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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.

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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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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.

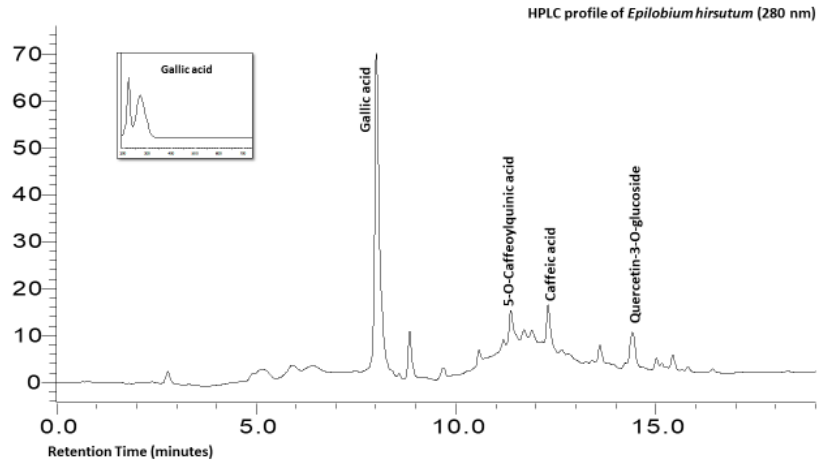

Figure 1. Phenolic composition of *Epilobium hirsutum*

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.

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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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