125 mg/mL, 0.25 mg/mL and 0.25 mg/mL, respectively [42]. The methanol extract, prepared from the stem of *Acer pentapomicum* Stewart ex Brandis, and its hexane, chloroform, ethyl acetate, butanol and aqeous subextracts were tested for their antibacterial effects by disc diffusion susceptibility assay, and showed their effects dose-dependent. All samples had more or less an activity against *E. coli* and *Klebsiella pneumoniae*. The methanol extract and its ethyl acetate and butanol subextracts were active against *S. aureus* at all concentrations, while chloroform subextract had no effect [43]. In an investigation on the inhibitory effects on bacterial growth of the ethanolic (50%) extracts from leaves of 5 *Acer* species (*A. platanoides, A. campestre, A. rubrum, A. saccharum* and *A. truncatum*), the *A. campestre* extract elicited a good activity against all studied microorganisms. It had an effect against *S. aureus, S. epidermidis, Enterococcus* spp., *E. coli, K. pneumoniae* and *P. aeruginosa* strains at the concentration of 1 mg/mL, 1 mg/mL, 4 mg/mL, 4 mg/mL, 8 mg/mL and 2 mg/mL, respectively [23]. In this present study, an activity occured only against *E. coli* and *P. aeruginosa* among studied bacteria, but the effective concentrations were detected better than in previous study. These antimicrobial test results were obtained as like as the antioxidant test results. Both alcohol extracts showed high antimicrobial activity among other samples, especially against *C. albicans*. The butanol subextract of syrup showed no effect against any microorganism, while the alcohol extract obtained by 48-hour maceration and ethyl acetate subextract of syrup had an antimicrobial effect on three microorganisms (*E. coli*, *P. aeruginosa* and *C. albicans*). It is noteworthy that both alcohol extracts show higher antifungal activity (78 μg/mL and 156 μg/mL) against *C. albicans* than other extracts and also important that the alcohol extract obtained by 24-hour maceration has higher activity than the alcohol extract obtained by 48-hour maceration. In the light of this, it can be concluded that the compounds with lower or no activity could be extracted with the exposure of alcohol for a long time. It appears that the total phenolic content is not a criterion for antimicrobial activity. As a matter of fact, while the MIC value of alcohol extract obtained by 24-hour maceration was the highest, its phenolic content was quite the fewer than other extracts. It was also concluded that most of the compounds that play a role in the activity of this extract were not phenolic compounds.

4. CONCLUSION

The knowledge on *Acer campestre* is quite few because of the limited investigations in literature. Promising results have been obtained in both antioxidant and antimicrobial tests. Similar to previous studies, parallelism was observed in both activity test results. The high antioxidant results suggest a good potential of this species for anti-inflammatory activity. It is interesting that the plant shows higher activity than the syrup content in the market. It is thought that a significant contribution to the use of the plant in treatment of various inflammation disorders, will be made when working in this direction and studying the role of tannins in activities of the plant.

Acknowledgements

The authors declare that, this study was a graduation thesis of Yavuz Selim Bal (Advisor: Burcu Sen-Utsukarci).

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 author(s).

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