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In vitro anticoagulant and antiinflammatory activities of *Geastrum fimbriatum* Fr., namely as Earthstar fungus

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Abstract: Mushrooms have great potential to be used as food and pharmaceutical sources. Most of the non-edible mushrooms contain biologically active metabolites that are functional for modern medicinal applications. Within the present study, anticoagulant and antiinflammatory activities of *Geastrum fimbriatum* Fr. (Syn. *Geastrum sessile* (Sowerby) Pouzar), a mushroom naturally grown in Turkey, were investigated. The *in vitro* anticoagulant activity of the ethanolic extract obtained with a soxhlet apparatus determined by activated partial thromboplastin time (APTT) and prothrombin time (PT) assays using commercial reagents. The antiinflammatory activity of the extract was determined by lipoxygenase inhibition assay. When compared with the negative control DMSO, *G. fimbriatum* extract exhibited significant anticoagulant effects in the APTT test that evaluates the intrinsic coagulation pathway. The ethanolic extract found to prolong the coagulation time. However, no inhibition was observed in the PT test which evaluates the extrinsic coagulation pathway, The extract showed 12.92% inhibition on the lipoxygenase enzyme activity. Overall, *G. fimbriatum* ethanolic extract exhibited potent antiinflammatory activity besides being a potential source of anticoagulant. Further analysis is required to evaluate the medical use of *Geastrum* mushrooms from a pharmaceutical point of view.

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1. INTRODUCTION

Health problems in heart and blood vessels and also thrombosis are the major causes of death in the World [1]. It is reported by World Health Organization [2] that an estimated 17.5 million people died from cardiovascular diseases (CVDs) in 2012, representing 31% of all global deaths. It is supposed that 7.4 million of those people died due to coronary heart disease and 6.7 million died because of stroke [2]. CVDs are a group of disorders of the heart and blood

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vessels such as coronary, cerebrovascular and rheumatic heart diseases, deep ven thrombosis and pulmonary embolism [2]. Most require anticoagulant therapy is generally required for thromboembolic processes. Recently researchers are focusing on developing specific and potent anticoagulant and antithrombotic agents [1]. Anti-coagulants that mentioned above present some restrictions to their use, and some are used under a critical control due to hemorrhagic risk and limitation in the administration, however, they have effective use [3]. That's why investigating new, safer, and more effective anti-coagulant drugs, with less hemorrhagic risk and fewer side effects or interaction with drugs and food has been gaining much importance [4].

Inflammation is a complex biological process, induced by microbial infection or tissue damage [5]. There are different types of rheumatic disorders such as rheumatic fever, rheumatoid arthritis, ankylosing spondylitis, polyarthritis nodosa, systemic lupus erythematosus and osteoarthritis, which are called as inflammatory diseases [6]. Inflammation is a body defense reaction which tries to eliminate or limit the spread of injurious agent. Inflammatory reaction involves various components that can contribute to the associated symptoms and tissue injury. These components may be listed as oedema, leukocyte infiltration, and granuloma formation [7].

Arachidonic acid is released from the cell membranes due to the cell damage associated with inflammation [8]. There are two metabolic pathways that arachidonic acid undergoes: the cyclooxygenase (COX) pathway involving cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) to produce the prostaglandins and thromboxanes [9]; and the lipoxygenase (LOX) pathway, involving 5-lipoxygenase (5-LOX), 12-lipoxygenase (12-LOX) and 15-lipoxygenase (15-LOX), to produce the leukotrienes and hydroperoxy fatty acids [10,11].

Lipoxygenases are involved in the biosynthesis of leukotrienes which have a key role in several inflammatory diseases such as cancer, arthritis, asthma and allergic diseases. For this reason, lipoxygenase inhibitors may be potentially used with their medicinal benefits in prevention of these inflammatory cases [12].

Lipoxygenases (LOXs) (LOX; EC 1.13.11.12) are the group of non-heme iron-containing dioxygenases which catalyze the biosynthesis of leukotrienes. Leukotrienes take part as initiators of inflammation so their inhibition is directly related to anti-inflammatory activity [13].

Mushrooms have been used since long times for the treatments of various diseases, but still it has not been approved in main stream science as drugs or medical treatments [14-16]. Both fruiting body and mycelia of different mushroom known to contain different compounds such as flavonoids, alkaloids, polysaccharides, polyglucans, polyphenol, steroids, terpenoids, polyketides and dietary fibers which exert several pharmacological activities [17-18]. *Geastrum* species are mushrooms of the class Gasteromycetes in which the hymenium is enclosed until spores are matured. This genus has cosmopolitan distribution especially in the sandy soil forests of Asia, Africa, Australia, Europe, Mexico, North America and South America [19-21]. Medicinal uses of *Geastrum* species have not been well documented but some members of this class have been reported to have bioactive potentials [22-23]. In the study reported the rare appearance of *Geastrum* species and its ethnomedicinal uses as wound healing by ethnic tribes of Northern Odisha, India [24]. Further, some species of *Geastrum* have also been reported to have antimicrobial activity from southern India [25].

In this study the anticoagulant and antiinflammatory activities of the ethanolic extract of *G. fimbriatum* were investigated. To our knowledge, this is the first study related to the anticoagulant and antiinflammatory activities of this mushroom.

2. MATERIALS AND METHODS

2.1. Mushroom Collection and Extraction Procedure

The mushroom samples were collected from Golcuk village (Gediz-Kutahya), Turkey and identified by Dr. Alli (Figure 1). The samples were brought to the laboratory, dried in an incubator at 40°C. The dried and powdered mushroom samples (20 g) were extracted with ethanol (Merck) (400 ml) using the Soxhlet apparatus at 4 h. The extract was evaporated and then kept in small sterile opac bottles under refrigerated conditions until used [26,27].

Figure 1. *Geastrum fimbriatum* Fr. (Syn. *Geastrum sessile* (Sowerby) Pouzar)

2.2. Anticoagulant Activity

Activated partial thromboplastin time (APTT) and prothrombin time (PT) were performed using commercial human plasma (purchased from Sigma Aldrich). 10 mg/ml concentration of the *G. fimbriatum* extract was prepared with pure dimethyl sulphoxide (DMSO). The APTT and PT were measured via Pacific Hemostasis™ APTT and PT reagents (Thermo Fisher Scientific) according to the manufactures guidelines.

In the APTT test, the calcium chloride (0.02 M) prewarmed at 37°C. 100 µl of commercial plasma (Sigma, P9523) and 10 µl of the *G. fimbriatum* extract (10 mg/ml) transferred to the tube and also prewarmed at 37°C. After then, 100 µl of APTT-XL added to the tube and mixed. The plasma-reagent mixture was incubated at 37°C for 3-5 minutes (activation time). Forcibly added 100 µl prewarmed calcium chloride and coagulation times in the tubes were recorded [28,29].

In the PT test, 100 µl of commercial plasma (Sigma, P9523) and 10 µl of the *G. fimbriatum* extract (10 mg/ml) were transferred to the test tube. The mixture was incubated at 37°C for 1 min and then 200 µl of heated Thromboplastin-DS (at 37°C) added to the tube. Immediately the stopwatch was operated and coagulation times in the tubes were recorded [28,29].

Acetylsalicylic acid (0.5 mM final concentration) and pure DMSO were used as positive control and negative control, respectively.

2.3. Lipoxygenase Inhibition Assay

15-Lipoxygenase (15-LOX) inhibition activity was assayed using the Lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's guidelines. 15-LOX from *soybean* was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Linolenic acid was used as a substrate [30].

Residual 15-LOX activity was determined after pre-incubation for 5 min at room temperature with 100 µg/ml *G. fimbriatum* extract. Firstly, 10 µl of *G. fimbriatum* extract (100 mg/ml), 90 µl of 15-LOX, 10 µl of 0.1 M Tris-HCl buffer (pH 7.4) and 10 µl of 1 mM linolenic acid were added to a plate and incubated for 5 min. Then, the reaction was stopped by adding 100 µl chromogen and the plate was incubated in a shaker for 5 minutes and the absorbance was measured at 550 nm. The 100% initial activity was obtained from the well which incubated with DMSO. Nordihydroguaiaretic acid (NDGA) at 100 µM final concentration was used as a positive control. Data analysis was performed according to the instructions supplied with the assay kit.

3. RESULTS

In this study, the anticoagulant and antiinflammatory activities of the ethanolic extract of *G. fimbriatum* were investigated. To carry out coagulation tests, two standard *in vitro* tests, including PT and APTT tests were applied. These two tests cover both intrinsic and extrinsic blood coagulation pathways. This assay was made with commercial human plasma, using acetylsalicylic acid as a positive control. The anticoagulant activity of the *G. fimbriatum* extract was shown in Table 1.

Table 1. The anticoagulant activity of *G. fimbriatum* extract at 10 mg/ml concentration

Sample	PT (sec)	APTT (sec)
G. fimbriatum extract	22	67
Negative control (DMSO)	24	50
Acetylsalicylic acid	23	71

The *G. fimbriatum* extract exhibited notable anticoagulant effects in the APTT test that was applied to evaluate the intrinsic coagulation pathway, compared with the negative control DMSO. The extract prolonged the time of coagulation. On the other hand, in the PT test that was applied to evaluate the extrinsic coagulation pathway, no inhibition was observed with the extract.

The antiinflammatory activity of the *G. fimbriatum* extract was determined with the lipoxygenase inhibition capacity. The extract showed 12.92% inhibition on the LOX enzyme activity (Table 2).

Table 2. The LOX inhibition activity of *G. fimbriatum* extract

Sample	Concentration	Inhibition (%)
G. fimbriatum extract	100 µg/ml	12.92
NDGA	100 µM	40.00

4. DISCUSSION

Enzyme promoting processes are involved in the blood coagulation [31]. At the end of these processes; fibrin is formed from fibrinogen and blood is transformed to gel state from collosol state. An enzyme, the thrombase, effects the hemostasis and blood coagulation [32]. In order to explain the possible hemostatic mechanism, the compounds were tested for PT and APTT assays. PT is mainly applied to evaluate the extrinsic coagulation pathway while APTT is related to the intrinsic coagulation pathway [33]. When APPT prolongs, it suggests the inhibition of the intrinsic and/or the common pathway. On the other hand, PT prolongation gives information about the inhibition of the extrinsic and/or the common pathway [34].

Anticoagulants play a role in the prevention and treatment of thromboembolic disorders [35-36]. Anticoagulant drugs consisting of heparin and its derivatives, and vitamin K antagonists, have been the main anticoagulants in clinical practice. Despite their efficacy, major and life-threatening side effects of these agents have also been reported [37,38].

Anticoagulant drugs become crucial when there is a failure throughout the hemostasis; which leads to blood coagulation that could cause a vessel occlusion; unfractionated heparin (UFH), coumarin, low molecular weight heparin (LMWH), and the most recent drug introduced in the market - the synthetic pentasaccharide fondaparinux are the current medical options [3].

The use of anticoagulants is under a critical control as there are some restrictions for their use and also it needs to pay attention due to hemorrhagic risk and limitation in the administration [3]. Nowadays researchers focus on developing new, safer, and more effective anti-coagulant drugs, with less hemorrhagic risk and fewer side effects or interaction with drugs and food [4].

Most pharmaceutical drugs have side effects that may be experienced alongside their therapeutic actions [39]; for example, warfarin, a commonly used anticoagulant drug, demonstrates side effects that include bruising, bleeding gums, red or dark brown urine, red or black bowel motions, nosebleeds, haemoptysis, dyspnoea and dysphagia, heavier than usual menstrual periods, excessive wound bleeding, dark or blood stained vomit, severe headache and dizziness, unexplained pain, swelling and discomfort [40].

However, traditional remedies have long been known to have fewer adverse effects [41]. Therefore, the authors claim that the use of herbs with therapeutic effects and minimal side effects, could be the best option for the prevention and treatment of a wide range of diseases including cardiovascular disorders [42].

LOXs are the group of enzymes involve in the biosynthesis of leukotrienes that play an important role in the pathophysiology of various inflammatory diseases. The LOXs are classified with respect to their positional specificity of arachidonic acid oxygenation (5-LOX, 9-LOX, 12-LOX, 15-LOX)[43].

LOX enzymes are directly related to inflammatory and allergic reactions due to the formation of the leukotrienes (LTs). LTs values may increase in the case of allergic rhinitis, rheumatoid arthritis, psoriasis, asthma and colitis ulcerosa. Inhibition of the lipoxygenase pathway prevents the production of LTs. As a useful tool for cancer treatment, therapeutic strategies for inhibition of L\isoforms and/or their biologically active metabolites may be biologically and pharmacologically targeted by using lipoxygenase inhibitors [44].

Medicinal mushrooms are recognized with their extracts that profound health benefits and functionally used for traditional therapies. There are extensive studies that demonstrating the *in vitro* or *in vivo* biological activities of extracted and/or purified mushroom species such as antitumor and immunomodulating activity [45,46], anticancer [47,48], antioxidant [49], antiviral [50], antihypercholesterolemia [51], antidiabetic [52] effects.

There are drugs in the market that are used to treat antiinflammatory disorders but most of them are not toxically free. The use of antiinflammatory drugs may lead to gastrointestinal problems which is a problem for medical industry [53]. Thus, investigation of a natural antiinflammatory drug with functional properties is a necessity for pharmaceutical technology. Similar to our study, Guerradore et al. [54] reported that *Geastrum saccatum* extract inhibited the enzyme cyclooxygenase and had a promising antiinflammatory activity. *Geastrum* species have also antioxidant [54,55], antibacterial [56], and cytotoxic [54] activities. Although there are not any studies about *G. fimbriatum*, a nature muschroom growing in Mugla. The present study presents an important view about the anticoagulant and antiinflammatory activities of *G. fimbriatum* that potentially active.

5. CONCLUSION

Studies with mushrooms have been developing recently and it is figured out that potent properties of secondary metabolites from different mushroom species show great biological activites. Besides being an important dietary source, mushrooms are strong pharmaceuticals that reveals multiple therapies to diseases. There is a growing interest in active metabolites that are obtained from natural sources as an alternative to synthetic drugs. Anticoagulant and antiinflammatory characteristics of an agent enhance its functionality. In the present research, the potential use of mushroom extract as an anticoagulant and/or antiinflammatory agent has been evaluated. From the results of the study, it can be concluded that *G.* ***fimbriatum*** extract is endowed with effective anticoagulant and antiinflammatory activities. Comprehensive studies may be required to investigate the health-related activites of this mushroom.

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Effects of Nitrate toxicity on free Proline accumulation, chlorophyll degradation and photosynthetic efficiency in the green alga *Chlorella vulgaris*

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Abstract: The effects of high nitrate concentrations on alterations in maximal photochemical efficiency of PSII ratio (Fv/Fm), chlorophyll content, chlorophyll degradation, growth rate and proline accumulation in *Chlorella vulgaris* Beyerinck [Beijerinck] were investigated in this study. The Fv/Fm ratio was observed in response to the increased nitrate concentration. The Fv/Fm ratio decreased in *C. vulgaris* following 44 h in 12.99 mM $NaNO_3$-enriched media. Experimental results showed that, growth rates and chlorophyll content declined by increasing nitrate concentration. The decrease in the ratio of chlorophyll a/b with enrichment of high nitrate concentration (6.5 mM and 12.99 mM $NaNO_3$) was also caused by a decrease in chlorophyll a and an increase in chlorophyll b concentration in *C. vulgaris* cultures. The results showed that 6.5 and 12.99 mM nitrate concentration increased proline content in treated cells, which suggests that nitrate stress lead to proline accumulation in *C. vulgaris.*

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1. INTRODUCTION

Nitrate occurs naturally in mineral sources and animal wastes, and anthropogenically as a by-product of agriculture and human wastes [1]. Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) are the most common ionic (reactive) forms of dissolved inorganic nitrogen in aquatic ecosystems. $NaNO_3$ is commonly used as a nitrogen source in algae culture mediums. Nutrient concentrations play an important role in the growth of phytoplankton. The nitrogen sources that are considerably important for phytoplankton growth are nitrate and ammonium [2, 3]. It is a constituent element of amino acids and thus of protein, and nucleic acids (DNA and RNA). It typically makes up around 4% of the dry weight of plant matter, and around 3% of the human weight. It is one of the biggest component of animal waste, usually in the form of urea, uric acid, ammonium compounds and derivatives of these nitrogenous products, which are essential

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nutrients for all plants that are unable to fix atmospheric nitrogen. Much higher nitrate concentrations have been found in aquatic ecosystems that were strongly contaminated by agricultural and urban activities [4,5]. Enrichment of nitrogen and phosporus in aquatic enviroment can lead blooming of algae. Previous studies have also reported that nitrate could affect photosynthesis, growth and can cause cellular toxicity of phytoplankton [6, 7, 8].

Under different stress conditions, cells also tend to accumulate low intracellular molecular weight compounds in plants. Proline is such a compound that accumulates in higher plants and algae as a response to osmotic stress such as drought, high levels of salinity and osmolarity. In addition, air pollution causes an increase in the intracellular proline levels of a variety of higher plants [9]. Different roles have also been proposed for proline accumulation as an adaptive response; it has been suggested that proline may function as an osmoticum [10], a sink of energy and reducing power [11], a nitrogen-storage compound [12], a hydroxyl-radical scavenger [13] and a compatible solute that protects enzymes [14].

In higher plants, plant growth generally decreases when nitrogen supply exceeding 10 mM, a value considered on the threshold of toxicity for some species [15, 16]. Previous studies demonstrated that excessive nitrogen fertilization causes osmotic stress, in which reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) are produced [17]. ROS are highly toxic and can cause serious damages on lipid, protein and nucleic acid metabolisms and then inhibit plant and algae growth [18]. Although, there are some findings about high nitrate concentration lead to inhibition of phytoplankton growth, effects of high nitrate stress on chlorophyll content, chlorophyll degradation and free proline accumulation is still unexplored. In order to gain some insights on toxicity of nitrate in green algae, this study examined the effects of nitrate stress on growth rate, photosynthesis, and chlorophyll degradation. In addition, the objective of the present work is to determine the relationship between the nitrate toxicity and proline accumulation.

2. METHOD

2.1. Cultures and materials

Chlorella vulgaris was obtained from EGE-MACC culture collection, Ege University, Izmir, Turkey. Five flasks of 100 ml *C. vulgaris* were used for the experiment. *C. vulgaris* culture was grown photoautotrophiclly in Rudic Medium (RD) [19] at 31°C in under 12:12 dark:light illumination. Illumination provided by daylight fluorescence tubes at 20 μmol photons $m^{-2}s^{-1}$. Air was supplied to the culture. Continuous aeration was provided by bubbling air using a blower. Microalgae cells were harvested by centrifugation and transferred to a fresh medium, grown under the same conditions for a day for adaptation. Then the concentrations of 3.1 mM, 6.5 mM and 12.99 mM $NaNO_3$ were added to the nitrate stress groups, respectively. The cultures were sampled at 18^{th}, 24^{th} and 44^{th} hours. All the experiments were repeated three times.

2.2. Cell density

Absorbance at 663 nm was determined with UV-Vis spectrophotometer for all groups. Relative cell density demonstrated at different absorbances in Table 1. Specific growth rates (μ) were calculated using the equation $\mu=\ln(X_t/X_0)/t$, where X_0 is the initial cell density, X_t is the cell density after t hours.

2.3. Measurement of photosynthetic efficiency

Assay of photosystem II (PSII) activity was performed by fast chlorophyll fluorescence according to Strasser et al. [20], using Handy-Pea from Hansatech (King's Lynn, England). Five drops of cell suspension were pipetted on a piece of filter paper fixed in the clips of the

Handy-Pea and incubated in darkness for 15 min. Cells were kept moist and control showed the normal induction curves (Kautsky-effect) and values of the Fv/Fm ration of approximately 0.78. Each experiment was performed by five replicates.

2.4. Determination of pigment content

About 20 mg cells were extracted in 2 ml 90% acetone. Completely homogenized cells were subjected to quick centrifugation and supernatant was transferred to fresh tube. The absorbance of supernatant was taken at 664 and 647 nm in UV–Vis spectrophotometer. Chlorophyll is a light sensitive pigment hence light was avoided and all steps were carried out in dark. Each experiment was performed five times. Chlorophyll content was calculated using following formula [21]:

$$\text{Chla } (\mu g/ml): 11.93A_{664} - 1.93A_{647}$$

$$\text{Chlb } (\mu g/ml): 20.36A_{647} - 5.50A_{664}$$

2.5. Determination of Proline content

Proline content of the pellet was measured following the method of Bates et al. [22] with some modification of the extraction procedure according to Bačkor et al. [23]. Algae cells were permeabilized by 2 ml of DMSO at 40ºC for 1 h. Cell extracts were vortexed with glass beads 1 mm diameter for 30 s, and 2 ml of 3% sulfosalicylic acid was then added to each tube. After 10 min., extracts were separated from cell debris by centrifugation at 400 x g for 20 min. Two milliters of supernatant containing proline were pipette and subsequently treated with acid-ninhydrin at 90ºC for 1 h. The reaction was then terminated in an ice bath and the colored complex extracted in toluene. Absorbance was recorded at 520 nm. The standard curve for proline was prepared by dissolving proline in 3% sulfosalicylic acid to cover the concentration range 0.1-20 µg ml^{-1}. Each experiment was performed three times.

2.6. Statistical analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) or Student's t-test followed by *posthoc* Tukey test as appropriate (SPSS for Windows version 11.0)**.**

3. RESULTS

The effects of different nitrate concentrations on growth were shown in Table 1. Specific growth rates in 3.1 and 6.5 mM nitrate-enriched media for 24 h were approx. 1.7 and 1.2-fold that in 12.99 mM nitrate-enriched group, respectively. In addition, the specific growth rates and maximum cell densities of algae at 6.5 and 12.99 mM nitrate-enriched showed a significantly decrease ($p< 0.05$) in comparison with control groups after 44 h. The toxicity of nitrate at 12.99 mM was also evidently greater than at 6.5 mM (Table 1).

Photoinhibition measured as a permanent reduction in maximal PSII efficiency (Fv/Fm), increased with increasing level of nitrate in nutrient medium and treatment duration (Figure 1). The results of Fv/Fm measurements (Table 2) indicated a progressive photoinhibition by the values closed to 29.73% for 24 h in 6.5 mM nitrate-enriched media. After 44 h, a significantly decrease Fv/Fm (62.16 %) was measured in samples treatment with 12.99 mM nitrate ($p< 0.05$).

Table 1. Maximum cell densities and specific growth rates of the alga Chlorella vulgaris cultivated with a nutritional level of nitrate (control) and nitrate supplement of 3.1, 6.5, and 12.99 mM.

Duration (hours)	**Nitrate concentrations (mM)**	**Specific growth rates**	**Maximum cell densities (x10^5 cell mL^{-1})**
18	3.1	0.42	2.523±*112*
	6.5	0.32	2.132±*110*
	12.99	0.303	**1.931±*52***
24	3.1	0.41	2.706±*127*
	6.5	**0.3**	**1.918±*63***
	12.99	**0.25**	**1.219±*56***
44	3.1	0.39	2.534±*123*
	6.5	**0.28**	**1.472±64**
	12.99	**0.23**	**0.973**±68

Table 2. The Fv/Fm ratio of the alga Chlorella vulgaris cultivated with a nutritional level of nitrate (control) and nitrate supplement of 3.1, 6.5, and 12.99 mM.

Groups		Fv/Fm			Photoinhibition (%)		
	n	18 h X±SD	24 h X±SD	44 h X±SD	18 h X±SD	24 h X±SD	44 h X±SD
Control	5	0.74±0.03	0.73±0.16	0.74±0.14	1.34±0.05	2.67±0.026	1.34±0.02
3.1 mM $NaNO_3$	5	0.72±0.22	0.72±0.04	0.73±0.03	2.71±0.03	2.7±0.34	1.36±0.04
6.5 mM $NaNO_3$	5	0.61±0.32	0.6±0.18	**0.52±0.08^a**	**17.57±1.2^a**	**18.92±1.4^a**	**29.73±1.10^a**
12.99 mM $NaNO_3$	5	**0.56±0.03^a**	**0.45±0.12^a**	**0.28±0.23^b**	**24.33±0.07^a**	**39.19±0.012^{ab}**	**62.16±1.03^{ab}**

On nutritional *C. vulgaris* media and 3.1 mM nitrate supplemented media, the pigment levels were not significantly different (Table 3), but they did differ between groups in the presence of different nitrate concentration. There are significant ($p<0.05$) differences determined on Chl*a*, Chl*b* and Chl*a/b* levels between control group and nitrate treated groups, with the only exception of the 3.1 mM nitrate treated group (Table 3 and 4). It was observed that chlorophyll a content, and chlorophyll a/b ratio of the 6.5 mM and 12.99 mM nitrate-enriched media treated *C. vulgaris* for 44 h were reduced by 43.7%, 68.65% and 55.37%, 82.51% respectively.

Table 3. Chlorophyll a (Chla) (mg g^{-1} dw cell mass) and Chlorophyll b (Chlb) (mg g^{-1} dw cell mass) contents of the alga Chlorella vulgaris cultivated with a nutritional level of nitrate (control) and nitrate supplement of 3.1, 6.5, and 12.99 mM.

Groups		Chl*a*			Chlb		
	n	*18 h X±SD*	24 h X±SD	44 h X±SD	*18 h X±SD*	24 h X±SD	44 h X±SD
Control	*5*	*6.02±0.05*	*5.68±0.21*	*6.14±0.03*	*1.86±0.23*	*1.84±0.4*	*2,02±0.12*
3.1 mM $NaNO_3$	*5*	5.92±0.42	5.19±0.29	5.62±0.02	1.98±0.01	1.82±0.17	**1.69±0.19^a**
6.5 mM $NaNO_3$	*5*	**4.18±0.04^a**	**3.88±0.09^a**	**3.457±0.03^b**	**2.14±0.05^a**	**2.73±0.05^b**	**3.582±0.07^a**
12.99 mM $NaNO_3$	*5*	**3.01±0.2^b**	**3.20±0.04^a**	**2.74±0.24^a**	**3.09±0.1^a**	**3.61±0.06^b**	**4.95±0.04^b**

Table 4. Chlorophyll a /Chlorophyll b (Chla/b) ratio of the alga Chlorella vulgaris cultivated with a nutritional level of nitrate (control) and nitrate supplement of 3.1, 6.5, and 12.99 mM.

Chl*a/b*	*n*	18 h X±SD	24 h X±SD	44 h X±SD
Control	*5*	*3.23±0.05*	3,08±*0.14*	*3.03±0.04*
03.1 mM $NaNO_3$	*5*	2.97±0.24	2,83±0.34	3.32±0.23
6.5 mM $NaNO_3$	*5*	**1.95±0.12[a]**	**1.42±0.01[a]**	**0.95±0.01[a]**
12.99 mM $NaNO_3$	*5*	**0.97±0.06[a]**	**0.88±0.02[a]**	**0.53±0.13[a]**

Nitrogen-induced proline accumulation in the *C. vulgaris* cells are presented in Figure 1. The proline content of *C. vulgaris* arisen by increasing level of nitrate in nutrient medium and treatment duration. Proline accumulation in groups of enhanced media with 6.5 mM and 12.99 mM nitrate for 44 h were approx. 8 and 11 times greater than control group, respectively.

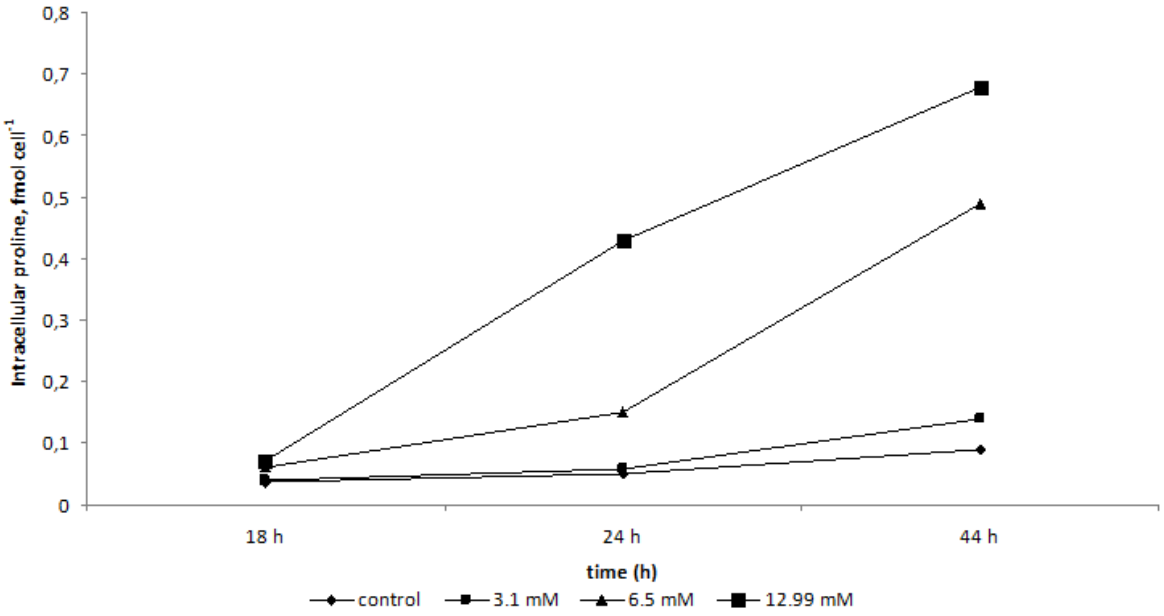

Figure 1. Proline accumulation levels according to nitrate concentrations at different time periods.

The results showed a positive correlation between proline accumulation and photoinhibition (Figure 2) also with chlorophyll degradation and growth inhibition (Figure 3).

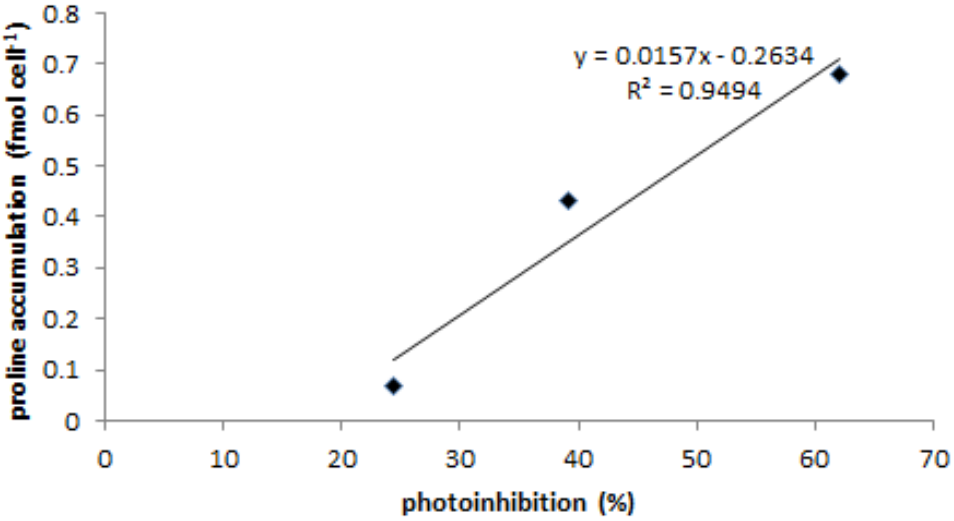

Figure 2. Relationship between proline accumulation and photoinhibition (%).

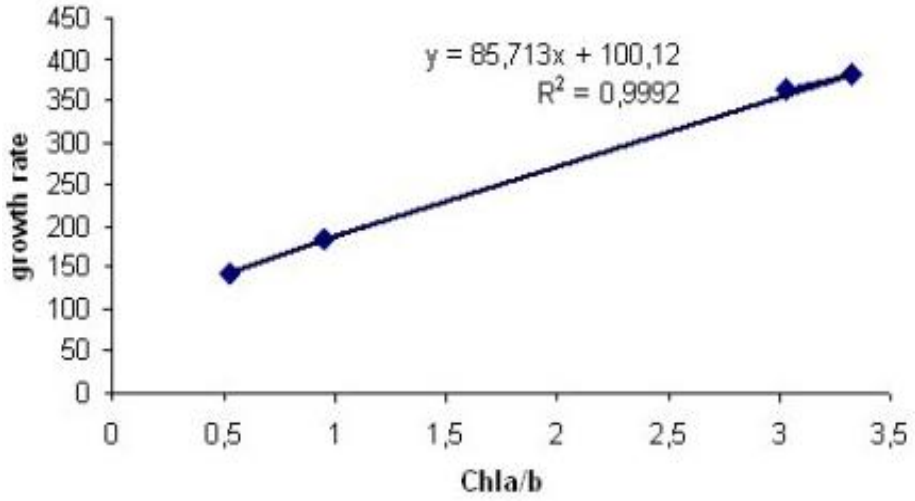

Figure 3. Relationship between chlorophyll degradation and growth rate of *Chlorella vulgaris*.

4. DISCUSSION

Nitrate is an important macronutrient and can serve as a good nitrogen source for phytoplankton. Menéndez [8] reported that moderate levels of fertilizing nitrate could increase the maximum rate of net photosynthesis in green alga *Chaetomorpha linum*. Touzet et al. [24] also found that the dinoflagellate *Alexandrium minutum* showed higher maximum cell densities with increasing nitrate concentrations. Similarly, Leong et al. [25] showed a positive correlation between the growth rates of *Alexandrium tamarense* and nitrate concentrations. However, Shi et al. [26] observed that there was a negative effect of excess nitrate on the growth of *A. tamarense*, and Hwang and Lu [27] also reported *A. minutum* exposed to excess nitrate had a low growth rate. These findings suggested that, although nitrate was a good nitrogen source, excess nitrate supply is harmful to phytoplankton and could depress growth, which is in agreement with the results of our present study.

Nitrate is converted to ammonium in two successive steps catalysed by nitrate reductase (NR) and nitrite reductase (NiR), and when nitrite formed by NR, the accumulation of

intracellular nitrite of alga will appear. Chen et al. [28] demonstrated that *Microcystis aeruginosa* grown under high nitrate concentrations showed a significant increase in NR activities, which is consistent with the results of Sivasankar and Oaks [29] and Crawford [30], where the activities of NR increased with nitrate raise. Chen et al. [28] observed a significant increase in intracellular nitrite content under high nitrate concentrations in *M. aeruginosa.* In general, excessive nitrate can be stored as non-toxic form of nitrogen in cells [31], but nitrite is an inorganic monovalent anion which affects the process of photosynthesis significantly. It is known that the inhibition of photosynthetic electron transport can [32, 33], change intracellular pH and damage cell membranes of algae [34, 35]. Therefore, it is possible that the increase of intracellular nitrite resulted in the decrease of growth and chlorophyll degradation at high nitrate concentrations [28]. In the present work, significant variations of the maximal potential quantum yield of PSII complex (Fv/Fm) were obtained in exposure to high dosages of nitrate in *C. vulgaris.* In particular fluorescence parameter results, a good indicator of physiological stress caused by heavy load of nitrate. *C. vulgaris* cells were cultivated in 6.5 mM and 12.99 mM nitrate-enriched media for 44 h, exhibited distinct photoinhibition at the end of the experiments. Photoinhibition rates close to 62% were recorded in 12.99 mM nitrate-enriched media after 44 h of exposure.

Chlorophyll is very sensitive to stress-initiated oxidative processes such as photo-oxidation [36-38]. Chlorophyll intermediate molecules are also potential chloroplast signals that could regulate photosynthetic gene expression, growth rates, and cell-death processes [39]. Our present results showed that decreasing growth rates are closely correlated with decreasing chlorophyll a/b ratio following to supplemental different nitrate concentration (Figure 2). Chlorophyll b is formed from chlorophyll a by the oxidation of the methyl group on ring II to the aldehyde [37] and the ratio of chlorophyll a/b is more sensitive to modification than chlorophyll a+b. In green plants, antenna size is determined by the amount of light-harvesting chlorophyll a/b protein complex that is associated with the photosystems [4]. Conversion of chlorophyll b to chlorophyll a not only impacts the chlorophyll a/b ratio but also is the first step of chlorophyll degradation. Our present results confirmed that supplemental nitrate causes a significantly increase in chlorophyll b and a concomitant decrease in chlorophyll a, consistent with accelerated conversion of one to the other (Table 4).

In response to environmental stress in plants, proline accumulation normally occurs in cytosol where it contributes substantially to the cytoplasmic osmotic adjustment [40-42]. Binzel et al. [41] showed that the glutamate pathway for proline biosynthesis is predominant under stress conditions such as high salinity and nitrogen limitation. In algae and higher plants, intracellular proline has been also regarded as an osmoprotectant that accumulates in relation to the level of intracellular potassium ions [9]. Wu et al. [43] showed that the enhanced intracellular proline accumulation during the exposing to heavy metals related to a mechanism of protection against osmotic change.

Despite the presence of a strong correlation between stress tolerance and accumulation of proline in higher plants, this relationship may not be universal. For example, Lutts et al. [44] demonstrated that the accumulation of proline in rice leaf may be a symptom of salt injury rather than an indication of salt tolerance. De-Lacerda et al. [45] assessed the proline accumulation and distribution during shoot and leaf development in two sorghum genotypes. As a result they suggested that proline accumulation was a reaction to salt stress, not a plant response associated with tolerance. Similarly, Sánchez et al. [46] also found that the metabolic response of proline under nitrogen toxicity is reflected primarily in seeds of *Phaseolus vulgaris*. In the present work, proline accumulation in 12.99 mM nitrate-enriched media was significantly

higher than the 3.1 mM and 6.5 mM nitrate-enriched media for 24 h and 44 h. The present observations showed a positive relationship between nitrogen toxicity and proline accumulation.

In conclusion, the present work demonstrates that high nitrate stress decreases the growth rate and maximal photosystem II efficiency (Fv/Fm) in *C.* while chlorophyll degradation and proline accumulation increased according to treatment duration. Photoinhibition (%) data demonstrated that there is a possitive correlation between nitrogen toxicity and proline accumulation (Figure 2). In addition, our chlorophyll degradation and growth inhibition results are supporting this hypothesis (Table 1 and 4, Figure 3). These results define, proline accumulation as a bioindicator of nitrogen toxicity in the cells of *C. vulgaris.*

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Essential oil composition and antibacterial activities of *Gypsophila* species

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Abstract: Essential oil composition of *Gypsophila turcica* Hamzaoğlu, *Gypsophila pinifolia* Boiss. & Hausskn., *G. tuberculosa* Hub.-Mor., *G. eriocalyx* Boiss. and *G. laricina* Schreb. were analyzed by means of gas chromatography-mass spectrometry (GC-MS). Thirty six, fourty four, sixty six, forty one and sixty one compounds were identified in the essential oils of *G. turcica*, *G. pinifolia*, *G. laricina*, *G. tuberculosa* and *G. eriocalyx* respectively. The major components were determined hentriacontane (12.93 ± 0.4%), 1-octadecanol (8.97 ± 0.1%), hexahydrofarnesyl acetone (6.9 ± 0.09%) and pentacosane (6.63 ± 0.08%) in *G. turcica* oil, hexadecanoic acid (17.6 ± 0.4%), 1-tetradecanol (7.6 ± 0.1%) and phytol (5.63 ± 0.05%) in *G. pinifolia* oil, octacosane (6.83%), eicosanal (6.19%), triacontane (6.03%) and heneicosane (5.78%) for *G. eriocalyx*, hexadecanoic acid (25.3%, 27.0%) and hentriacontane (13.0%, 12.6%) for *G. tuberculosa* and *G. laricina*, respectively. Antibacterial activity of *G. eriocalyx*, *G. tuberculosis* and *G. laricina* were investigated against Gram negative (*Escherichia coli*) and Gram positive (*Staphylococcus aureus*) bacteria.

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1. INTRODUCTION

Gypsophila is the 3th biggest genus in family of Caryophyllaceae to Turkey. *Gypsophila* species are annual, biennial or perennial herbaceous plants. This genus are distributed mainly in Mediterranean and Iran-Turan areas in Turkey. *Gypsophila* has 56 species in 10 sections and 33 species are endemic to Turkey [1]. By this way, it has made a significant contribution to the biodiversity of Turkey [2]. *Gypsophila turcica* is perennial plant and it was described as a new species in 2012 [3].

Gypsophila species are rich source of triterpene saponin especially in root parts [4,5]. Triterpene saponin from this genus are used commercially as medicines, detergent, adjuvants and cosmetics [5,6]. Root and barks of the genus used as analgesic, sedative, antipyretic,

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antiinflammatory, emetic and insecticidal in Turkey [7]. Biological activities of the genus seem to be associated with triterpene saponin. Due to the various beneficial biological activities, *Gypsophila* was the focus of studies that described the phytochemistry of the genus extensively.

According to study from Iran, antimicrobial activity and chemical constituents of the essential oils from flower, leaf and stem of *Gypsophila bicolor* were investigated. The main components of the essential oil from flower were germacrene-D (21.2 %), *p*-cymene (20.6 %), bicyclogermacrene (17.6 %), γ-dodecadienolactone (13.7%) and terpinolene (9.4 %). The main components of the essential oil from leaves were germacrene-D (23.4 %), terpinolene (14.5 %), bicyclogermacrene (7.5 %), γ-dodecadienolactone (6.8 %), *p*-cymene (6.7 %) and *cis*-β-ocimene (6.3 %). The main components of the essential oil from stems were γ-dodecadienolactone (28.5 %), bicyclogermacrene (14.8 %), germacrene-D (12.6 %), *p*-cymene (12.5 %), terpinolene (11.6 %) and *trans*-β-ocimene (4.2 %). The essential oils had moderate effect on Gram-positive and Gram negative bacteria, but had significant effect on the fungi [8].

As summarized above *Gypsophila* species have very high medicinal and commercial importance and also contains interesting natural substances. However, according to our literature survey we have not encountered any reports on the essential oil composition of *Gypsophila* species from Turkey. Additionaly, there is no report on antibacterial activity of essential oils of *G. eriocalyx*, *G. laricina* and *G. tuberculosa*. This prompted us to investigate the essential oil composition and antibacterial activity of *Gypsophila* genus. To the best of our knowledge this is the first report on the essential oil composition and antibacterial activity of *Gypsophila* genus.

2. MATERIALS AND METHODS

2.1. Plant Materials

Plant materials were collected during the flowering period; *G. pinifolia* on 17.07.2016 from Aşağı Ulupınar town between Darende and Malatya (1300 m), *G. turcica* on 17.07.2016 from Jipsli Hills Zara-Baglama village in Sivas (1760 m), *G. laricina* on 17.07.2017 from Ucpinar, Sarkisla in Sivas (1740-1800 m), *G. tuberculosa* on 16.07.2015 from Aşağı Ulupınar town between Darende and Malatya (1480 m) and *G. eriocalyx* on 20.07.2015 from Jipsli Hills Soğuk Çermik way in Sivas (1440 m) in Turkey by Çelik and Budak. Voucher specimens have been deposited in the Herbarium of Bozok University (Voucher no: Bozok HB 3310 and Bozok HB 3309 for *G. pinifolia* and *G. turcica* respectively), Turkey.

2.2. Isolation of the Essential Oils

Aerial parts of the air dried plants subjected to hydrodistillation for 3 h, using a Clevenger-type apparatus to produce essential oils. Condenser of the Clevenger was attached to a microchiller that set to 4°C. Essential oil yields obtained from *G. pinifolia*, *G. turcica*, *G. laricina*, *G. eriocalyx* and *G. tuberculosa* 0.03;0.01;0.01;0.01;0.03% (v/w), respectively. The oils were recovered with 1 mL *n*-hexane and preserved in amber vials under -20°C until the day they were analyzed.

2.3. Gas Chromatography/Mass Spectrometry Analysis

The GC-MS analysis was performed with an Agilent 5975C Inert XL EI/CI MSD system operating in EI mode. Essential oil of *G. pinifolia* and *G. turcica* were diluted 1/65 and 1/100 (v/v) with *n*-hexane, respectively. Injector and MS transfer line temperatures were set at 250˚C. Innowax FSC column (60 m (x) 0.25 mm, 0.25 µm film thickness) and helium as carrier gas (1 mL/min) were used in both GC/MS analyses. Splitless injection was employed. Oven temperature was programmed to 60˚C for 10 min. and raised to 220˚C at rate of 4˚C/min. Temperature kept constant at 220˚C for 10 min. and then raised to 240˚C at a rate of 1˚C/min. Mass spectra were recorded at 70 eV with the mass range *m*/*z* 35 to 425.

2.4. Gas Chromatography Analysis

The GC analyses were done with an Agilent 6890N GC system. FID detector temperature was set to 300ºC and same operational conditions applied to a duplicate of the same column used in GC-MS analyses. Simultaneous auto injection was done to obtain the same retention times. Relative percentage amounts of the separated compounds were calculated from integration of the peaks in FID chromatograms. Identification of essential oil components were carried out by comparison of their relative retention indices (RRI) obtained by series of *n*-alkanes (C5 to C30) to the literature and with mass spectra comparison [11-27]. Mass spectra comparison was done by computer matching with commercial Wiley 8th Ed./NIST 05 Mass Spectra library, Adams Essential Oil Mass Spectral Library and Pallisade 600K Complete Mass Spectra Library. The analysis was carried out in triplicate and the results were given as the mean ± standard deviation.

2.5. Antibacterial Assay

Antibacterial activities of the essential oils were tested against two strains; Gram positive *Staphylococcus aureus* (ATCC 25923) and Gram negative *Escherichia coli* (ATCC 25922). For the antimicrobial tests, Luria-Bertani broth was used as a growth medium for bacteria.

In order to evaluate antibacterial activity, minimum inhibition concentration (MIC_{50}) values were determined by using broth dilution method. DMSO was used in stock solutions to enhance solubility of the essential oils. Serial dilutions of the stock solutions were prepared on a 96 well plate. After incubation at 37°C for 24 h, bacterial suspension concentrations were standardized to McFarland No: 0.5. Essential oils and bacterial cultures were mixed in the range of 1000-1,95 µg/mL as final concentration. It was paid attention to not exceed 1% final concentration for DMSO. After treatment, the bacteria were incubated at 37°C for 24 h. As negative control, essential oil-free solutions were used. Each test was repeated for three times. Growth analysis was done by using spectrophotometric measurements for MIC determination. Minimum inhibitory concentrations (MIC_{50}) were detected as the minimum concentration at which at least 50% of bacterial growth was missing.

3. RESULTS

Essential oil composition of *Gypsophila turcica*, *G. pinifolia*, *G. tuberculosa*, *G. eriocalyx* and *G. laricina*. were analyzed by means of gas chromatography-mass spectrometry (GC-MS). In order, thirty six, fourty four, sixty six, forty one and sixty one compounds were identified in the essential oils of *G. turcica*, *G. pinifolia*, *G. laricina*, *G. tuberculosa* and *G. eriocalyx* that represent 69.1%, 71.7%, 78.1%, 71.7% and 85.6% of the oil, respectively. The major components were determined hentriacontane (12.93%), 1-octadecanol (8.97%), hexahydrofarnesyl acetone (6.9%) and pentacosane (6.63%) in *G. turcica* oil, hexadecanoic acid (17.6%), 1-tetradecanol (7.6%) and phytol (5.63%) in *G. pinifolia* oil, octacosane (6.83%), eicosanal (6.19%), triacontane (6.03%) and heneicosane (5.78%) for *G. eriocalyx*, hexadecanoic acid (25.3%, 27.0%) and hentriacontane (13.0%, 12.6%) for *G. tuberculosa* and *G. laricina*, respectively. The essential oil composition of five *Gypsophila* species are given in Table 1.

Antibacterial of the oils were evaluated for one Gram (+) and one Gram (-) bacteria by using a broth microdilution assay. *G. eriocalyx* essential oil showed mild activity on *S. aureus* (250 µg/mL) but the oil showed very low activity against *E. coli* (1000 µg/mL). However, *G. tuberculosa* and *G. laricina* essential oils did not show any significant activity against tested grains. The results of antibacterial activity of *Gypsophila* species are given in Table 2.

The essential oil of *G. pinifolia*, *G. tuberculosa* and *G. laricina* had hexadecanoic acid in high amount unlike *G. turcica* and *G. eriocalyx*. Essential oils of *G. turcica*, *G. tuberculosa* and

G. laricina were rich in hentriacontane. But hentriacontane contained at low amount in *G. eriocalyx* and not detected in *G. pinifolia*.

Table 1. The essential oil composition of five *Gypsophila* species

				G.laricina	*G.tuberculosa*	*G. turcica*	*G.eriocalyx*	*G. pinifolia*	
No	**RRI[1]**	**RRI Lit.[2]**	**Compound**	**Mean (%)[3]**	**Mean (%)**	**Mean (%)**	**Mean (%)**	**Mean (%)**	**Id. Met.[4]**
1	1200	1200	Dodecane	-	-	-	-	0.6	RI, MS, Ac
2	1233	1244	2-pentyl furan	0.27	-	-	0.08	-	RI, MS
3	1300	1300	Tridecane	-	-	-	0.09	-	RI, MS, Ac
4	1398	1399	Nonanal	0.29	0.33	-	0.32	0.33	RI, MS
5	1401	1400	Tetradecane	0.16	0.26	0.4	0.35	0.8	RI, MS, Ac
6	1442	1443	Dimethyl tetradecane	0.06	-	-	-	-	RI, MS
7	1498	1466	α-cubebene	-	-	-	-	0.1	RI, MS
8	1499	1505	Dihydroedulan II	0.15	-	-	-	-	RI, MS
9	1502	1500	Pentadecane	0.15	0.26	0.6	0.1	0.2	RI, MS, Ac
10	1505	1506	Decanal	0.47	1.05	0.5	1.79	2.53	RI, MS
11	1510	1516	Theaspirane A	0.70	-	0.5	0.04	0.1	RI, MS
12	1525	1532	Camphor	0.04	-	-	-	-	RI, MS
13	1529	1535	Dihydroedulan I	0.14	-	-	-	-	RI, MS
14	1536	1541	Benzaldehyde	-	-	-	0.07	-	RI, MS
15	1543	1548	(*E*)-2-nonenal	0.12	-	-	-	-	RI, MS
16	1549	1553	Theaspirane B	0.64	-	0.4	0.18	-	RI, MS
17	1550	1553	β-Linalool	-	0.34	-	-	2.03	RI, MS
18	1558	1549	1-Tetradecene	0.08	-	-	0.07	-	RI, MS
19	1580	1562	Longifolene	-	-	-	0.09	-	RI, MS
20	1602	1600	Hexadecane	0.29	0.34	0.77	0.45	0.80	RI, MS, Ac
21	1603	1605	2-undecanone	-	-	0.03	0.06	-	RI, MS
22	1608	1612	β-caryophyllene	-	-	-	0.13	0.1	RI, MS
23	1612	1617	Undecanal	-	-	-	-	2.57	RI, MS
24	1612	1613	β-cedrene	-	-	-	0.13	-	RI, MS
25	1633	1638	β-cyclocitral	0.13	-	-	0.06	0.17	RI, MS
26	1635	1644	Thujopsene	0.04	-	-	0.1	-	RI, MS
27	1649	1654	1-Hexadecene	-	-	0.3	-	-	RI, MS
28	1653	1655	(*E*)-2-Decanal	0.25	-	-	0.45	0.3	RI, MS
29	1660	1664	Nonanol	0.1	-	-	-	-	RI, MS
30	1672	1671	(*E*)-β-Farnesene	-	-	-	0.13	-	RI, MS
31	1683	1687	α-Humulene	-	-	-	0.07	-	RI, MS
32	1693	1685	6,10-dimethyl-2-undecanone	0.1	-	-	-	-	RI, MS
33	1701	1700	Heptadecane	0.28	0.33	0.50	0.39	-	RI, MS, Ac
34	1703	1706	α-terpineol	-	-	-	-	0.80	RI, MS
35	1718	1722	Dodecanal	0.29	0.28	-	0.67	0.53	RI, MS
36	1735	1742	β-Selinene	-	0.23	-	-	-	RI, MS
37	1761	1763	Naphthalene	0.32	-	-	-	-	RI, MS
38	1764	1766	Decanol	-	-	-	0.23	0.23	RI, MS
39	1775	1779	(*E,Z*)-2,4-Decadienal	0.13	0.13	-	0.11	-	RI, MS

Table 1. Continues

40	1785	1786	Ar-curcumene	-	-	-	0.03	-	RI, MS
41	1802	1800	Octadecane	0.21	0.29	0.3	0.51	0.3	RI, MS, Ac
42	1804	1820	Isogeraniol	-	-	-	-	0.3	RI, MS
43	1815	1815	2-tridecanone	-	-	0.1	-	-	RI, MS
44	1823	1823	(*E*)-α-Damascenone	0.2	-	-	-	0.6	RI, MS
45	1824	1827	(*E*,*E*)-2,4-Decadienal	0.4	0.56	-	0.13	-	RI, MS
46	1826	1830	Tridecanal	-	-	-	0.51	2.3	RI, MS
47	1830	1838	(*E*)-β-Damascenone	0.36	0.18	-	-	1.63	RI, MS
48	1850	1857	Geraniol	-	-	-	-	1.23	RI, MS
49	1863	1868	(*E*)-Geranyl acetone	1.12	1.17	1.03	0.6	1.43	RI, MS
50	1879	1871	Undecanol	0.17	-	-	-	-	RI, MS
51	1886	1864	*p*-cymene-8-ol	0.08	-	-	-	-	RI, MS
52	1901	1900	Nonadecane	-	0.73	-	1.3	-	RI, MS, Ac
53	1931	1933	Tetradecanal	0.38	-	-	0.96	0.5	RI, MS
54	1953	1958	(*E*)-β-Ionone	1.03	0.52	0.5	0.73	0.6	RI, MS
55	1969	1973	1-Dodecanol	0.63	0.88	-	-	0.6	RI, MS
56	2003	2000	Eicosane	0.29	0.74	0.3	1.13	0.23	RI, MS, Ac
57	2005	2007	Caryophyllene oxide	0.29	-	-	-	-	RI, MS
58	2028	2036	2-pentadecanone	-	0.41	0.3	-	-	RI, MS
59	2037	2036	Pentadecanal	0.26	-	-	-	-	RI, MS
60	2039	2036	Hexadecanal	-	-	-	-	2.00	RI, MS
61	2043	2050	(*E*)-Nerolidol	0.05	-	-	-	-	RI, MS
62	2051	2056	13-Tetradecanolide	0.35	-	-	-	-	RI, MS
63	2104	2100	Heneicosane	-	0.55	0.5	5.78	0.27	RI, MS, Ac
64	2135	2131	Hexahydro farnesyl acetone	1.65	1.9	6.9	4.44	2.73	RI, MS
65	2145	2136	Hexadecanal	0.3	-	-	1.01	-	RI, MS
66	2145	2148	(*Z*)-3-hexeneyl benzoate	-	-	-	-	1.36	RI, MS
67	2170	2192	Nonanoic acid	0.5	-	-	-	-	RI, MS
68	2173	2179	Tetradecanol	-	-	0.7	0.68	7.6	RI, MS
69	2184	2186	Eugenol	-	-	-	-	0.1	RI, MS
70	2190	2144	Spathulenol	0.05	-	-	-	1.1	RI, MS
71	2190	2198	1-Docosene	-	2.21	-	-	-	RI, MS
72	2202	2200	Docosane	-	0.6	0.4	1.6	-	RI, MS, Ac
73	2225	2226	Hexadecanoic acid methyl ester	-	-	-	-	0.2	RI, MS
74	2240	2242	2-Heptadecanone	-	0.19	-	0.12	-	RI, MS
75	2278	2282	Decanoic acid	1.03	1.56	-	-	1.37	RI, MS
76	2290	2296	Isophytol	-	-	0.4	-	-	RI, MS
77	2302	2300	Tricosane	0.55	0.81	2.2	4.5	-	RI, MS, Ac
78	2318	2315	2,4-bis-tert-butylphenol	0.35	-	2.23	-	-	RI, MS
79	2338	2345	Galaxolide I	-	-	-	0.13	-	RI, MS
80	2345	2353	Galaxolide II	-	-	-	0.09	-	RI, MS
81	2355	2353	Octadecanal	0.28	-	0.9	1.71	-	RI, MS
82	2381	2384	1-Hexadecanol	-	-	-	0.4	-	RI, MS

Table 1. Continues

83	2384	2381	Farnesyl acetone	1.41	1.5	1.87	0.8	2.1	RI, MS
84	2402	2400	Tetracosane	0.31	0.63	0.6	0.5	-	RI, MS, Ac
85	2448	2471	Nonadecanal	0.2	-	-	-	-	RI, MS
86	2489	2492	Dodecanoic acid	3.51	7.55	-	0.17	1.9	RI, MS
87	2504	2500	Pentacosane	1.4	3.06	6.63	2.32	-	RI, MS, Ac
88	2555	2592	Diisobutyl phthalate	2.15	4.23	4.48	1.42	2.91	RI, MS
89	2585	2582	Eicosanal	2.07	-	-	6.19	-	RI, MS
90	2589	2607	1-octadecanol	-	0.88	8.97	0.63	-	RI, MS
91	2590	2617	Tridecanoic acid	0.23	0.37	-	-	-	RI, MS
92	2606	2600	Hexacosane	0.31	0.58	-	0.32	-	RI, MS, Ac
93	2618	2622	Phytol	1.76	1.1	2.7	2.59	5.63	RI, MS
94	2671	2676	Heneicosanal	1.97	-	-	-	-	RI, MS
95	2701	2704	Tetradecanoic acid	4.7	6.53	-	0.26	1.33	RI, MS
96	2706	2700	Heptacosane	0.7	1.97	1.3	3.40	1.27	RI, MS, Ac
97	2775	2783	1-Docosanol	0.31	-	0.8	-	-	RI, MS
98	2796	2794	1-Eicosanol	-	-	3.63	-	-	RI, MS
99	2795	2800	Octacosane	0.25	-	-	6.83	-	RI, MS
100	2806	2822	Pentadecanoic acid	1.4	1.69	-	-	0.60	RI, MS
101	2838	2857	Palmito-γ-lactone	0.21	0.41	-	0.25	-	RI, MS
102	2904	2900	Nonacosane	-	-	1.37	1.65	-	RI, MS, Ac
103	2918	2931	Hexadecanoic acid	27.03	25.3	-	4.64	17.6	RI, MS
104	2984	2990	Docosanal	0.22	-	0.73	-	-	RI, MS
105	3003	3000	Triacontane	-	-	2.4	6.03	-	RI, MS, Ac
106	3098	3100	Hentriacontane	12.63	13.0	12.93	1.20	-	RI, MS, Ac
			Total	**78.1**	**85.6**	**69.1**	**71.7**	**71.7**	

[1]RRI: Relative retention time indices calculated against *n*-alkanes (C5-C30).
[2]RRI Lit.: Relative retention time given in the literature for the compound in similar columns and analysis conditions.
[3]The results of the analysis.
[4]Identification method: RI: identification based on the relative retention times (RRI) of genuine compounds on the HP Innowax column and the literature data; MS: identification based on MS comparison with the database or the literature data, Ac: Identification is done according to RRI and MS values of the authentic compounds.

Table 2. Antibacterial activity [MIC_{50} (µg/mL)] of the essential oils of *G. eriocalyx*, *G. tuberculosa* and *G. laricina*

Strain	*G. eriocalyx* (µg/mL)	*G. tuberculosa* (µg/mL)	*G. laricina* (µg/mL)
E. coli	1000	>1000	>1000
S. aureus	250	>1000	>1000

4. DISCUSSION and CONCLUSION

Only mild antibacterial activity is observed on *G. eriocalyx* essential oil against *S. aureus*. The main compounds of essential oil of *G. eriocalyx* contained low amount or not detected in other *Gypsophila* species. Eicosanal is one of the main compound of *G. eriocalyx*. Antibacterial activity could be correlated with this compound. According to a study from Iran, *Gypsophila bicolor* was reported to contain germacrene-D, *p*-cymene, bicyclogermacrene, γ-dodecadienolactone, terpinolene, *cis*-β-ocimene and *trans*-β-ocimene [8] however these compounds were not detected in the *G. turcica*, *G. pinifolia*, *G. eriocalyx*, *G. tuberculosa* and

G. laricina. These differences in the previous literature and present data could be related to different collection times, climatic and soil conditions, ecological factors, methods and instruments employed in analysis or different genotypes. There are very few reports on the essential oil of *Gypsophila* species therefore it is difficult to produce a comment on the chemo-systematic position of this species according to current findings and the existing reports. We believe the results obtained from this research will stimulate further research on the chemistry of *Gypsophila* species.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Determination of anticancer effects of *Urospermum picroides* against human cancer cell lines

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Abstract: Plants continue to be a good source for developing effective anticancer agents. In this study, *in vitro* various biological effects of crude ethanolic extract from flowering parts of *Urospermum picroides* collected from the Muğla province of Turkey were investigated for the first time. Daudi, A549 and HeLa cancer cell lines and BEAS-2B normal cell line were used to identify the cytotoxic effect of the extract using MTT assay. The effect of the extract on cell cycle progression was detected by flow cytometric analysis. The level of VEGF, IL-1α, IL-6 and TNF-α secretion in the cells treated with the extract were measured using ELISA The extract caused a higher cytotoxic effect on Daudi cells with an IC_{50} value of 85.64 µg/mL than the other cells tested. The IC_{50} values in HeLa and A549 cells were determined to be 135.35 and 234.8 µg/ mL, respectively. The selective cytotoxicity was considered between Daudi and BEAS-2B (109.80 µg/mL) cell lines. In addition, the effect of the extract on cell cycle progression changes according to cell line used. Moreover, the extract decreased the level of secreted VEGF in treated A549 cells by 31%. In addition, the extract resulted in a significant decrease in the secretion of IL-1α, IL-6 and TNF-α cytokines in A549 and Daudi cells compared to the untreated cells. These findings suggest that the flowering parts of *U. picroides* may be a potential source for anticancer agents.

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1. INTRODUCTION

Cancer is an important health problem around the globe and significantly contributes to human deaths. The annual number of new cancer cases are increasing estimated to be 21.6 million by 2030 [1].

Because the drugs used for the treatment of cancer can cause serious side effects [2], it is important to develop new-selective anticancer agents. Plants have been used for treating various diseases throughout the ages and they continue to be a critical source of potent anticancer agents

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due to their contents [3]. Some of the plant derived compounds such as vincristine, vinblastine and paclitaxel are still used as chemotherapeutic agents in clinical cancer treatment [4]. Therefore, the anticancer potentials of the plants are being investigated all over the world to obtain new agents.

Urospermum picroides (L.) SCOP. ex. F. W. Schmidt is a species of the Asteraceae family [5] and also known as prickly golden fleece [6]. It belongs to the traditional Mediterranean diet and has anti-inflammatory activity [7]. The consumption of this plant was reported to reduce postprandial platelet aggregation in metabolic syndrome patients [8]. Previous studies demonstrated the antioxidant and antimicrobial activity [9,10] of this species as well as its phytochemical content [11,12] with potential biological properties. However, to our knowledge, there is no detailed study in the literature about the different biological activities of *U. picroides*. So, for the first time we aimed to determine the various biological effects of ethanolic extract from flowering parts of *U. picroides* on different cancer cell lines.

2. MATERIALS AND METHODS

2.1. Collection and extraction of plant material

U. picroides were harvested from Muğla province, Turkey, in the between June and July 2014. The plant species was identified by Dr. Fatma Güneş at the Department of Pharmaceutical Botany, Trakya University, Edirne. The voucher specimen was kept in their herbarium.

The fresh flowering parts of plant were firstly washed with the deionized water and air dried at room temperature under shade for about 15 days. The dried flowers were powdered and each 10 grams powder were extracted with 100 mL absolute ethanol (Merck, USA) using soxhlet apparatus for 10 h. The extract was then filtered through Whatman filter paper no: 1 and solvent was separated from the extract using a rotary evaporator (IKA, RV 10, USA). The ethanolic crude extract of *U. picroides* was stored at -20^{o}C in polyethylene tubes protected from light-until needed.

2.2. Cell culture conditions

A549 (lung adenocarcinoma), HeLa (cervix adenocarcinoma), Daudi (Burkitt's lymphoma) and BEAS-2b (normal bronchial epithelium) human cell lines were obtained from ATCC. The cell lines were grown in RPMI 1640 (Biochrom, Germany) medium with stable L-glutamine (Biochrom, Germany) contained 10% heat inactivated fetal bovine serum (FBS) (Biochrom, Germany) supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin (Biochrom, Germany). All cell cultures were incubated at 37^{o}C in a humidified atmosphere with 5% CO_2 and 95% air.

2.3. Cytotoxic assay

MTT [3 (4, 5 Dimethyl-2-thiazolyl)-2, 5 diphenyl-2H-tetrazolium bromide)] (Applichem, USA) assay [13] was used for determination of cytotoxicity of the extract on cell lines used in present study. The assay is based on the reduction of yellow tetrazolium salt MTT to purple formazan crystal by mitochondrial dehydrogenase in the viable cells [14]. Briefly, the exponentially growing cells were seeded at a density of $4x10^3$ cells per well in 180 µL of growth medium into 96-well plates (Greiner, Germany) as triplicate for each tested extract concentration. After 24 h of incubation, the cells were treated with the extract at different final concentrations (15.625 to 1000 µg/mL) for 72 h. The stock solution of the extract was prepared in 10% DMSO (Applichem, USA) in growth medium and diluted with growth medium to obtain serial dilutions. So, the cells were exposed to DMSO at less than 0.1% of the final concentration. The untreated cells were used as a control. Later, the medium was removed and 100 µL of fresh growth medium was added into each well. Then, the cells in each well were incubated with 10 µL of 5 mg/mL MTT in phosphate-buffered saline (PBS) for 4 h at 37^{o}C. At the end of 4 h, the

medium with MTT was gently discarded from the wells and formazan crystals formed in the cells were solubilized with 100 µL of DMSO by shaking at 150 rpm for 5 min. The absorbance (Abs) measurement of reduced MTT in each well was made at 540 nm using a microplate reader (ThermoScientific, Multiscan FC, USA). The following formula was used to calculate the percentage of cell viability for each concentration.

Cell viability %= (Mean Abs of treated cells/Mean Abs of untreated cells) x 100

2.4. Cell cycle analysis

Cell cycle detection in A549 and HeLa cells was performed by propidium iodide (PI) (Sigma-Aldrich, USA) staining using flow cytometry. Firstly, the cells were seeded at $5x10^5$ cells/well in 6-well plates and incubated for 24 h. Then, the cells were exposed to 500 and 200 µg/mL extract for 24 h. DMSO at 1% final concentration was used as a control. After treatment, cells were harvested by trypsinization, washed with cold PBS twice, fixed gently in absolute ethanol and stored at -20°C for 48 h. After centrifugation at 1200 rpm for 10 min at 4°C, cell pellets were washed with cold PBS and resuspended in 1 mL 0.1% (v/v) Triton X-100 (Amresco, USA) in PBS. Then, cell suspensions were treated with 100 µL of RNase A (200 µg/mL) (Applichem, USA) and incubated at 37°C for 30 min. Finally, 100 µL of PI (1 mg/mL) was added to each cell suspensions and cells were incubated for additional 15 min at room temperature in the dark. After staining, cells were analysed for cell cycle phases by BD FACSCanto flow cytometry (BD Biosciences, San Jose, CA) using ModFit LT 3.0 software.

2.5. Enzyme-linked immune sorbent assay (ELISA)

The supernatants of the cell cultures were used for quantification of Vascular endothelial growth factor (VEGF) in A549 and for quantification of cytokines (IL-1α, IL-6 and TNF-α) in both A549 and Daudi by using commercial human ELISA kits (Boster Biological Technology, USA). To obtain supernatants, cells were seeded in a 6 well-plate at a density of $2x10^5$ cells/well. After 1 h incubation at 37°C in CO_2 incubator, cells were treated with extract at 200 µg/mL for 6 h. The untreated cells were used as a control. Later, supernatants were collected from treated and untreated cells and centrifuged at 14000 rpm for 30 seconds. The obtained supernatants were aliquoted and stored at -20°C until use. A 100 µL of supernatant was analysed for VEGF or inflammatory each cytokine production via ELISA kits according to the manufacturer instructions. The absorbance in each well was read at a wavelength of 450 nm using a microplate reader. The level of secreted VEGF or each cytokine was interpolated from the standard curve prepared separately for each assay.

2.6. Statistical analysis

The results obtained from in this study were expressed as mean ± standard error (SE). Statistical analysis and data processing were performed by using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

3. RESULTS

3.1. Cytotoxic effect of the extract on different cell lines

The effect of the extract on cell viability was investigated on A549, HeLa, Daudi and BEAS-2B cell lines for 72 h by MTT assay. The extract was prepared at seven serial concentrations from 1000 to 15.625 µg/mL and tested against the cell lines used. The IC_{50} values (µg/mL) that causes 50% cell death were then calculated for each cell lines. As shown in Figure 1, cell viability, particularly in cancer cell lines was significantly inhibited by the extract in a concentration-dependent manner. However, the results showed that the extract at 15.625-62.5 µg/mL concentrations were less cytotoxic on normal BEAS-2B cells when compared to cancer cells tested. The extract exhibited the higher cytotoxicity against Daudi

cells with an IC_{50} value of 85. 64 µg/mL when compared to normal BEAS-2B cells (IC_{50} value= 109.8 µg/mL) and other cancer cells. The calculated IC_{50} values were 234.8 and 135.35 µg/mL for A549 and HeLa, respectively. So, Daudi cell lines were found to be the most sensitive line to the extract.

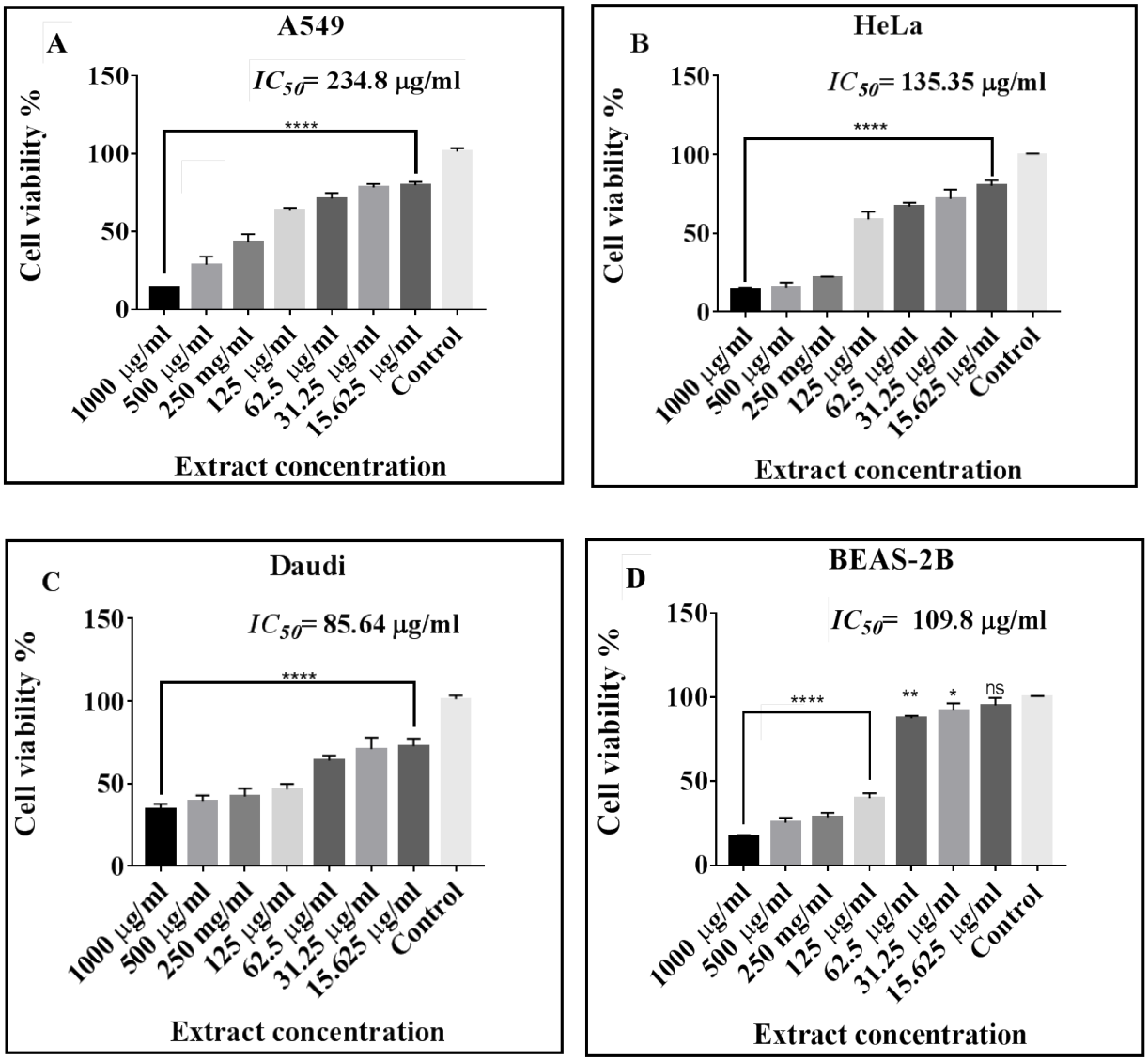
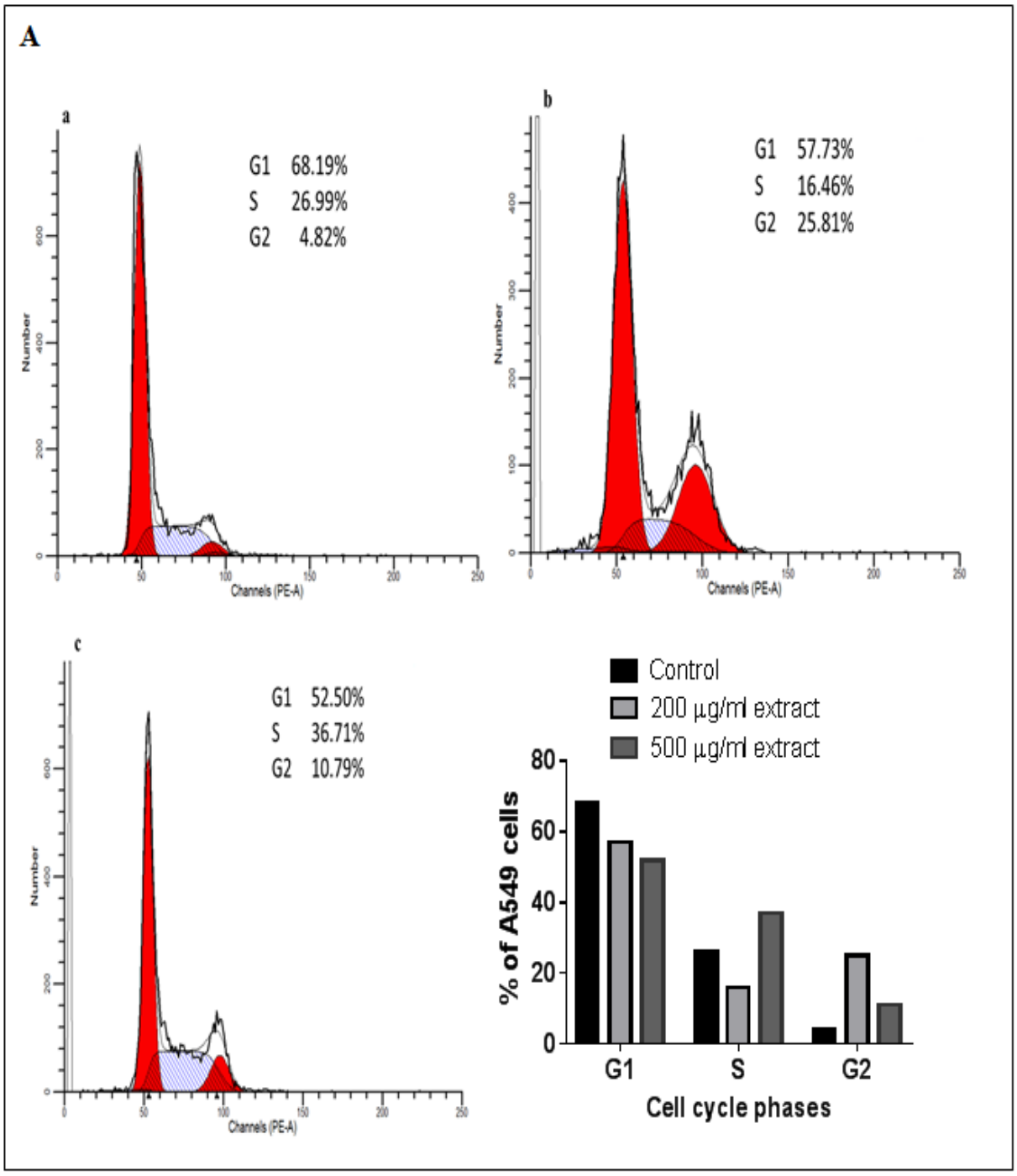


Figure 1. Cytotoxic effects of ethanol extract of flowering parts of *U. picroides* against A549 (A), HeLa (B), Daudi (C) and BEAS-2B (D) cell lines after an exposure time of 72 h. Cell viability was determined using MTT assay. The data represent mean ± SE of three independent experiments for each concentration. ****P< 0.0001, **P< 0.01, *P< 0.05 and ns: non-significant (P> 0.05) compared to control (ANOVA and Tukey's multiple comparison test).

3.2. Effect of the extract on cell cycle progression in A549 and HeLa cell lines

The flow cytometry analysis was performed to investigate cell cycle progression in A549 and HeLa cancer cells after treatment with extracts at 500 and 200 µg/mL for 24 h (Figure 2). The treatment with 500 µg/mL extract showed 5.97% and 9.72% increases of A549 cells in G2 and S phase compared to control, respectively and there was a concomitant decrease in the percentage of A549 cells in the G1 phase. The percentage of A549 cells in G2 phase after treatment with extract at 200 µg/mL significantly increased from 4.82 to 25.81 and it was accompanied by a decrease in the percentage of cells in G1 and S phase. In addition, the percentage of HeLa cells in G1 phase increased from 48.97 to 59.00 after treatment with the

extract at 500 µg/mL and there was a sharp decrease in the percentage of G2 and S phase. On the other hand, the treatment of HeLa cells with 200 µg/mL extract resulted in 4.19% and 5.06% increase of cells in S and G2 phase, respectively. These data demonstrated that the plant extract affect the cell cycle progression in the different phases according to the type of cell line tested.



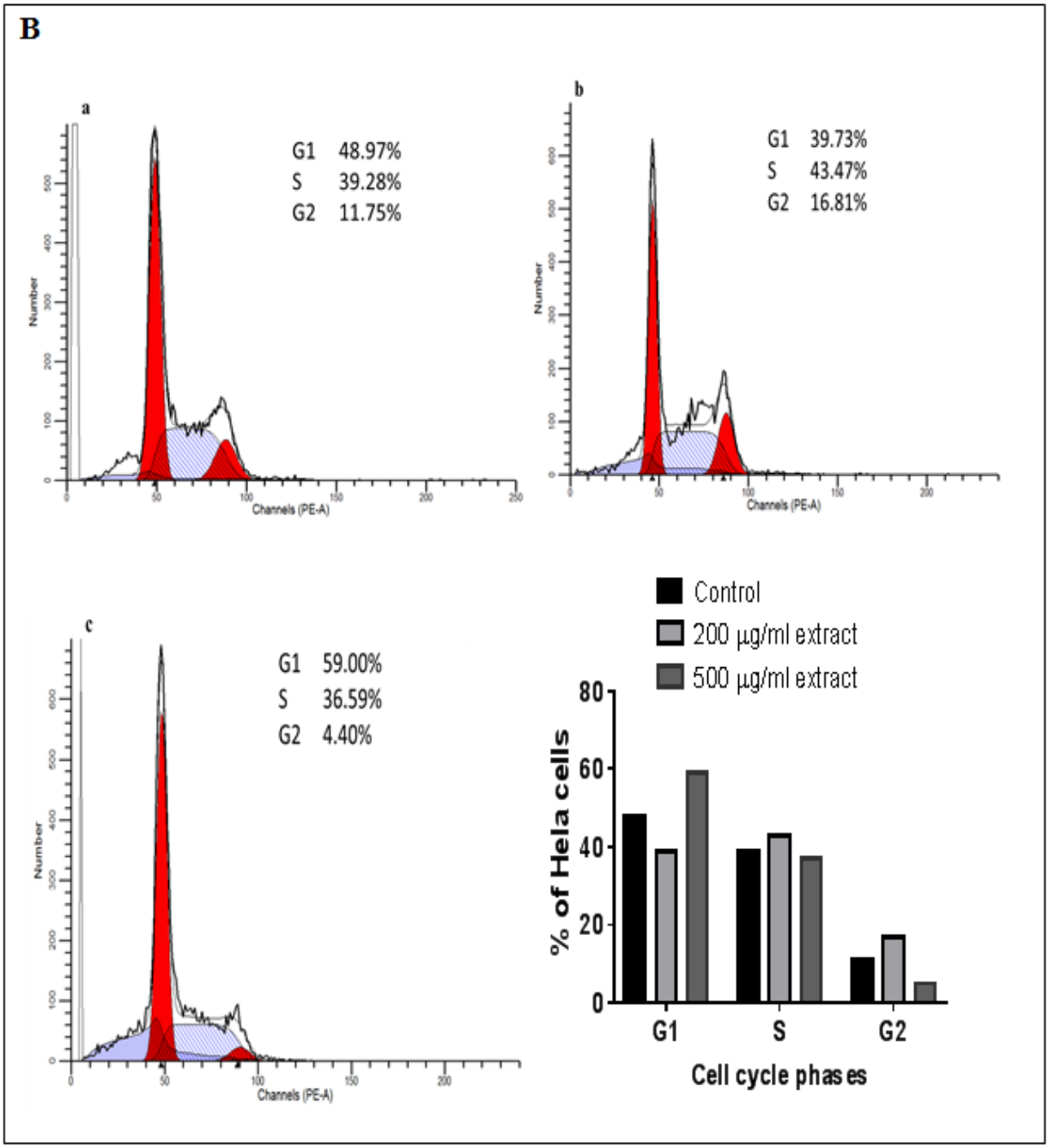


Figure 2. Effects of the extract on cell cycle distribution in A549 and HeLa cells. A549 (**A**) and HeLa (**B**) cells treated with 1% DMSO as control (a), 200 (b) and 500 (c) µg/mL extract for 24 h. Bar graph with percentages of cells at different cell cycle phases are shown (d).

3.3 Effect of the extract on VEGF secretion

It was reported that A549 cells released VEGF [15]. In this study, we investigated the VEGF levels in supernatants of A549 cell culture using human VEGF ELISA assay. Our findings showed that VEGF secretion was reduced by 31% in A549 cells treated with 200 µg/mL extract compared to untreated cells (Figure 3). This result indicate that the extract has antiangiogenic potential against A549 cells because the VEGF is an angiogenic factor [16].

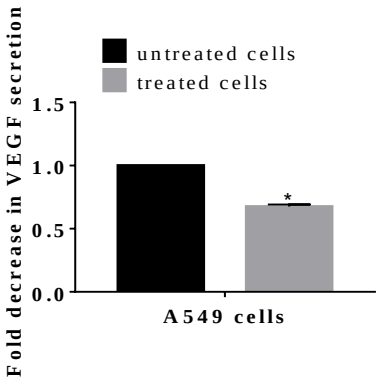


Figure 3. Effects of the extract on VEGF secretion of A549 cells. The VEGF concentration in supernatants of A549 cells treated with the extract at 200 µg/mL for 6 h was determined by ELISA. The values represent the mean of three independent experiments ± SE. The concentration of VEGF in control (untreated) cells is taken as 1-fold. The asterisks indicate statistical significance when compared to untreated cells. *P< 0.05

3.4 Effect of the extract on secretion of IL-1α, IL-6 and TNF-α cytokines

In this study, we also investigated the effect of the plant extract at 200 µg/mL on IL-1α, IL-6 and TNF-α secretion in A549 and Daudi cells by using ELISA. The results were graphically shown in Figure 4. The extract did not cause any increase in concentrations of IL-1α, IL-6 and TNF-α cytokines in treated cells compared to untreated cells. The highest inhibition effect of the extract was found in A549 for IL-6 secretion (12%) and Daudi for IL-1α secretion (15%). As a result, the plant extract does not induce secretion of cytokine that can promote cancer development and progression.

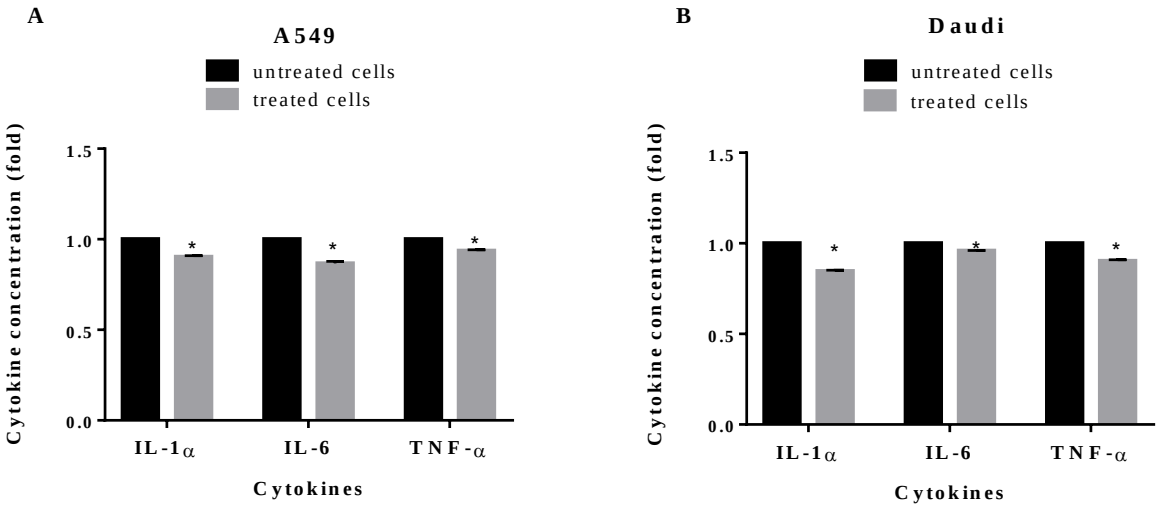


Figure 4. Effects of the extract on IL-1α, IL-6 and TNF-α secretion of A549 (A) and Daudi (B) cells. The cytokines concentration in supernatants of the cancer cells treated with the extract at 200 µg/mL for 6 h was determined by ELISA. The values represent the mean of three independent experiments ± SE. Results presented as fold of change in relation to the control (untreated) cells. The asterisks indicate statistical significance when compared to untreated cells. *P< 0.05.

4. DISCUSSION and CONCLUSION

Cancer is one of the most important diseases that can be fatal worldwide [2]. Because cancer cells can develop drug resistance in traditional therapies [17], the discovery of a new drug with anti-cancer mechanism is of great importance. For centuries, natural products especially plant-derived compounds have an important role in the development of drugs used in the treatment of cancer [18].

It was reported that *U. picroides* was commonly used in the traditional Mediterranean diet [7] and sold in the Dalmatia markets [6]. Interestingly, a significant information was not found that it was a common edible plant in the world.

There are various research studies on the chemical composition of *U. picroides* in the literature [11,12,19,20]. Also, the anti-inflammatory [7], antioxidant and antimicrobial activity [9,10] of *U. picroides* were reported. In addition, Fragpouli et al. [8] stated that the consumption of *U. picroides* meals significantly reduced the *ex vivo* platelet activating factor-induced platelet aggregation postprandially. However, to our knowledge, the cytotoxic and anti-inflammatory and antiangiogenic activity of ethanolic extract of flowering parts of *U. picroides* collected from Turkey were investigated for the first time on different cancer cell lines.

We first aimed to assess the cytotoxic activity of the extract on different cancer cell lines after treatment for 72 h. The extract was found to exhibit cytotoxicity at the different level according to the type of cell line tested and inhibit the viability of these cells in a dose-dependent manner. Daudi cells with an IC_{50} value of 85.64 µg/mLwas the most sensitive cell lines to the extract. However, the IC_{50} value of the extract against BEAS-2B normal cell line was calculated as 109.80 µg/mL. In addition, IC_{50} values in HeLa and A549 cells were found to be 135.35 and 234.8 µg/mL, respectively. In Egypt, El-Nabawy et al. [9] assessed the cytotoxic activity of different fractions of aerial parts and seeds of *U. picroides* on different cell lines. They reported that the seeds butanol fraction and the aerial parts ethyl acetate fraction were very cytotoxic to MCF-7 (IC_{50} value= 9.4±0.37 and 8.8±0.47 µg/mL, respectively), and to HePG-2 (IC_{50} value= 14.7±0.85 and 10.1±0.88 µg/mL, respectively) cells. So, it can be suggested that the different part of *U. picroides* and the type solvent used for extraction may cause cytotoxicity at the different level according to the cell type examined.

One of the hallmarks of cancer is uncontrolled cell division [21]. Because the normal cell regulation process in the human cancer cells are altered [22], it is important to investigate new anti-cancer drugs which can inhibit the different steps of the cell cycle. We examined the effect of the extract on cell cycle for 24 h. Our results showed that the extract caused cell cycle arrest for A549 cells and HeLa cells at different phase of cell cycle. These results indicate that the effect of the plant extract on cancer cell proliferation may occur by arresting the cell cycle.

The development of new blood vessels from pre-existing ones is called angiogenesis and this process is important in the growth and metastasis of cancer tissue [23]. VEGF is known as a key regulator of angiogenesis in cancer [24] so that VEGF is considered as a rational target for anticancer therapy. In the present study, we tested the effect of the extract on the secretion of VEGF in A549 cells after treatment for 6 h. Our results show that the treatment with the extract at 200 µg/mL reduced the level of VEGF secretion by 31% in A549 cells compared to untreated cells, suggesting the antiangiogenic potential of the extract against A549 cells.

It is known that the inflammatory cytokines play a critical role in tumor development [25]. The pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 may contribute the growth and the metastasis of cancer cells [26]. We finally investigated whether the extract altered the TNF-α, IL-1-α and IL-6 secretion in the A549 and HeLa cells after treatment for 6 h. The treatment with the extract at 200 µg/mL did not cause any increase in the secretion of pro-inflammatory cytokines examined in cancer cell lines used. Strzelecka et al. [7] investigated the

anti-inflammatory activity of the extracts of some traditional Mediterranean diet plants and reported that the extracts of plants including *U. picroides* showed promising anti-inflammatory properties. Thus, our results suggest that the plant extract may not promote the development of cancer associated with the pro-inflammatory cytokines.

As a result, this study demonstrated for the first time that the ethanolic extract from the flowering parts of *U. picroides* showed significant cytotoxic activity against different cancer cell lines. Daudi cells were the most sensitive to the extract than the other cells used. The extract resulted in an arrest of A549 and HeLa cells at different phases of cell cycle. In addition, the extract caused a significant decrease in the secretion of cytokines after 6 h treatment in comparison with the untreated cells. Therefore, these findings may provide an important contribution to obtain a new bioactive compound with anti-cancer potential from the flowering part of *U. picroides*. Future studies should evaluate the determination of the potential of novel agents for cancer therapy via in vivo and in vitro analysis.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Microwave-assisted synthesis of N-benzylidene-4-fluoroaniline and N-benzylidene-4-Nitroaniline and their inhibitory activities on hCA isoenzymes

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Abstract: In this study, N-benzylidene-4-fluoroaniline (3a), N-benzylidene-4-nitroaniline (3b) as a result of condensation of benzaldehyde (1) and 4-fluoroaniline (2a), 4-nitroaniline (2b) using a microwave method was synthesized. The structures of the synthesized compounds were characterized by Fouirer Transform İnfrared Spektrofotometre (FTIR spectroscopy), Nuclear Magnetic Resonance (NMR spectroscopy) and elemental analysis methods. The prepared compounds were tested for their inhibitory effects on carbonic anhydrase isoenzymes (hCA-I and hCA-II). The results showed that the synthesized compounds 3a and 3b had a strong inhibitory effect on hCA-I and hCA-II enzymes activity in vitro. By testing these compounds in other CA isoenzymes, more effective CA inhibitors can be formed. Thus, new therapeutic applications can be made for enzyme activators in the near future.

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1. INTRODUCTION

Schiff bases derived from the reaction of primary amines with carbonyl compounds are used in a wide range of different fields. [1]. Schiff bases are indicated by the general formula RCH = NR. R and R 'are alkyl or aryl substituents [2]. Schiff bases are widely used in various fields such as pharmaceutical preparation, agricultural and plastic industry, polymer production, electronics industry, cosmetics, dyestuff production due to their biological and structural properties [3]. Schiff bases have antifungal properties as fungicides, insecticides, chemotherap eutic, anticancer agents in antioxidants, antimutagenic and antiviral drugs [4-8]. Nitro and halo derivatives of Schiff bases are known to have antimicrobial and anti-tumoral activity.

For the first time in 1986 Gedye used microwave in organic synthesis. The synthesis of microwave methods in scientific studies has started to attract great interest in recent years [9]. The microwave is a low frequency electromagnetic energy state at the end of the electromagnetic spectrum between the radio and infrared waves in the frequency range 300-300.000 MHz [10]. Microwave chemistry continues its development with scientific studies [11-

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15]. Because of the direct heating of the substances in the microwave reactions, the products are obtained with pure and high yields in less time than the classical heating methods [16, 17].

Carbonic anhydrase enzymes (CA, EC 4.2.1.1) are metalloproteins containing zinc (Zn^{2+}) which catalyze the conversion of CO_2 to bicarbonate (HCO_3^-) and proton (H^+) release. In addition, the carbonic anhydrase enzyme plays a role in many important reactions such as transport of CO_2 in tissues, pH and CO_2 homeostasis, transport of ions, biosynthetic reactions, bone formation. CA enzymes have been found in many tissues such as salivary glands, muscles, brain, nerve myelin sheath, pancreas, prostate and endometrial tissues by histochemical methods [18].

$$CO_2 + H_2O \longrightarrow H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The carbonic anhydrase isoenzymes are encoded by five different family of genes that are evolutionally independent, including α-, β-, CA-, δ- and ε- CA. Six teen different CA isoenzymes and CA-linked proteins (CARPs) were identified in the mammals. hCA-I and hCA-II are two main cytosolic CA isoforms found in mammalian red blood cells [19, 20].

For this reason, in this study it is aimed to synthesize Schiff base derivative N-benzylidene-4-fluoroaniline (**3a**), N-benzylidene-4-nitroaniline (**3b**) by micro wave method and investigate the inhibition potentials on CA-I and hCA-II isoenzymes.

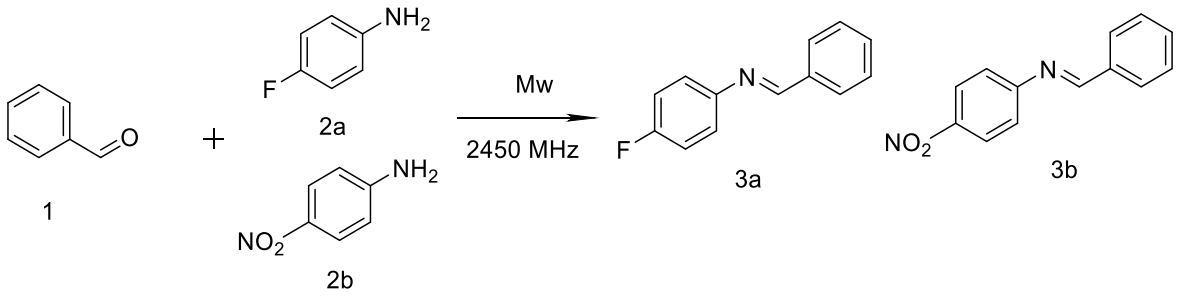
2. MATERIALS and METHODS

2.1. Chemicals

Benzaldehyde, 4-fluoroaniline are commercially available (Merck, Sigma-Aldrich) and were used without further purification. Sepharose-4B, sulphanylamide, L-tyrosine, Tris, Na_2SO_4, protein assay reagents, and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck. Reactions were monitored via thin-layer chromatography (TLC). ^{1}H NMR and ^{13}C NMR spectra were recorded on a 400 (100) MHz Varian spectrometer using $CDCl_3$. Column chromatography was performed on silica gel 60 (70–230 mesh ASTM), and TLC was carried out on silica gel (254–366 mesh ASTM). Melting points were determined on a capillary melting apparatus (Buchi 530) and are uncorrected. Infrared (IR) spectra were obtained from solutions in 0.1-mm cells with a Perkin-Elmer spectrophotometer (Waltham, MA). Elemental analyzes were performed on a Leco CHNS-932 apparatus.

2.2. General Method for Synthesis of 3a, 3b

4-floroanilin (**2a**) (1mmol), 4-nitroanilin (**2b**) (1mmol) was added into the benzaldehyde (**1**) (1 mmol) mixture, and then the reaction mixture was exposed to microwave radiation at 2450 MHz. The progress of the reaction was monitored by thin layer chromatography (TLC) (Runner phase, *n*-hexane-ethylacetate (4: 1) was used). It was determined that the reactions were completed in 8 minutes for all aniline derivatives. The crude products **3a** ve **3b** were obtained in pure form (Scheme 1).



Scheme 1. The synthesis of the compounds **3a, 3b**

2.3. Purification of Carbonic Anhydrase Isozymes (hCA-I and hCA-II) from Human Erythrocytes by Affinity Chromatography

Fresh human blood was obtained from Atatürk University, Blood Center, kept at 4°C and used within 2-3 days at most. Blood samples were centrifuged for 15 minutes at 2500 rpm to separate the erythrocytes, and the remaining plasma and leucocytes layers were carefully removed and discarded. Subsequently, the remaining erythrocytes were washed twice with 0.9% NaCl solution and the upper portions were discarded. Then, the erythrocytes were hemolyzed with pure water at 0°C and mixed for half an hour, and the cell membranes were separated by centrifuging the hemolyzate at 20,000 rpm at 4°C for half an hour. The pH was adjusted to 8.7 with solid Tris. Thus, the hemolyzate came to be applied to the colonic [21].

The affinity was prepared on gel Sepharose-4B matrix. After activating Sepharose-4B with CNBr, L-tyrosine was covalently attached as the extension arm. Tyrosine was then coupled by diazotization of the sulfanilamide as the ligand. The hemolysate was applied to the prepared Sepharose-4B-L-tyrosine-sulfanylamide affinity column equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA I and hCA II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were stored at 4 ^oC [22].

2.4. Hydratase Activity Assay

Carbonic anhydrase activity was determined using the Wilbur-Anderson Method modified by Rickli et al. [23]. This method is based on the determination of the pH change from the H^+ ion resulting from the hydration of CO_2 to the bromine thymol blue indicator and measuring the permeation rate. The time (tc) interval was determined between addition of CO_2 solution and occurrence of a yellow-green color. The same interval was recorded without enzyme solution (to). The activity was calculated from the formula**.** One Wilbur – Anderson Unit = (to- tc) / tc

2.5. Inhibition Assays

In order to calculate the IC_{50} values of hCA I and hCA-II enzymes, inhibitor effects of Schiff bases derivatives were run at different concentrations, keeping the substrate concentration constant on hydratase activity. The activities of the enzymes in the uninhibited medium were used as 100% activity. Hydratase activities were measured in the presence of different concentrations of enzymes, and % activity values were calculated. IC_{50} values were calculated for each inhibitor using the scheme of % Activity- [inhibitor] [24].

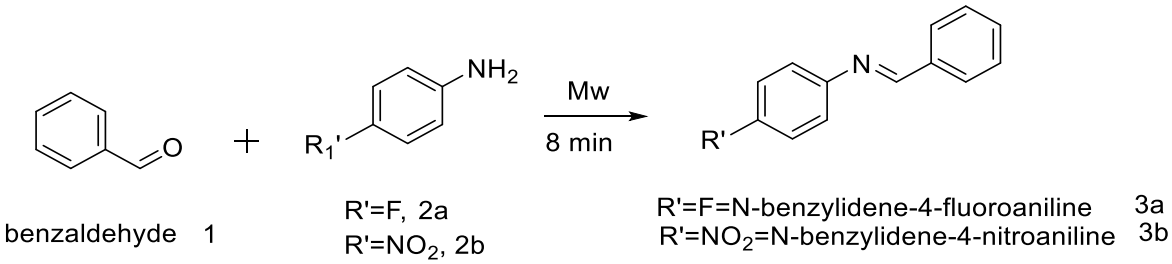
2.6. Protein Determination

The protein assay for all purification steps was performed according to the Bradford method [25]. This method is based on the principle that coomassie brilliant blue G-250 binds

to proteins in a phosphoric acid medium. The resulting complex shows maximum absorbance at 595 nm. The sensitivity of this method is between 1 - 100 micrograms.

3. RESULTS

N-benzylidene-4-fluoroaniline (3a), N-benzylidene-4-nitroroaniline (3b), were prepared from reaction of benzaldehyde (1), 4-floroaniline (2a), and 4-nitroaniline (2b) by microwave method (Scheme 2). It was determined that the reactions were completed in 8 minutes for two Schiff bases derivatives. Its physical properties, analytical and spectral data of the imine's compounds are summarized below.



Scheme 2. The synthesis route of the compound (**3a**,**3b**)

3.1. N-Benzylidene-4-fluoroaniline (3a)

Yield: 95 %, M.p. 104 °C, yellow solid.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 H (s, 1H), 7.90 (dd, *J* = 7.0, 2.6 Hz, 2H), 7.48 (dd, *J* = 5.2, 2.0 Hz, 3H), 7.21 (dd, *J* = 8.6, 5.0 Hz, 2H), 7.08 (t, *J* = 8.5 Hz, 2H) (Figure 1). ^{13}C NMR (400 MHz, $CDCl_3$) δ 160.4, 131.7, 129.0, 129.0, 122.6, 122.5, 116.2, 115.9, 29.9 (Figure 2). FTIR ($CDCl_3$, cm^{-1}): 3408 (aromatic CH), 3058 (aromatic CH), 2923, 2852 (CH=N), 1627 (aromattic C=C), 1505, 1261, 1222, 1094 (C-F), 801, 700 cm^{-1} (Figure 3). $C_{13}H_{10}FN$ Anal. calc. for: C,78.37; H, 5.06; N, 7.03 % Found: C, 78.71; H, 4.870; N, 7.093 %

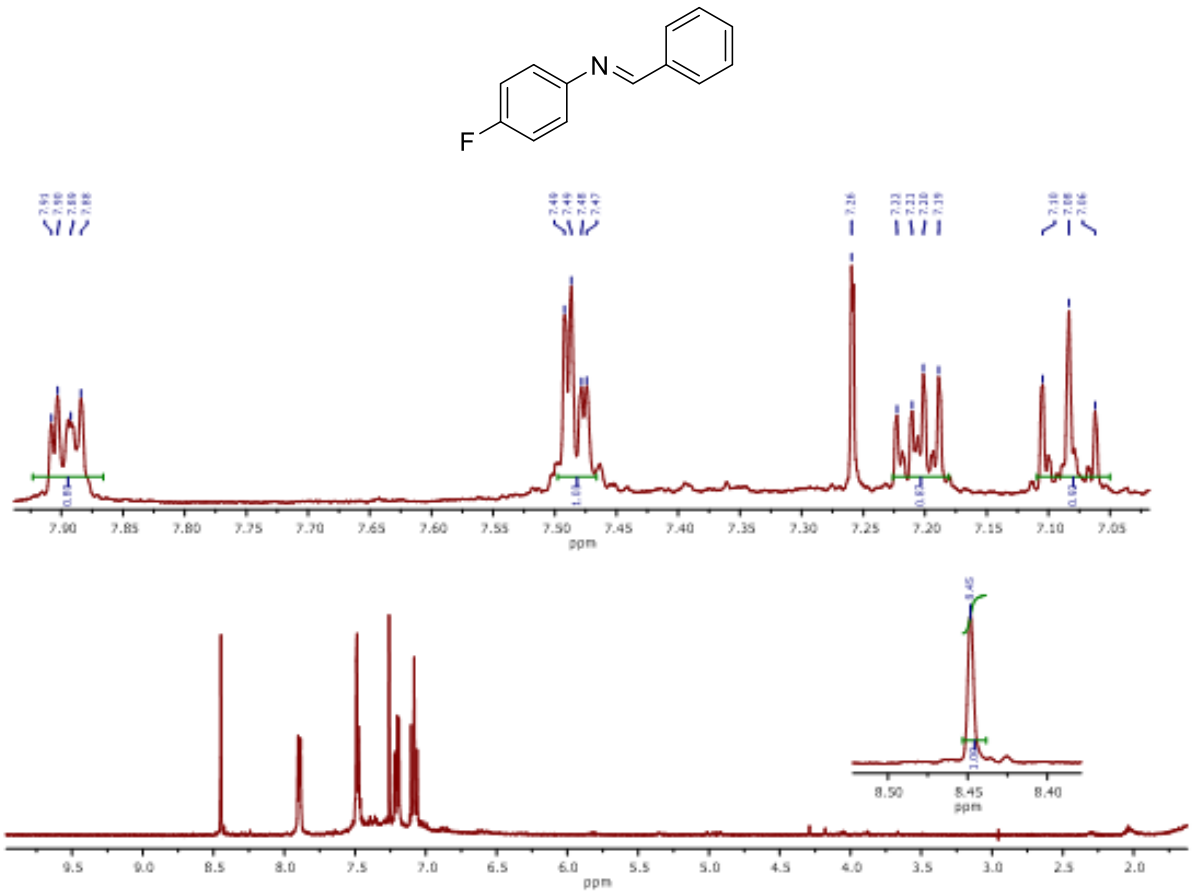


Figure 1. ^{1}H-NMR spectrum (400 MHz) of Compound **3a** ($CDCl_3$, δ ppm)

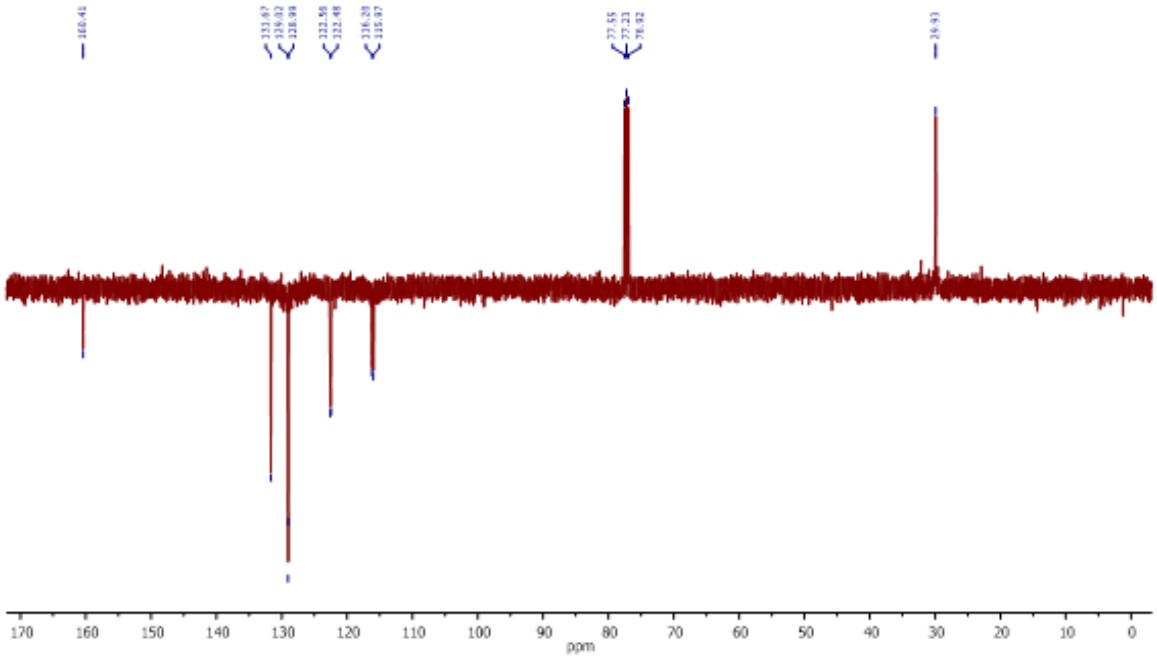


Figure 2. ^{13}C-NMR Spectrum (400 MHz) of Compound **3a** ($CDCl_3$, δ ppm)

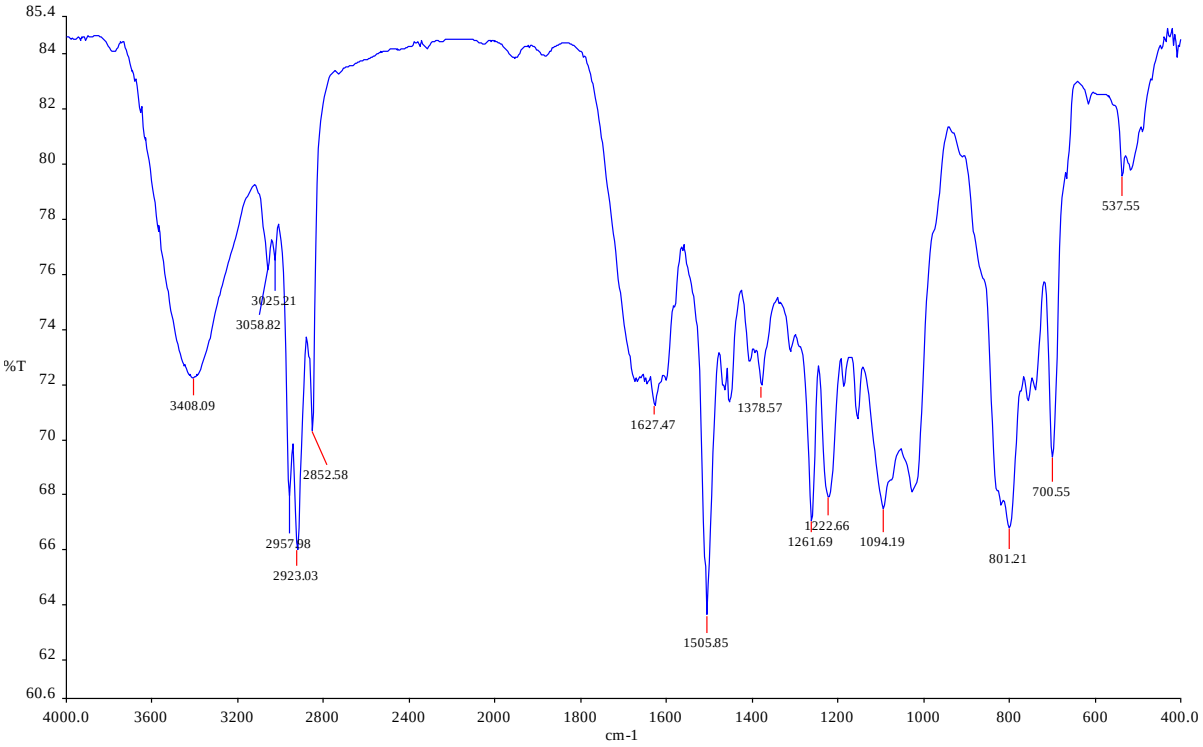


Figure 3. FTIR spectrum of Compound **3a** ($CDCl_3$, cm^{-1})

3.2. N-Benzylidene-4-nitroaniline (3b)

Yield: 93 %, M.p. 115°C, yellow solid**.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.27 (d, *J* = 8.8 Hz, 2H), 7.92 (d, *J* = 7.1 Hz, 2H), 7.61 – 7.45 (m, 3H), 7.25 (d, *J* = 8.9 Hz, 2H). (Figure 4).^{13}C NMR (400 MHz, $CDCl_3$) δ 162.95, 135.37, 132.43, 129.30, 128.99, 126.35, 125.05, 121.27, 113.37, 77.35, 77.03, 76.72. (Figure 5). FTIR ($CDCl_3$, cm^{-1}): 3482 (aromatic CH), 3369 (CH=N), 1629 (aromatic C=C), 1586, 1470, 1304, 1114, 838, 752, 697, 488 cm^{-1} (Figure 6). $C_{13}H_{10}N_2O_2$ Anal. calc. for: C,69.02; H, 4.46; N, 12.38; O, 14.14 % Found: C, 69.50; H, 4.370; N, 12.02; O, 15.10 %

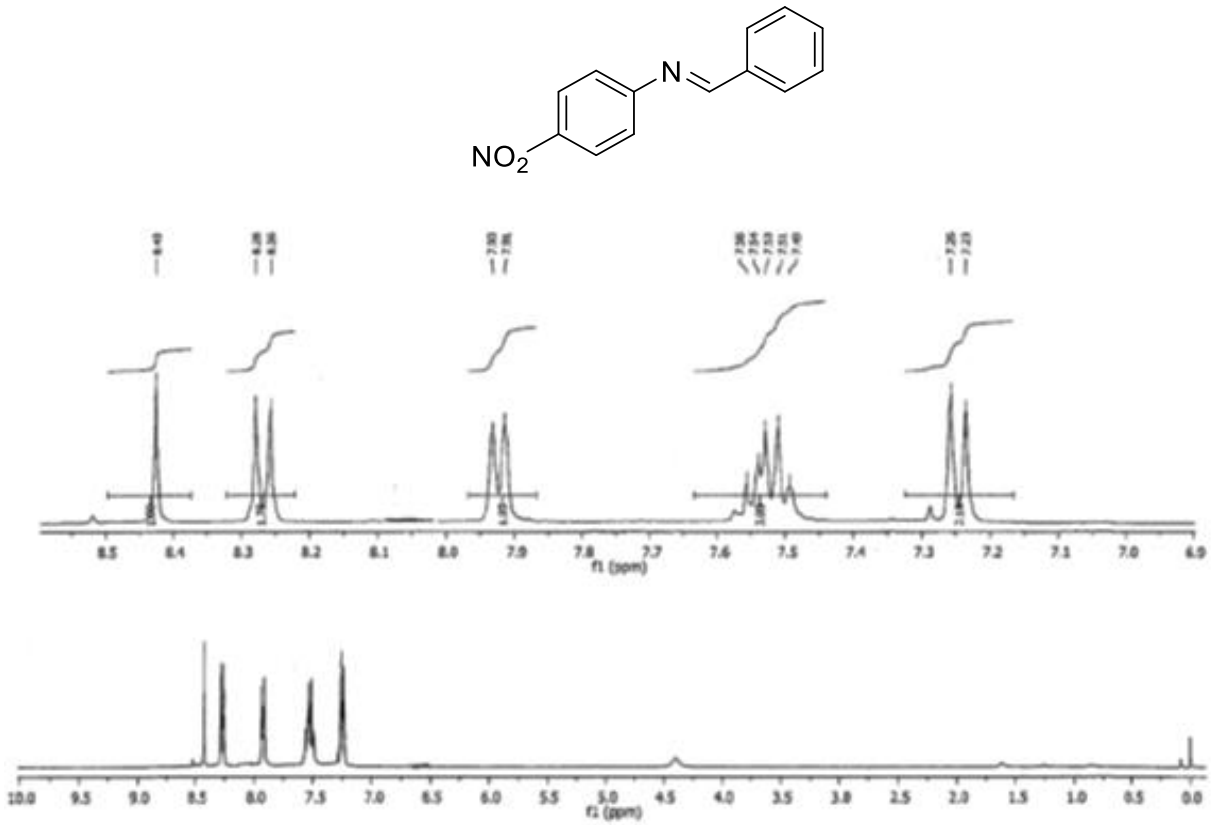


Figure 4. ^{1}H-NMR spectrum (400 MHz) of Compound **3b** ($CDCl_3$, δ ppm)

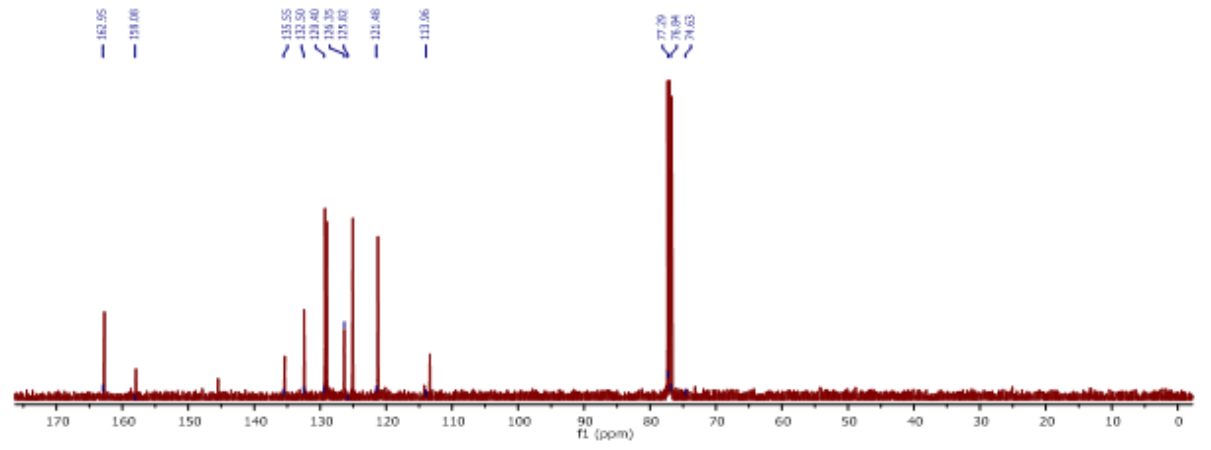


Figure 5. ^{13}C-NMR Spectrum (400 MHz) of Compound **3b** ($CDCl_3$, δ ppm)

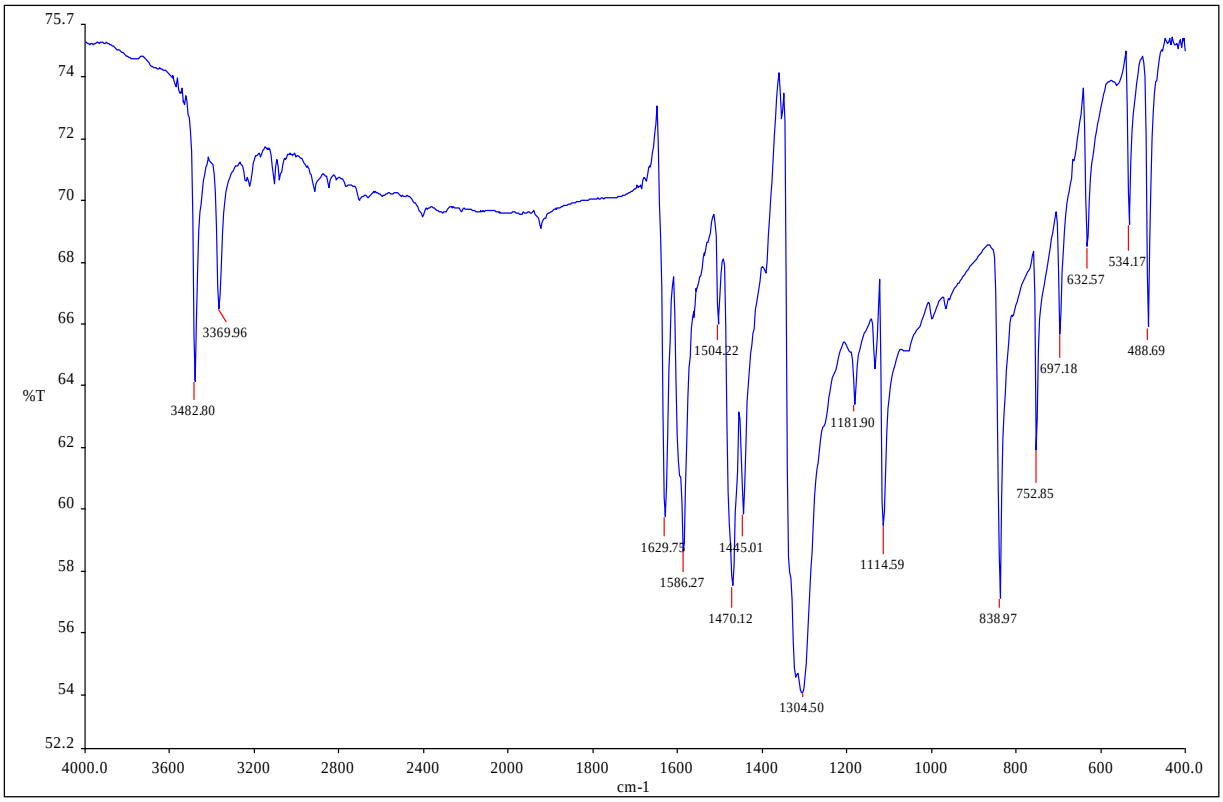


Figure 6. FTIR spectrum of Compound **3b** ($CDCl_3$, cm^{-1})

3.3. Purification Results of Carbonic Anhydrase Isoenzymes (hCA-I and hCA-II)

hCA-I and hCA-II isoenzymes were purified in one step using human blood Sepharose 4B-L tyrosine-sulphanylamide affinity chromatography. hCA-I was purified with specific activity of 2953.1 fold and 60.2 %, hCA-II purified with 797.33 specific activity, 7497.2 fold and 52.3% yield (Table 1).

Table 1. Purification of hCA I and hCA II from human erythrocytes

Purification steps	Volume	Activity	Total activity		Protein	Specific activity	Purification layer number
	(mL)	(EU/mL)	(EU)	(%)	(mg/mL)	(EU/mg)	
Hemolysate	100	44.67	4467	100	1.64×10^3	0.27	-
hCA-I	45	59.8	2691	60.2	0.075	797.33	2953.1
hCA-II	35	66.8	2338	52.3	0.033	2024.24	7497.2

The carbonic anhydrase enzyme has been purified many times from numerous different organisms and the effects of various pesticides, chemicals and drugs on enzyme activities have been investigated. In this study, hCA-I and hCA-II isoenzymes were purified by Sepharose 4B-L-tyrosine-sulphanylamide affinity chromatography and kinetic studies for schiff base (**3a, 3b**) were carried out by hydratase activity method using CO_2 as substrate. For two isoenzymes this was seen as a highly effective activator. The inhibitory effects on the activity of the isoenzymes were tested in vitro, the IC_{50} values were calculated from the % Activity- [inhibitor] plot drawn for compound (**3a, 3b**) and the results are given in (Figure 7), (Figure 8).

Chemical	**CA-I** IC50	**CA-II** IC50
$C_{13}H_{10}FN$	1,82	1,85
Acetazolamide (AAZ)	5.2	6.1

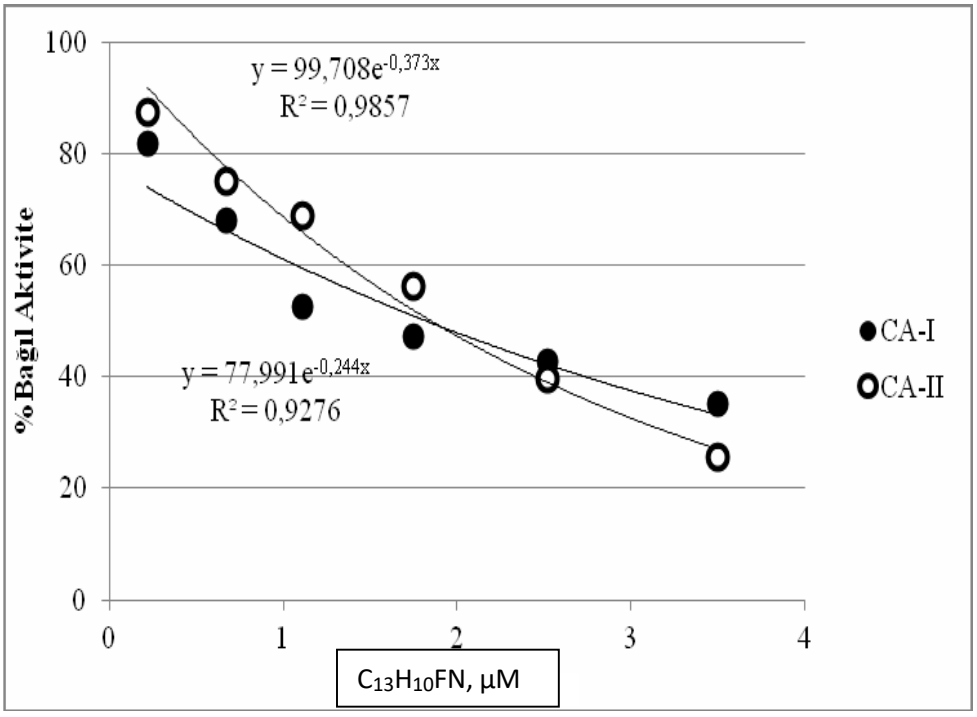


Figure 7. IC_{50} values for the in vitro inhibition of hCA I and hCA II with synthetized *N*-Benzylidene-4-fluoroaniline (**3a**)

Chemical	**CA-I** IC_{50}	**CA-II** IC_{50}
$C_{13}H_{10}N_2O_2$	1,85	2,07
Acetazolamide (AAZ)	5.2	6.1

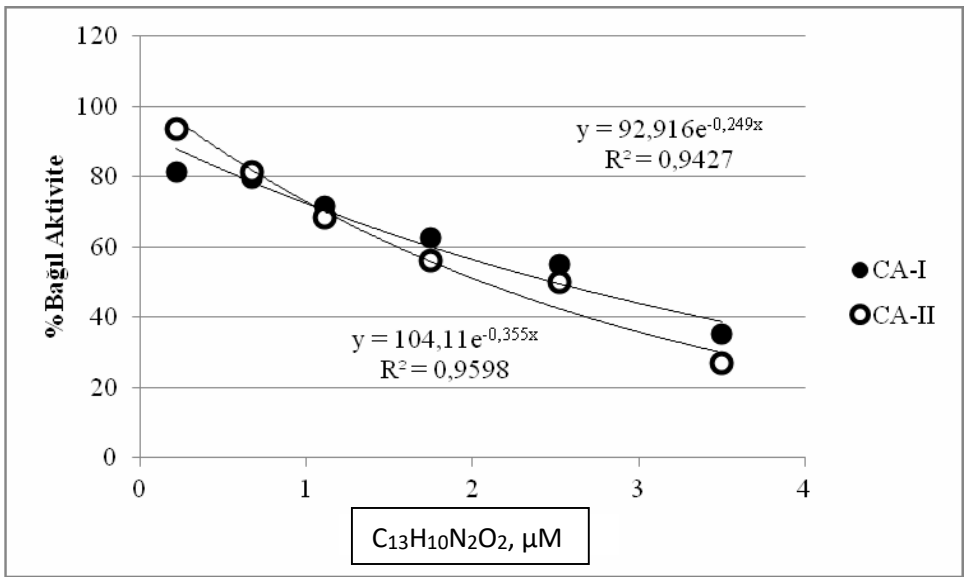


Figure 8. IC_{50} values for the in vitro inhibition of hCA I and hCA II with synthetized N-Benzylidene-4-nitroaniline (**3b**)

4. DISCUSSION

In vitro effects of $C_{13}H_{10}FN$, $C_{13}H_{10}N_2O_2$ on human activities of hCA-I and hCA-II enzyme purified from human erythrocytes revealed that inhibitory activity against acetazolamide reference compound was higher. The most effective effect on hCA1 and hCAII isoenzymes has IC_{50} values of 1.82 mM and 1.85 mM, respectively, of the $C_{13}H_{10}FN$, compound. It is thought that the newly synthesized Schiff bases show strong inhibition effect on hCAI and hCAII isoenzymes and they will serve as a reference for the formation of new inhibitors of CA enzyme [26-27].

The pharmacological effects of synthesized compounds **3a** and **3b** can be improved clinically for hCA I and II. The novel therapeutic applications of these enzyme inhibitors or activators will be directed to the design of prodrugs and drugs in the health sector.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Cobalt+Salt-Stressed *Salvia officinalis*: ROS Scavenging Capacity and Antioxidant Potency

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Abstract: *Salvia officinalis* L. (Lamiaceae) is one of the most widespread herbal species used in the food processing industry and for culinary and medicinal purposes. This work aimed to investigate changes in plant growth, water content, lipid peroxidation, H_2O_2, proline, and enzymes related to reactive oxygen species (ROS) detoxification including superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). Phenolic contents and antioxidant capacity values such as ferric ion reducing antioxidant power (FRAP), cupric ion reducing antioxidant capacity (CUPRAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging were studied under stress conditions of salt, cobalt and a combination of the two. No significant differences were found in relative water content and chlorophyll fluorescence under salt, cobalt and their combination. However, the osmotic potential and relative growth rate were enhanced with salt+cobalt compared to salt-treated plants. Salt and cobalt individually stimulated high antioxidant activity. High APX and GR activities were associated with the high proline accumulation in the sage plants under the combined effect of salt+cobalt. The combination decreased lipid peroxidation (TBARS), while H_2O_2 content was increased. This increase with the combined salt+cobalt effect may be associated with the decrease in CAT activity. Moreover, a strong correlation was found between TPC and TF content and antioxidant capacity measured via FRAP, CUPRAC and DPPH. The TPC, TF and antioxidant capacity values also increased under the salt+cobalt combination, suggesting an increase in antioxidant content in the sage leaves. Therefore, the combination of salt and cobalt improved the stress tolerance of *S. officinalis*.

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1. INTRODUCTION

Common sage (*Salvia officinalis* L.) is one of the most widespread herbal species used in both culinary and medicinal preparations [1]. Like most other medicinal plants, sage is traditionally used as a medicine because of its diverse biologically active compounds, namely antioxidants. Rosmarinic acid and carnosol are the main compounds of all the antioxidant phenolic extracts isolated from *S. officinalis* [2]. The importance of this species for medicinal and cosmetic use and in the food processing sector warrants an investigation into the response

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of sage plants to undesirable environmental conditions like salinity, drought, heat and heavy metals.

Plants are living organisms and they need certain mechanisms to survive during their lifetime because of exposure to abiotic/biotic factors under field conditions. Unfavorable abiotic stress conditions cause electrons in a high energy state to be accepted by molecular O_2 [3]. The changes in the metabolite levels of the plants result in reactive oxygen species (ROS) production [4]. In order to scavenge and detoxify overproduction of ROS, plant cells have developed various effective defense systems including enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and non-enzymatic antioxidants such as glutathione, ascorbic acid, carotenoids, flavonoids and tocopherols [5,6].

Recent studies have revealed that more information is needed to understand the response to a combination of different abiotic stresses rather than to each individual stress alone [7]. In fact, crops are subjected to combined stress factors in the field. However, the positive or negative interactions of these abiotic factors require further investigation. Crops need to tolerate multiple stresses with a minimal effect on their growth and development. Zandalinas et al. [8] reported an unknown interaction between some abiotic stress factors according to the Stress Matrix. Therefore, novel combined effects of abiotic factors, especially in terms of the role of ROS in signaling are unexplored topics. Salt and cobalt are among these abiotic stress factors whose positive or negative interactions are not fully described in the literature in terms of physiological and biochemical changes. Soil salinity causes huge detrimental effects that restrict the growth, productivity and yield in plants [9]. Apart from awareness of the destructive effects of salinity in plants, the exact role of cobalt in plants is still under debate. Although cobalt has been recognized as a micronutrient in animals and certain microorganisms, until recently there has been no conclusive evidence of its essential role in higher plants [10]. Moreover, cobalt has been reported as a heavy metal pollutant in plants [10,11]. In addition, there is little information about the combined effect of salt and cobalt on plants. For example, the growth and mineral composition of tomato [12] and wheat [13], the endogenous hormone levels and proline content of barley [14] and the macro and micro element contents of maize [15] have all been evaluated under conditions of salt stress along with the application of different concentrations of cobalt. However, no studies have been conducted on ROS formation and the antioxidative defense system in *S. officinalis* grown under a combined salt and cobalt treatment.

As an important aromatic and medicinal plant, sage was selected for this study. Previous studies investigating *S. officinalis* have mostly focused on antioxidant activity and phenolic content. However, there is little information about the physiological and biochemical behavior of *S. officinalis* regarding ROS formation and detoxification under salt stress. This study aimed to examine the tolerance potential of *S. officinalis* when subjected to combined salt and cobalt based on changes in the values of relative plant growth (RGR), leaf relative water content (RWC), osmotic potential, chlorophyll fluorescence (Fv/Fm), lipid peroxidation (TBARS), hydrogen peroxide (H_2O_2) content, proline level, activities of antioxidant enzymes such as SOD, CAT, POX, APX and GR and antioxidant capacity.

2. MATERIALS and METHODS

2.1. Plant Material and Stress Applications

Salvia officinalis L. seeds were used in this study. The seed surfaces were sterilized with 70% ethanol for 5 min, rinsed in sterile deionised water and then immersed in 5% commercial bleach for 15 min. Finally, the seeds were washed at least five times with sterile dI-H_2O in order to remove the bleach. The seeds were then germinated in the dark at 22 °C and 70% humidity.

After germination, uniformly germinated seeds were selected and cultivated in organic media made of peat moss, perlite and sand at a ratio of 1:1:1. The seedlings were grown at 27/22 °C (day/night) and a relative humidity of 70% in a controlled plant greenhouse. After thirty days, the pots were randomly separated into four groups: Control, Salt (NaCl), Cobalt ($CoCl_2$) and Salt+Cobalt (Figure 1). For the salt and cobalt treatments, 100 mM NaCl and 500 µM $CoCl_2$ were used, respectively. The concentrations were chosen according to preliminary experiments to induce physiological processes without killing the sage plants. Mature leaves were harvested after two weeks and immediately frozen in liquid nitrogen and stored at -80 °C for further analyses.

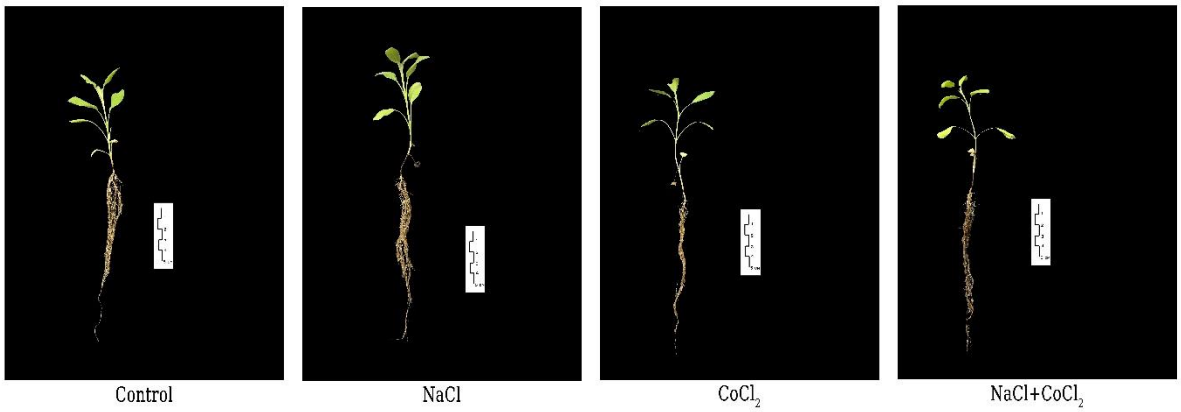

Figure 1. Morphological effects of salt (NaCl), cobalt ($CoCl_2$) and their combination (NaCl+$CoCl_2$) on growth of *Salvia officinalis* L. (Scale bar, 5 cm).

2.2. Growth Analyses

Six random plants from each group were used for the growth analyses and separated into leaves and root fractions on Day 0 (before stress applications). They were then subjected to 14 days of stress treatment. The samples were dried in an oven at 70 °C for 72 h after which dry weight (DW) was determined. The relative growth rate (RGR) of the leaves was calculated from the dry mass data taken initially (Day 0) and at final harvest (Day 14) according to Hunt et al. [16].

2.3. Lipid Peroxidation

Lipid peroxidation levels, determined as thiobarbituric acid reactive substances (TBARS), were detected according to Heath and Packer [17]. The amount of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.4. Hydrogen Peroxide Content

Hydrogen peroxide (H_2O_2) content was measured according to the method described by Liu et al. [18] and determined at 410 nm in 1.5 ml of the reaction mixture containing 0.1% (v/v) $TiCl_4$ and the appropriate extract. A standard curve prepared using known concentrations of H_2O_2 was used for calculations and the results were defined as l mol of H_2O_2 per gram of fresh weight.

2.5. Proline Content

Determination of free proline content was done according to Bates et al. [19]. Leaf samples were homogenized in 3% sulphosalycylic acid and the extracts were assayed for proline using the acid-ninhydrin method. The proline contents were determined using a standard curve prepared using known concentrations of proline as µmol proline g^{-1} FW.

2.6. Antioxidant Enzyme Extractions and Assays

For antioxidant enzyme activity assays, leaf samples were ground to a fine powder in an ice-cold mortar with liquid nitrogen and then homogenized in ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). Ascorbate (2 mM) was added to the homogenization buffer for the APX activity assays. The protein content in the enzyme extract was assayed with bovine serum albumin (BSA) as the standard [20]. The SOD (EC.1.15.1.1) activity was measured using the method of Beauchamp and Fridovich [21] and assayed according to its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm. One unit of SOD was defined as the amount of enzyme needed for inhibition of the NBT photoreduction rate by 50%. The POX (EC.1.11.1.7) activity was determined according to the method described by Mika and Lüthje [22]. One unit of POX activity was defined as the amount required to decompose 1 µmol H_2O_2 per min^{-1}. The CAT (EC 1.11.1.6) activity was estimated according to the method of Aebi [23]. One unit of CAT activity was defined as the amount needed to decompose 1 µmol H_2O_2 per min^{-1}. The APX (EC 1.11.1.11) activity was measured according to Nakano and Asada [24]. One unit of APX was defined as the amount needed to oxidize 1 µmol ascorbate per min^{-1}. The GR (EC 1.6.4.2) activity was determined according to the method described by Foyer and Halliwell [25]. One unit of GR was defined as the amount required to reduce 1 µmol GSSG per min^{-1}.

2.7. Total Phenolic Compounds (TPC) and Flavonoid (TF) Contents

The TPC content of the leaves was determined by the Folin-Ciocalteu (FC) method [26] and the TF content was determined according to the $AlCl_3$ colorimetric assay [27]. Gallic acid and quercetin were used, respectively, to prepare the calibration curves. Briefly, for the TPC content determination, 500 µL of the extract and the same amount of deionized water were mixed with 2% Na_2CO_3 (w/v) and 2 N FC reagent. After 30 min at room temperature (25 °C), a blue-purple color was formed as a result of the reaction and the absorbance was measured at 750 nm. The TPC content was expressed as µg gallic acid equivalents (GAE)/mg extract. For the TF content determination, a total of 1 mL reaction mixture was prepared by mixing the aqueous extract and 2% (w/v) $AlCl_3$ (1:1) in methanol. This was incubated at room temperature for 30 min and the absorbance of the reaction mixture was then measured at 415 nm. The TF content was expressed as µg quercetin equivalent (QE)/mg extract.

2.8. Antioxidant Capacity Values

The ferric reducing antioxidant power (FRAP) assay was conducted according to the procedure described by Benzie and Strain [28]. The cupric ion reducing antioxidant capacity (CUPRAC) was determined following the method of Apak et al. [29]. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was measured according to the colorimetric assay described by Blois [30]. Briefly, the FRAP reagent was freshly prepared [300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6- tripyridyl-s-triazine) and 20 mM FeCl-$6H_2O$ (10:1:1, v/v)] and kept at 37 °C. Next, 100 µL of each sample was mixed with 2900 µL of the FRAP reagent and the reaction mixture was incubated at 37 °C in the dark for 30 min. The absorbance of the mixture was measured at 593 nm against a blank. For the CUPRAC determination, 1 mL of extract was mixed with 1 mL of $CuCl_2$ (10 mM), 1 mL of acetate buffer (1 mM, pH 7.0) and 1 mL neocupraoine (7.5 mM). The total reaction mixture was gently shaken and then incubated in the dark at room temperature. After 30 min, the absorbance was measured at 450 nm. For the DPPH determination, 1 mL of DPPH solution (1 mg/30 mL) was freshly prepared and mixed with 100 µL of the extract. The mixture was then incubated for 30 min in the dark. Absorbance of the mixture was measured at 520 nm. The results of these three antioxidant capacity values were expressed as nmol trolox equivalent (TE)/mg extract.

2.9. Statistical Analysis

All analyses were performed according to a completely randomized design. Each experiment was repeated as two biological replicates and three technical replicates (n = 6). The results were expressed as means and error bars were used to show standard error of the mean (± SEM). All data obtained were subjected to one-way analyses of variance (ANOVA) and the significant differences among all treatments were compared using Duncan's multiple range test, with P <0.05 considered as significantly different.

3. RESULTS

3.1. Leaf Relative Water Content (RWC) and Relative Growth Rate (RGR)

The RWC of the *S. officinalis* leaves did not change significantly with salt, cobalt or the combined salt and cobalt, as compared to the control plants (Figure 2A). However, the treatment significantly inhibited the plant growth (Figure 2B). Salt stress reduced the RGR of the *S. officinalis* by 56.5% when compared with the control plants, while cobalt alone and the combination of salt and cobalt reduced RGR by 30.4 and 39.1%, respectively. On the other hand, cobalt caused a 60% increase in RGR compared to the salt-stressed leaves.

3.2. Chlorophyll Fluorescence and Leaf Osmotic Potential

Like RWC, no significant effect of salt and cobalt treatments alone on chlorophyll fluorescence of the *S. officinalis* was found in the present study (Figure 2C). However, significant differences were detected in osmotic potential level (Figure 2D). Salt and the combined salt and cobalt reduced leaf osmotic potential in this species. This decrease was 2.1-fold with salt and 29.3% with the combination of salt and cobalt, as compared to the controls. Interestingly, cobalt caused a 27% increase in osmotic potential.

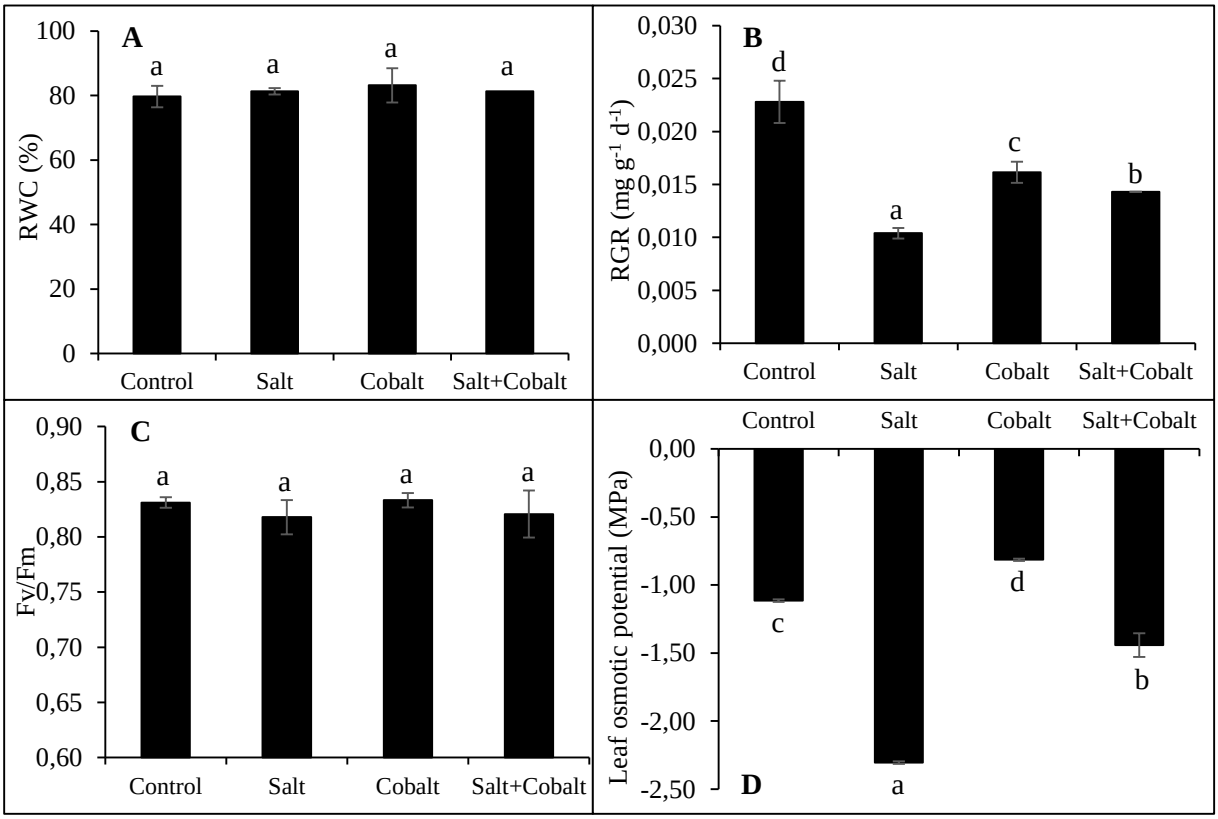

Figure 2. (A) Relative water content (RWC); (B) relative growth rate (RGR); (C) chlorophyll fluorescence (Fv/Fm); and (D) leaf osmotic potential of *Salvia officinalis* L. grown under control, salt, cobalt and the combination of salt and cobalt. (Vertical bars indicate ± SE; values sharing the same letter are not significantly different at P < 0.05)

3.3. Lipid Peroxidation, H_2O_2 and Proline Content

Lipid peroxidation (as indicated by TBARS content), H_2O_2 and proline contents are shown in Table 1. The TBARS level of the *S. officinalis* increased with the same intensity (4.9%) in both salt and cobalt treatments alone as compared to the control plants. On the other hand, the TBARS were reduced by 2.1% in the *S. officinalis* leaves under the combination of salt and cobalt.

There was no significant change in H_2O_2 content under salt stress, as compared to the control plants. However, the H_2O_2 content was significantly reduced by 30.6% with the cobalt treatment. Moreover, the combination of salt and cobalt increased the H_2O_2 content by 7% as compared to the controls.

In the *S. officinalis* leaves, the salt and the combination of salt and cobalt treatments enhanced the proline content by 32.6 and 72.6%, respectively when compared to the controls, while there was no significant change in proline level with the cobalt treatment.

Table 1. TBARS, H_2O_2 and proline contents of *Salvia officinalis* L. grown under control, salt, cobalt and the combination of salt and cobalt.

	TBARS (nmol g^{-1} FW)	H_2O_2 (µmol g^{-1} FW)	Proline (µmol g^{-1} FW)
Control	2.43 ± 0.01 c	16.39 ± 0.04 b	1.90 ± 0.03 a
Salt	2.55 ± 0.00 b	16.73 ± 0.02 b	2.52 ± 0.05 b
Cobalt	2.55 ± 0.00 b	11.38 ± 0.48 a	1.96 ± 0.06 a
Salt+Cobalt	2.38 ± 0.01 a	17.53 ± 0.19 c	3.28 ± 0.02 c

Values indicate mean ± SE; values in the same column sharing the same letter are not significantly different at $p < 0.05$.

3.4. Antioxidant Enzyme Activities

The amount of SOD activity increased with both salt and cobalt applications (Figure 3A). The SOD activity in *S. officinalis* subjected to salt alone was increased by 3.7-fold, while cobalt increased it by 2.1-fold, as compared to the control plants. The combined effect of salt and cobalt also increased the SOD activity when compared to the controls, but this effect never reached the high levels produced under salt treatment.

The POX activity was enhanced significantly by salt, cobalt and their combination (Figure 3B). Compared to the control plants, these increases of POX activity levels were detected as 2.2-fold for salt, 2.6-fold for cobalt and 1.7-fold for the combination of salt and cobalt.

As with the SOD and POX activities, salt and cobalt alone enhanced CAT activity in the *S. officinalis* leaves (Figure 3C). Significant increases in CAT activity by 3- and 7-fold were demonstrated by the salt and cobalt treatments, respectively, while no change was caused by the combination of salt and cobalt as compared to the controls.

The APX activity in the *S. officinalis* leaves was increased by 54.5% with the salt and cobalt treatments alone in (Figure 3D). Moreover, the combination of salt and cobalt increased APX activity by 90.9% when compared to the control plants.

As with the other enzymes, cobalt increased the GR activity by 66.7% in the *S. officinalis* (Figure 3E). Surprisingly, GR activity was reduced by 26.7% with the salt stress as compared to the control group. On the other hand, the combination of salt and cobalt enhanced the GR activity by 33.3%.

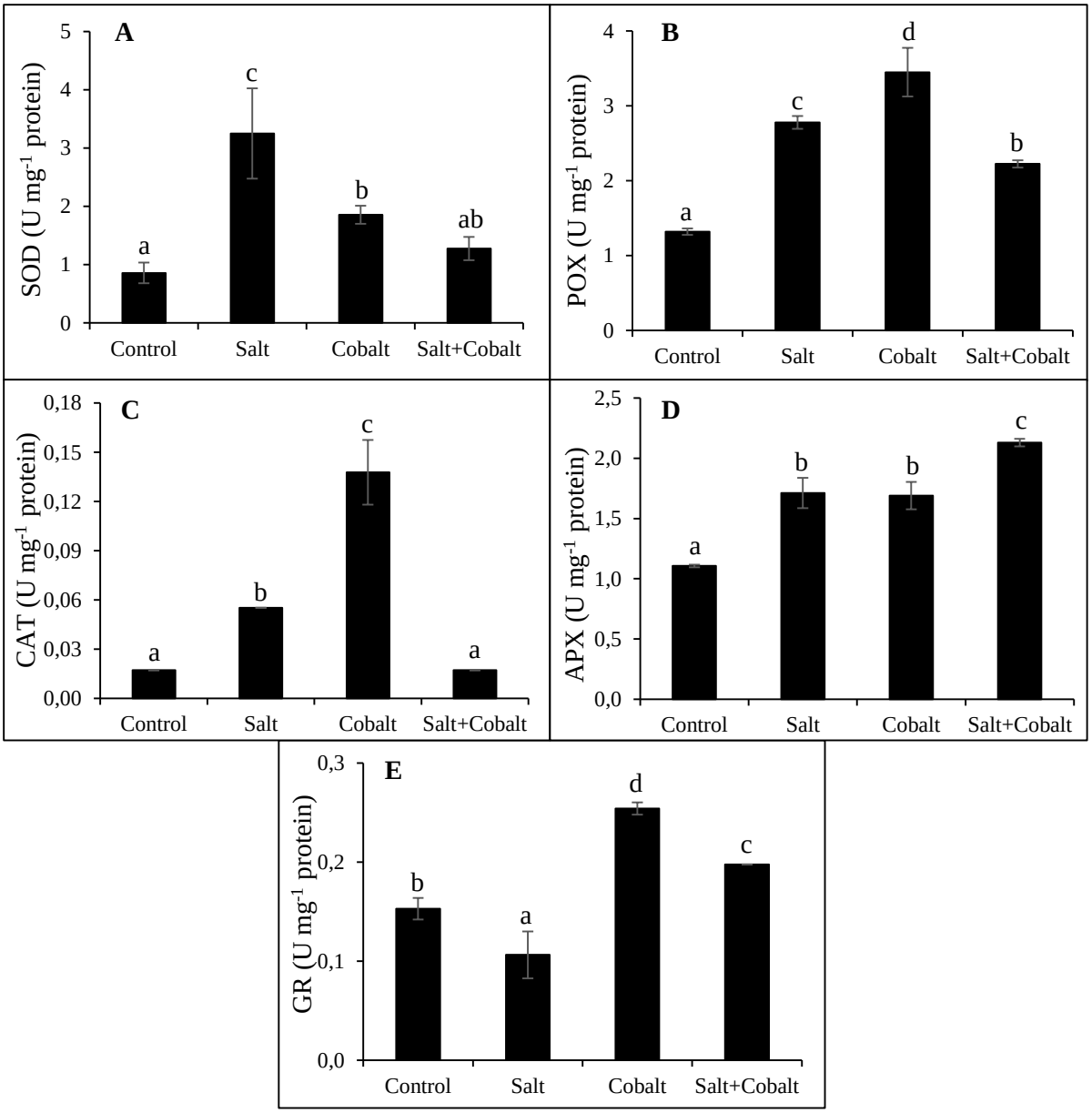

Figure 3. (A) Superoxide dismutase (SOD); (B) peroxidase (POX); (C) catalase (CAT); (D) ascorbate peroxidase (APX); and (E) glutathione reductase (GR) activities of *Salvia officinalis* L. grown under control, salt, cobalt and the combination of salt and cobalt. (Vertical bars indicate ± SE; values sharing the same letter are not significantly different at $P < 0.05$)

3.5. Total Phenolic Compounds (TPC) and Total Flavonoid (TF) Contents

The concentrations of TPC and TF of *S. officinalis* leaves grown under salt, cobalt and their combination are shown in Table 2. Significant increases in both TPC and TF contents were observed under salt stress, which were 45.3 and 13% higher than in the control plants. Moreover, cobalt alone also increased TPC and TF contents by 15.3 and 7.7%, respectively, as compared to the controls. Although the combination of salt and cobalt enhanced TPC content by 21.1%, this combined effect did not cause any significant change in the TF content.

Table 2. Antioxidant capacity values of *Salvia officinalis* L. grown under control, salt, cobalt and the combination of salt and cobalt.

	TPC (µg GAE/mg extract)	TF (µg QE/mg extract)	FRAP (nmol TE/mg extract)	CUPRAC (nmol TE/mg extract)	DPPH (nmol TE/mg extract)
Control	87.0 ± 1.88 a	8.82 ± 0.16 a	86.5 ± 0.43 a	201.2 ± 1.00 a	157.8 ± 1.88 a
Salt	126.4 ± 1.42 d	9.97 ± 0.06 c	131.7 ± 2.72 d	317.4 ± 10.6 c	251.1 ± 0.61 c
Cobalt	100.3 ± 1.71 b	9.50 ± 0.19 b	93.4 ± 0.68 b	212.3 ± 2.33 a	244.2 ± 1.69 b
Salt+Cobalt	105.4 ± 1.53 c	9.03 ± 0.23 a	111.1 ± 1.28 c	236.1 ± 5.79 b	250.7 ± 0.35 c

GAE: Gallic acid equivalent; QE: Quercetin equivalent; TE: Trolox equivalent
Values indicate mean ± SE; values in the same column sharing the same letter are not significantly different at $P < 0.05$.

3.6. Antioxidant Capacity Values (FRAP, CUPRAC and DPPH)

The FRAP, CUPRAC and DPPH activities were measured to determine antioxidant capacity and are shown in Table 2. As with the TPC and TF contents, salt and cobalt treatments alone caused significant increases; however, salt resulted in higher increases than cobalt. Salt stress enhanced FRAP, CUPRAC and DPPH activities by 52.3, 57.8 and 59.1%, while cobalt increased them by 8, 5.5 and 57.8%, respectively, when compared to the non-treated control plants. Compared to the control group, the combination cobalt and salt treatment caused significant increases of 28.4, 17.3 and 58.9% in FRAP, CUPRAC and DPPH values.

4. DISCUSSION

Abiotic stresses applied individually cause detrimental effects in terms of the growth and productivity of plants. However, a combination of these abiotic stresses produces greater harmful effects [7]. To counter the stress, plants undergo a process of stress acclimation [4]. Salt and cobalt are two important abiotic factors which can restrict plant growth and yield. The injurious effects of salt have been reported in most studies [9,31]; however, information is lacking about cobalt. Although some previous research has reported cobalt to be an essential micronutrient [14,15], it is one of the heavy metals found in contaminated soils [10]. Moreover, Karuppanapandian and Kim [32] reported that cobalt caused irreversible damage to plant cells, resulting in the reduction of plant growth and decline in crop productivity and quality. Plants can survive if they are able tolerate stress conditions via ROS detoxifying antioxidant defense mechanisms.

In our study, the effect of the salt and cobalt combination on *S. officinalis* was very different from that of salt or cobalt applied individually. As in previous studies in the literature [9,31], salinity reduced RGR and osmotic potential while increasing TBARS and proline levels in the leaves. In higher plants, the level of cobalt concentration causing toxicity differs widely among species or genotypes within a species [33]. In this study, cobalt alone increased the osmotic potential and TBARS content while at the same time causing a reduction in H_2O_2 content in the *S. officinalis* leaves. Contrary to these findings, cobalt induced a significant reduction in growth and a dramatic increase in lipid peroxidation in barley plants [34]. Although salt and cobalt alone caused a significant reduction in the growth of *S. officinalis*, the combination of salt and cobalt increased RGR as compared to the salt-treated plants. The RWC and Fv/Fm were not significantly affected by salt, cobalt or their combination. In the present study, our results showed that plants grown under a combination of salt and cobalt accumulated more proline than plants grown under salt and cobalt alone. Moreover, the *S. officinalis* leaves maintained their water content and photosynthesis levels in order to protect the other vegetative parts of the plant. Only in the salt stressed plants did proline content increase while osmotic potential decreased. In contrast to our results, using cobalt resulted in increments in the growth, yield and quality of maize [15], tomato [12] and *Nigella sativa* [35] under salt stress. Moreover,

a correlation between H_2O_2 accumulation and a decrease in CAT activity was determined in the sage plants under the combined effect of salt and cobalt. The lower levels of TBARS found in the sage leaves suggested that they may have exhibited better protection against oxidative damage under the combined stress of salt and cobalt.

Antioxidant responses of different plant species to salt or cobalt application have been examined in a number of works [9,10,34]. However, none of these studies have included positive or negative interaction responses to a combination of salt and cobalt. Except for GR activity under salt stress, salt and cobalt treatments, both individually and in combination, led to an increase in SOD, POX, CAT, APX and GR activity in *S. officinalis* leaves. The SOD activity in salt-treated plants was higher than in those treated with cobalt alone or the combined stress, whereas increases in POX, CAT and GR activities were more pronounced only in the cobalt-treated plants. However, cobalt alone did not induce H_2O_2 formation. These increases in POX, CAT and GR may indicate the importance of cobalt for the redox balance of *S. officinalis* cells. Parallel to these results, salt stress alone markedly increased the CAT activity of *Salvia miltiorrhiza* leaves, but not the SOD activity [36]. Furthermore, in this study, CAT activity significantly diminished under combined stress. The increased H_2O_2 content may have been the reason for that reduction. The results of reduction in growth and osmotic potential and increase in TBARS and H_2O_2 content suggest that the sage plants used in this study exhibited an effective defense mechanism against the negative effects of combined cobalt and salt stress. Similarly, increases in activities of antioxidative enzymes were also detected in cobalt-treated barley seedlings [34]. However, there is little information about antioxidative defense enzymes in plants grown under a combination of cobalt and salt stress.

Abiotic stresses stimulate polyphenol synthesis and their accumulation [37] and these polyphenolic compounds participate in plant protection against ROS. Most studies in the literature are concerned with the polyphenolic compounds and the strong antioxidant activities of *S. officinalis* as a medicinal plant under either non-stressed conditions or one type of stress only [38,39]. In the present study, *Salvia officinalis* exhibited higher TPC and TF content and antioxidant capacity values under salt stress. Hence, salinity caused strong radical scavenging activity against FRAP, CUPRAC and DPPH in the sage leaves. Moreover, the correlation between the phenolic and antioxidant activity in the sage used in this study suggests that these compounds are involved together in this activity under salt stress. Total phenol and flavonoid contents and DPPH and FRAP assays for antioxidant activity were measured under non-salt-treated normal conditions and it was found that the activity of the extracts depended mainly on the extraction solvent and harvesting season [40]. The non-salt-treated methanol extract of *Salvia verticillata* subsp. *amasiaca* displayed high DPPH, FRAP and CUPRAC activities [41]. Similar to our results, Taârit et al. [39] reported higher phenolics and significant DPPH quenching activity in the same herb under salinity. Moreover, in other sage species (*Salvia sclarea* L.) 75 mM NaCl caused a reduction in phenolic contents and an increase in DPPH radical scavenging activity [42]. In another study with most findings similar to those of the present study, salt stress increased the total polyphenol content of *Salvia mirzayanii* and induced antioxidant synthesis [43]. On the other hand, other cobalt-related studies on sage plants were mainly concerned with heavy metal accumulation [44,45]. Exogenous application of cobalt to sage plants was rarely covered in the literature. In our study, cobalt alone also increased the TPC, TF and antioxidant capacity values in sage. However, this increase never reached the high levels detected under salt and combined stress. To the best of our knowledge, this is the first study conducted on the activities of total phenolic and flavonoid contents and antioxidant capacity values (FRAP, CUPRAC and DPPH) of sage plants under combined salt and cobalt.

5. CONCLUSION

The results of this study showed that the responses of plants exposed to combined stress were different from those determined under each stress individually. There was a possible positive interaction between the salt and cobalt treatments. The RGR and osmotic potential were less affected when cobalt and salt+cobalt stresses were applied. Apart from reduction in growth and osmotic potential, the combined stress markedly increased the H_2O_2 content and lipid peroxidation in the sage. The proline content of the sage was enhanced under the salt stress, but not as high as that under the combined stress. The activities of antioxidant defense enzymes increased under all stress conditions, except for CAT under the combined stress. However, APX and GR in particular were suppressed under the combination of salt and cobalt as compared to salt alone. Moreover, sage has a high antioxidant capacity, as estimated via FRAP, CUPRAC and DPPH, and is rich in phenolic compounds under both salt and salt+cobalt stress. In summary, the combination of cobalt and salt treatments alleviated the negative effects of salt stress in *S. officinalis*.

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Antioxidant and Antibacterial Effects of Some Medicinal Plants of Iran

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Abstract: Medicinal plants used in the treatment of diseases earlier times are potential sources of new drugs. The present study was undertaken to study the chemical composition, antioxidant and antibacterial activity of certain medicinal plants of Iran by gas chromatography and mass spectrometry (GC/MS), DPPH and disk diffusion method. According to the results of GC/MS, there are 46 kinds of chemical compounds including mucilage, fatty acids, flavonoid and diterpenes in flower of *Echium khuzistanicum*. There are aldehydes (7.9%), phenols (7.5%), fatty acids (5.8%) and furfural (5.4%) in the methanol extract of *Echinops cephalotes*. Furfural, steroids, vitamin B and flavonoids are the main compounds of *Marrubium anisodon*. Results of the antibacterial test showed that *Staphylococcus aureus* and *Bacillus subtilis* were more sensitive to methanol extract of *Echium khuzistanicum* root. *Pseudomonas auruginosa* was more sensitive to DMSO extract of *Marrubium anisodon* at 600 mg/ml concentration. Maximum flavonoid and phenol contents were belonging to *Echinops cephalotes*. *Marrubium anisodon* showed the best DPPH free radical scavenging activity.

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1. INTRODUCTION

Plants can produce a variety of chemicals so that new compounds always are discovered and extracted from plants. Each of these compounds may have therapeutic effects like antibacterial and antioxidant activities [1].

The antioxidant system in plants and animals comprise both-low molecular mass and high molecular mass antioxidants. Low molecular mass antioxidants described to date include water-soluble compounds such as reduced glutathione, ascorbic acid, and lipid-soluble ones such as carotenoids (including β-carotene), retinol, α-tocopherol. They usually operate as free radical scavengers. Various compounds of a plant such as fibres, carotenoids, phenols, flavonoids, isoflavones ,and ascorbic acid eliminate free radicals and have antimutagenic and antioxidant activity[2]. The ability of elimination of active oxygen makes these compounds converted to factors which protect human from ailments like cancer[3]. In recent years, there are considerable attention towards the identification of plants with the antioxidant ability [4].

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Several drug resistances have been observed in human pathogenic microorganisms due to the excess usage of commercial antimicrobial drugs in the treatment of infection [5]. Also in all countries including developed countries, the prevalence of foodborne diseases are still considered a serious issue. So, a permanent search is needed to discover effective methods and materials to treat the food infection caused by microorganisms [6]. We need to identify and introduce new medicinal and aromatic plants with effective natural antibiotics, high biological value and low side effects [7].

Echium khuzistanicum Mozaff is a biennial plant of Boraginaceae family which grows in the southwest of Iran [8]. In about 150 species of Boraginaceae, naphthoquinone pigments such as alkannin and shikonin derivatives exist in roots. Alkannin (S enantiomer) and shikonin (R enantiomer) and their derivatives have a lot of medicinal properties like anti-allergic, antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory and wound healing [9-15]. Shikonin plays a significant role in the treatment of the obesity, intestinal ulcers, skin diseases, cancers ,and AIDS [16].

Marrubium anisodon K. Koch is a plant of the Lamiaceae. There is the various activity such as antioxidant and anti-inflammatory effect in this genus [17]. *Echinops cephalotes* DC is a plant of the Asteraceae. This genus is remarkable regarding chemical composition, tens of alkaloids extracted from various parts of them which used in industry, agriculture, and medicine. Alkaloids, saponins, polyphenols, carotenoids, and phytosterols were detected in this genus [18]. The aim of this study is the assessment of the chemical composition, antioxidant and antibacterial activity of these plants of Iran for the first time.

2. MATERIAL AND METHODS

2.1. Plant Material

M. anisodon and *E. cephalotes* were collected from the medicinal plant garden of Hamadan (Natural Resources Department, Hamedan Natural Resources and Agriculture, Education and Research Center, Medicinal Plant Garden, Hamedan, Iran) and *E. khuzistanicum* was planted in a greenhouse. The plant's seeds were collected from Alhaii region around Ahwaz (the southwest of Iran). The plants were identified by the botanist, Dr. Dinarvand (Faculty member, Natural Resources Department, Khuzistan Natural Resources and Agriculture, Education and Research Center, Ahvaz, Iran).

2.2. Extraction of Plant Material

The samples were dried at the room temperature in the dark and further was ground in a mortar. About 10 grams of each plant powder extracted in 100 ml of methanol by soxhlet till the solvent in siphon tube of an extractor become colorless. The extract was concentrated at temperature below 40°C and was used for determination of flavonoids, phenols, free radical scavenging, antibacterial activity and GC/MS analysis [19].

2.3. Determination of Total Flavonoid

Flavonoids were determined using Aluminum chloride[20]. 0.5 ml of extract solution (1mg/ml) with 1.5 ml methanol, 0.1 ml Aluminum chloride (10%), 0.1 ml Potassium acetate (1M) and 2.8 ml distilled water was mixed. After 30 min, sample absorption was read at 415nm by a double beam Lambda 45 UV–visible spectrophotometer. The total flavonoid content was determined using a standard curve of quercetin (R^2= 0.981). Total flavonoid content is expressed as µg of quercetin equivalents/ 100 mg of sample. Total flavonoid was calculated by using the following equation:

$$\text{Absorbance} = 0.0077 \text{ quercetin } (\mu g) - 0.0532$$

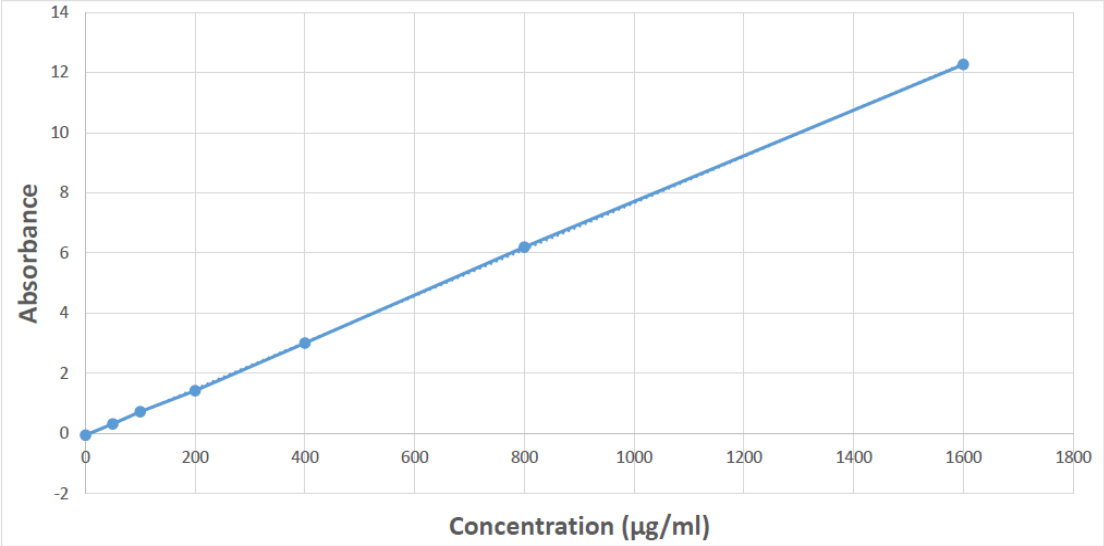

Figure 1. Quercetin standard calibration curve

2.4. Determination of Total Phenols

Total phenolic content in each extract was determined by using Folin-Ciocalteu reagent [21]. 200 microliter of extract (1mg/ml) was mixed with 1ml (1N) Folin-Ciocalteu reagent (Sigma-Aldrich, Germany) and 5.8 ml of distilled water, followed by 3ml 20% Sodium Carbonate (Na_2Co_3) 3min later. The mixture was shaken for two h at room temperature in the dark, and absorbance was measured at 165nm. All tests were performed in triplicate. Gallic acid (Sigma-Aldrich, Germany) used as a standard. The concentration of total phenolic compounds (TPC) was determined as µg gallic acid equivalents (GAE)/mg extract by using the following equation obtained from a standard gallic acid graph ($R^2 = 0.9877$):

$$\text{Absorbance} = 0.0012 \text{ gallic acid } (\mu g) - 0.0034$$

2.5. Free Radical Scavenging Activity

Free radical scavenging activity was determined by using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The Ascorbic acid was used as a standard control. To study inhibition percent of DPPH, 50µl of the extract with different concentration (0.2, 0.4, 0.6, 0.8, 1mg/ml) mixed with 5ml of DPPH (0.0004%) and after 30 min, the absorption was measured at 517nm [22]. The percentage of inhibition (I) was calculated as:

$$I = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}]$$

IC_{50} values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radical.

2.6. Assessment of Antibacterial Effects

Six human pathogenic bacteria were used including gram-positive bacteria of *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 19615 and gram-negative bacteria of *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028 and *Pseudomonas aeruginosa* ATCC 9027 prepared from Reference Center of Bu-Ali Sina Hospital (Hamedan, Iran). The antibacterial effect of the extracts was examined by the disk diffusion method. Small paper disks (prepared from Padtan Teb Co. with a diameter of 6.4 mm) soaked in different concentration of the plant extract with 100, 200 and 600 mg/ml concentration. In this method, a suspension with the dilution equal to 0.5 McFarland standard was prepared by 24-hour culture of bacteria. At the next stage, 0.2 ml of the bacterial suspension

was added to each plate and surface-cultured by a sterile swab [23]. Then the disc containing 20 µl of the extract was placed on the medium using sterile forceps tip. The plates were incubated for 24 hours at 37 ° C [24]. The solvent was only used as the negative control and ten µg antibiotic gentamicin disc (Padtan Teb Co.) as the positive control. After incubation, the diameter of the inhibition halo was measured using a ruler and recorded. The antibacterial test of the extracts was done in triplicate for each concentration, and completely randomized design and ANOVA test were employed at 5% level. The results were expressed in means ± SEs.

2.7. Gas Chromatography and Mass Spectrometry

The chemical composition of the extracts was identified by GC/MS (Agilent 6890N gas chromatography coiled with Agilent 5973N mass detector). 1µl of each extract was injected. The separation of extract was performed using an HP-5 column of 30m in length and 0.25 mm in diameter and 0.25 µm in stationary phase thickness. The analysis conditions were shown in Table 1.

Table 1. Temperature program of analysis

Rate(˚C/min)	Temperature ˚C	Hold (min)
-	60.00	0.00
5.00	150.00	0.00
10.00	250.00	0.00

The solvent delay was 5 min, and the identification of the compound was based on comparing their mass spectra with those recorded in the Wiley 7n mass spectra database and with literature reports.

2.8. Statistical Analysis

All the experiments did with three replicates for each sample of plants. A completely randomized design was employed at 1% level.

3. RESULTS

3.1. Chemical Composition of Plant Extracts

To analyze results accurately and given that the chemical composition of these plants is not detected, after extraction, chemical composition of plants were checked by GC/MS. The amount and type of chemical compounds were achieved by comparing the data from GC/MS with information of libraries.

3.2. Chemical Composition of *Marrubium anisodon*

In methanol extract of this plant's aerial parts, 86 compounds were identified by GC/MS (Figure 2). The compounds present in this plant (with more than one percent), their retention time (RT), molecular formula, molecular weight (MW), and concentration (peak area %) are presented in Table 2. Furfural, steroids, vitamin B and flavonoids are the main compounds of *M. anisodon*. According to the results, furfural is the most abundant compound (20.43%). Furfural is the natural product of lignocellulose degradation. Also, furfural is obtained from dehydration of pentose sugars during cellulose depolymerization under acidic conditions [25]. Furfural and its derivatives are the main flavors of foods. Furfural at low concentrations (1-12 mM) inhibits microorganisms [26]. About 13.26% of this plant extract is cyclopentane which is in steroid structure. The extract consists of lactose (9.53%) and inositol vitamin (8.55%). This plant extract has flavonoids such as 4H-pyran-4-one (5.42%). Fatty acids such as the dodecanoic acid (1.036%) and pentadecanoic acid (1.55%), alkaloids such as alpha-pyrrolidone (2.21%) and cyclic isoprenoids such as cyclotetradecan (2.32%) were detected by GC/MS.

Previous studies have reported that there are some compounds such as diterpenes, sterol, derivatives of caffeic acid and flavonoids in this genus [27]. One acylated flavonoid glycoside and two tetrasaccharides phenylethanoid glycosides, velutinosides I-II, have been isolated from *Marrubium velutinum* shoot [28]. Marrusidins A and B are two labdane-type diterpenes isolated from the chloroform extract of *Marrubium anisodon* along with polyodonine [29]. The methanol extract of the plant showed a 27.7% inhibitory activity of acetylcholine esterase used for the treatment of the disease Alzheimer. This inhibitory effect was attributed to the components that are functionally or structurally similar to tacrine [30]. According to the results of GC / MS, it is possible that this effect of the plant is related to alkaloids such as alpha-pyrrolidine, which need to be tested, and confirmed in the laboratory. This family plants have been used to treat dandruff and hair regrowth [31]. According to the presence of vitamin B7 in this plant, it can be concluded that this plant is a good candidate for the treatment of hair loss and alopecia.

3.3. Chemical Composition of *Echium Khuzistanicum* (Flowers)

According to GC/MS results, 46 compounds were found in the methanol extract of *E. khuzistanicum* flower. Each of these compounds made a peak on chromatogram (Figure 3). The plant compounds with more than 1% are shown in Table 3 including the mucilage, fatty acids, flavonoids and diterpenes. According to the results, glucose is the highest compound in the flower extract of this plant (22.32%). Mucilage in *Borago officinalis* is hydrolyzed to glucose, galactose, arabinose and allantoin [32-36] so the glucose present in the extract of this plant can be obtained by hydrolysis of mucilage [37]. Mucilages are carbohydrates with very complex chemical structures and high molecular weights. One of the most important medicinal properties of the mucilage is their anti-inflammatory property. It is used to treat gastrointestinal ulcers (stomach and intestines) and infections of the throat mucous [38]. In flower extract of this plant, 11.23% of 9, 12, 15-octadecatrien-1-ol was found. This compound is also present in the *spartium junceam* extract. Fatty acids such as a capric acid (12.6%), octadecanoic acid

Table 2. The compounds present in *M. anisodon* (more than 1%)

No.	Name of the compounds	RT[a]	MF[b]	MW[c] g/mol	Peak area%
1	Furancarboxaldehyde	13.63	$C_5H_4O_2$	96.09	20.43
2	Cyclopentane	26.41	C_5H_{10}	70.1	13.26
3	Lactose	26.64	$C_{12}H_{22}O_{11}$	342.3	9.53
4	Neo-inositole	24.86	$C_6H_{12}O_6$	180.16	8.55
5	4H-pyran-4-one	11.08	$C_5H_4O_2$	96.085	5.42
6	12-methyl-E,E-2,13-octadecadien-1-ol	20.09	$C_{19}H_{36}O$	280.496	3.22
7	Cyclotetradecan	16.17	C14H28	196.37	2.32
8	Alpha-pyrrolidone	9.21	C_4H_7NO	85.106	2.21
9	Propanoic acid	8.05	$C_3H_6O_2$	74.07854	1.88
10	Pentadecanoic acid	25.11	$C_{15}H_{30}O_2$	242.3975	1.55
11	Dodecanoic acid	24.47	$C_{12}H_{24}O_2$	200.32	1.03

a: Retention Time, b: Molecular Formula, c: Molecular Weight

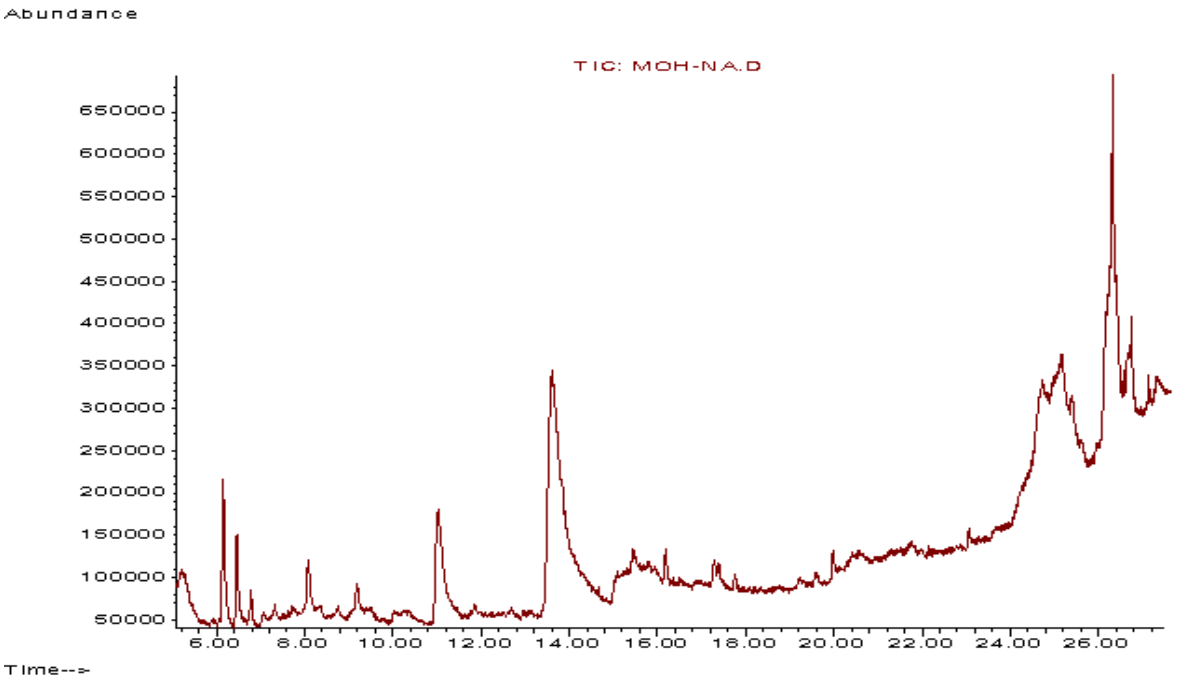

Figure 2. The chromatogram of *M. anisodon*

(3.75%) and butanoic acid (1.62%), alkaloids such as indole (2.57%), phenolic compounds such as 4-vinyl-2-methoxy-phenol (4.4%), diterpenes such as phytol (5.7%) and flavonoids such as 4H-Pyran-4-one (1.91%) and 3-Hepten-2-one (1.54%) are found in the extract. Capric acid is a 10-carbon fatty acid found in palm and coconut oil and less in animal fats and milk. This oil reduces insulin resistance and balances insulin level in humans. 36.7% of furfural and 1.99% of the sulfur compound such as dimethyl sulphone compound are also found in this plant. It is used as a food additive to maintain the quality and taste of food and treat parasitic infections and carriers of drugs (Jacob et al., 1999). Researchers try to increase the level of this fatty acid in transgenic plants [39]. Diterpene available in this plant is an alcoholic non-cyclic diterpene with antibacterial, anticancer, anti-inflammatory and diuretic effects (Furumoto, 2002). Delorme et al. (1977) reported that *Echium amoenum* has anthocyanins (13%), flavonoids (0.15%) and a small amount of alkaloids [40]. Javadzade (1995) reported that *Borago officinalis* have mucilage, tannins, Na, Ca and K. Due to the presence of different materials such as mucilage, flavonoids, phenolic compounds, diterpenes and useful fatty acids in this plant, it could be a good candidate for the treatment of many diseases and it is necessary to examine the effects of secondary metabolites of this plant.

3.4. Chemical composition of *Echinops cephalotes*

According to the results, 76 kinds of the chemical compounds found in the methanol extract of *E. cephalotes* (Figure 4). The compounds present in this plant (with more than one percent), their retention time (RT), molecular formula, molecular weight (MW), and concentration (peak area %) are presented in Table 4. Aldehydes (7.9%), coniferol (4.8%), fatty acids (5.8%) and furfural (5.4%) are found in *E. cephalotes*. According to the results, tridecanedinal is the most abundant compound in this plant (7.9%). The presence of alkaloids, saponins, plant sterols, polyphenols, and carotenoids has been detected in different parts of the echinops genus [18]. Diisodecyl ether compound derived from streptomyces had the anti-bacterial effect and was found in this plant [41]. Benzenemethanol is a type of benzyl alcohol present in many plants and an aglycone with antioxidant effect [42]. There are toxic compounds such as DDMP and benzyl alcohol in this plant so it is necessary to examine its toxicity.

3.5. Total phenol and flavonoids

The total phenol content in the samples varied from 21.24 to 177.19 µg gallic acid /mg dr.wt. Maximum amount phenol was found in *E. cephalotes* while the lowest amount was observed in the shoot of *E. khuzistanicum*.

Flavonoids are regarded as one of the most widespread group of natural constituents found in plants. The value of flavonoid content varied from 323.59 to 1305.61 µg QC/100 mg dr.wt. Maximum flavonoid content was determined in *E. cephalotes* and leaves of *E. khuzistanicum* (Table 5).

3.6. DPPH

The antioxidants are known to mediate their effect by directly reacting with ROS quenching them and chelating the catalytic metal ions. The radical scavenging activity was found to be high in *M. anisodon* followed by the flower of *E. khuzistanicum* (Table 6). IC_{50} values in *M. anisodon* and the flower of *E. khuzistanicum* are lower than ascorbic acid.

3.7. Antibacterial Effects

Data statistical analysis showed a significant difference at the level of 1%. In Tables 7-10, the diameter of the halo preventing the growth in the presence of the extracts is shown. Gentamycin was a positive control (Table 11).

3.8. Antibacterial Effects of *Echium Khuzistanicum*

The methanol extract of the root had maximum inhibition of gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. The growth inhibition was increased by increasing the extracts' concentration. *Bacillus subtilis* halo diameter in the presence of methanol extract of the root was 24.33±0.57, and in the bacteria, *Staphylococcus aureus* was equal to 24.16±1.6. These halos' diameter were also bigger than with the positive control (Gentamicin). The extracts had the lowest effect on *Pseudomonas aeruginosa*.

Table 3. The compounds present in *E. khuzistanicum* (more than 1%)

No.	Name of the compounds	RT[a]	MF[b]	MW[c] g/mol	Peak area%
1	alpha-D-Glucopyranoside	20.43	$C_7H_{14}O_6$	194.1825	22.32
2	Decanoic acid	25.57	$C_{10}H_{20}O_2$	172.268	12.60
3	9,12,15-Octadecatrien-1-ol	25.81	$C_{18}H_{32}O$	264.453	11.23
4	2-Furancarboxaldehyde	13.83	$C_5H_4O_2$	96.09	7.36
5	Phytol	18.57	$C_{20}H_{40}O$	296.539	5.7
6	4-vinyl-2-methoxy-phenol	11.26	$C_9H_{10}O_2$	150.18	4.40
7	d-Talonic acid lactone	12.31	$C_6H_{10}O_6$	178.14	4
8	Octadecanoic acid	26.28	$C_{18}H_{36}O_2$	284.48	3.75
9	Indole	9.07	C_8H_7N	117.15	2.57
10	Dimethyl sulfone	13.62	$C_2H_6O_2S$	94.13	1.99
11	4H-Pyran-4-one	13.79	$C_5H_4O_2$	96.085	1.91
12	Butanoic acid	11.06	$C_4H_8O_2$	88.11	1.62
13	3-Hepten-2-one	21.61	$C_7H_{12}O$	112.172	1.54

a: Retention Time, b: Molecular Formula, c: Molecular Weight

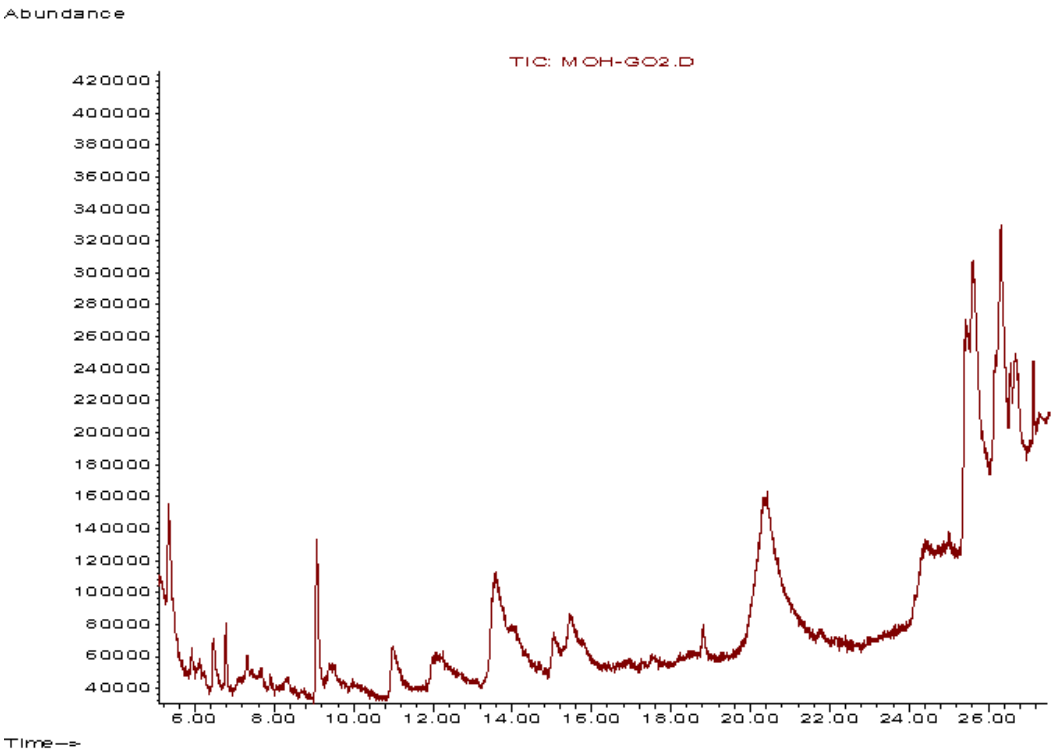

Figure 3. The chromatogram of *E. khuzistanicum*

Table 4. The compounds present in *E. cephalotes* (more than 1%)

No.	Name of the compounds	RT[a]	MF[b]	MW[c] g/mol	Peak area%
1	Tridecanedial	26.47	$C_{13}H_{24}O_2$	212.333	7.9
2	*Capronic acid*	25.14	$C_6H_{12}O_2$	116.16	5.82
3	11,13-Dimethyl-12-tetradecen-1-ol acetate	27.13	$C_{18}H_{34}O_2$	282.4614	5.76
4	2-Furancarboxaldehyde	13.83	$C_5H_4O_2$	96.09	5.46
5	Octadecanoic acid	26.47	$C_{18}H_{36}O_2$	284.48	4.91
6	Coniferol	25.61	$C_{10}H_{12}$	180.201	4.87
7	Phytol	18.57	$C_{20}H_{40}O$	296.539	4.15
8	2,2,3-Trimethyloxirane	11.21	$C_5H_{10}O$	86.132	4.08
9	2-Ethyl-2-hexen-1-ol	25.43	$C_8H_{16}O$	128.212	3.47
10	Benzenemethanol	24.38	$C_7H_{10}O$	110.156	2.94
11	2-Methyl-2-pentenal	13.90	$C_6H_{10}O$	98.145	2.59
12	Hexanoic acid	24.52	$C_6H_{12}O_2$	116.16	2.22
13	1-Methoxy-3-hydroxy methyl octane	24.81	$C_{10}H_{22}O_2$	174.281	2.13

a: Retention Time, b: Molecular Formula, c: Molecular Weight

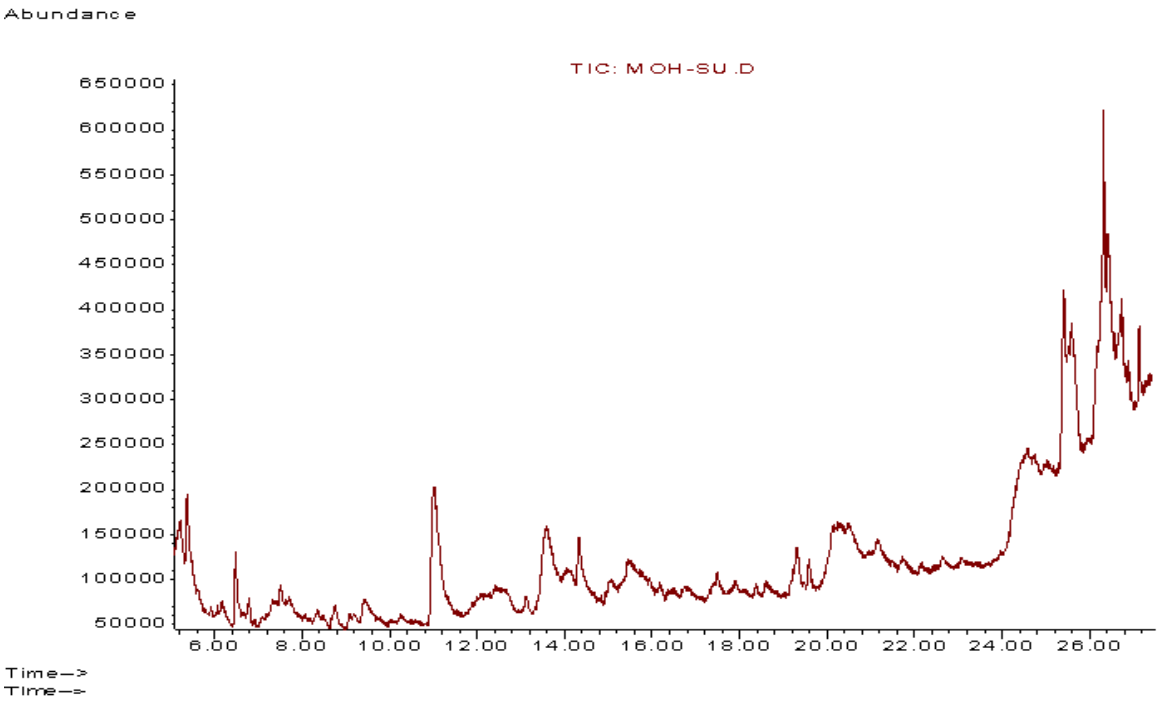

Figure 4. The chromatogram of *E. cephalotes*

Table 5. The total phenol content (µg gallic acid/mg dr.wt) and the total flavonoid content (µg QC/100 mg dr.wt)

Plant sample	*E. cephalotes*	*E. khuzistanicum* Leaf	*E. khuzistanicum* Root	*E. khuzistanicum* Shoot	*E. khuzistanicum* flower	*M. anisodon*
Phenol content	177/19±7.49[a]	60.96±1.16 [c]	28.13±0.82[d]	21.24±.083[d]	73.71± 2.44[c]	109.8±6.5[b]
Flavonoid content	1305.61[a]	1305.61[a]	323.59±4.52[b]	336.91±4.52[b]	543.69±20.61[b]	369.18±6.75[b]

Table 6. The inhibition percent (I) and IC_{50}

Variables	Ascorbic acid	*E. cephalotes*	*E. khuzistanicum* Leaf	*E. khuzistanicum* Root	*E. khuzistanicum* Shoot	*E. khuzistanicum* flower	*M. anisodon*
I	95.92±0.72[a]	7/26±0.72[e]	13.6±0.46 [e]	69.36±0.30[d]	69.08±0.08[d]	71.23± 0.28[c]	72.48±0.24[b]
IC_{50} (mg/ml)	0.39	6.94	3.67	0.72	0.72	0.14	0.14

Table 7. The diameters of clear zone (mm) in the presence of *E. khuzistanicum* shoot extracts

Bacterium	Methanol			Ethanol			DMSO		
	Concentration (mg)								
	100	200	400	100	200	400	100	200	400
S. aureus	0	10.5±0.96	20.33±1.02	0	0	11.83±0.76	8.83±1.04	11.5±0.5	13.5±0.5
B. subtilis	0	8.5±1	20.83±0.28	0	0	0	8.5±0.5	10.5±0.5	12.83±0.76
S. pyogenes	0	8.5±0.5	14±1	0	0	0	0	0	0
E. coli	8.23±0.25	16.83±1.06	23.5±0.78	0	0	12.83±0.28	0	0	0
P. aeruginosa	0	0	10±0.5	0	0	11.83±0.76	0	0	0
S. typhimurium	7.96±1.37	15±1	20.5±1.32	0	0	12.83±0.28	0	0	12.83±0.28

Table 8. The diameters of clear zone (mm) in the presence of *E. khuzistanicum* leaf extracts

Bacterium	Methanol			Ethanol			DMSO		
	Concentration (mg)								
	100	200	400	100	200	400	100	200	400
S. aureus	0	0	11.5±1.32	0	8.5±0.5	11.83±0.28	0	9.5±1.8	14.5±0.5
B. subtilis	0	6.66±0.57	11.16±0.76	7.16±0.28	7.83±0.28	12.16±0.28	0	17.83±1.2	20±1
S. pyogenes	0	0	0	0	0	0	0	0	0
E. coli	0	0	11.16±1.75	±0.58	±0.59	13.5±0.5	8.16±0.28	10.33±1.1	16.16±0.3
P. aeruginosa	0	0	8±0.5	0	0	0	0	0	8.16±0.28
S. typhimurium	0	0	17±1.32	0	9.66±0.28	16.66±0.76	8.66±0.28	13±0.5	15.16±1.6

Table 9. The diameters of clear zone (mm) in the presence of *E.khuzistanicum* flower extracts

	Methanol			Ethanol			DMSO		
Bacterium	Concentration (mg)								
	100	200	400	100	200	400	100	200	400
S. aureus	0	8.5±0.5	9.83±0.28	0	7.5±0.5	9.33±0.28	0	12.16±1.75	16.33±0.76
B. subtilis	0	0	10.83±0.76	0	0	0	0	18.33±0.28	11.5±0.5
S. pyogenes	0	8.83±0.76	9.5±0.5	0	0	0	0	0	0
E. coli	0	0	0	0	0	0	0	8.66±0.28	10.83±0.76
P. aeruginosa	0	0	0	0	0	0	0	0	0
S. typhimurium	0	0	0	0	0	0	9.66±0.76	14±1	15.66±0.57

Table 10. The diameters of clear zone (mm) in the presence of *E. khuzistanicum* root extracts

Bacterium	Methanol			DMSO			Ethanol		
	Concentration (mg)								
	100	200	400	100	200	400	100	200	400
S. aureus	12.16±1.6	16.16±1.89	24.16±1.6	10.83±0.76	14.5±0.5	20.83±0.76	0	0	0
B. subtilis	0	13.16±0.76	24.33±0.57	±110	14.33±2.08	21.66±1.15	0	0	0
S. pyogenes	9.66±0.28	13.66±0.57	21.83±0.76	0	10±0.5	13.16±0.76	0	0	0
E. coli	0	11.83±0.76	18.66±0.78	9.66±0.57	15.16±0.28	19.33±1.52	0	0	0
P. aeruginosa	0	0	12.66±1.o6	0	0	8.16±0.28	0	0	0
S. typhimurium	10.83±0.76	17.5±0.5	21±1	13.5±0.5	18.83±0.76	22±0.5	0	0	0

Table 11. The diameters (mm) in the presence of the gentamicin antibiotic as a positive control.

Bacterium	Diameter (mm)
S. aureus	17.5±0.76
B. subtilis	20.83±0.28
S. pyogenes	19±0.18
E. coli	16.25±0.52
P. aeruginosa	15±0.5
S. typhimurium	17±0.57

3.9. Antibacterial Effects of *M. anisodon*

The halo diameter of standed-growth in the presence of extracts was put in 10 groups based on mean comparison with Duncan test. The highest inhibition was seen in *Pseudomonas aeruginosa* at the concentration of 400 mg/ml of DMSO extract.

4. DISCUSSION

E. cephalotes had by far the highest amount of phenol among the plants in this study which may be due to the presence of coniferol (4.87%). Plant organs in *E. khuzistanicum* had different total phenolic content, however, the flower and leaf contain the higher phenol concentration than the other organs. This could be due to the presence of 4-vinyl, 2-methoxy phenol (4.4%) in flower of *E. khuzistanicum*. Maximum flavonoid content was determined in *E. cephalotes* and leaves of *E. khuzistanicum*. Given the importance of flavonoid compounds in the treatment of human diseases and the prevention of lipid oxidation in foods, high amounts of flavonoid in *E. cephalotes* and leaves of *E. khuzistanicum* is significant. *M. anisodon* and flower of *E. khuzistanicum* have high antioxidant activity in consensus with previous reports on the antioxidant activity in the genus of Marrubium and Echium [43-45]. There was no correlation between total phenolic content and antioxidant activity.

This activity occurs because of some compounds like phenols, flavonoids, and alkaloids in these plants. In general, the inhibitory effect on free radical DPPH depends on the type of solvent extraction, its polarity, separation method, purification of active components and method of measurement [46]. Molecular structure and position of the hydroxyl group on molecule determined antioxidant activity in flavonoid compounds [47]. Antioxidant activity in the plant was often evaluated by considering phenolic compound content. However, the antioxidant potential of the extracts does not solely depend on it. Terpenes are another major group of chemicals showed the antioxidant potential against DPPH radical scavenging activity

which could be an additional contributory factor for antioxidant activity of extracts [48]. In flower of *E. khuzistanicum* and *M. anisodon* terpenes were detected (Tables 2 and 3). *E. cephalotes* have low percent inhibition effect on free radical DPPH despite the highest amount of phenol. It is possible to conclude that the antioxidant capacity observed doesn't only come from the phenolic contents but can occur because of some other phytochemicals such as ascorbic acid, tocopherol, terpenes and the synergistic effects of them, which also affect the total antioxidant capacity. On the other hand, various kinds of phenolic compounds depending on their structure show the different antioxidant activities. The extract of *E. cephalotes* possibly has different type of phenolic compounds with different antioxidant capacities [49].

The beneficial medicinal effect of a plant is due to the secondary metabolites in the plant [50-53]. There are alkaloids, flavonoids and phenolic compounds in the methanol extract of *E. khuzistanicum* flower according to GC/MS analysis that similar results have been described by Tiwari et al. (2011) [50]. The *E. khuzistanicum* root has an excellent antibacterial effect which can be attributed to the pigment (shikonin or alkannin) in the root of this family for which antibacterial properties was reported [54]. The antibacterial properties were reported in some genus of this family, for example, Tabata et al. (1982) showed that quinone derivatives of callus culture of *Echium lycopsis* have antimicrobial properties [54] and aqueous extract of *Echium amoenum* flower has anti-viral and anti-bacterial properties [55-56].

The type of solvent that is used in the extraction determines to a large extent the active compounds that are extracted from the plant [57]. The traditional physicians used the aqueous solvent for extraction, but the results of the research showed that the organic solvent in comparison with the aqueous solvent contains more anti-microbial compounds. Most of the active antimicrobial compounds that have been identified so far are not soluble in water, so organic solvents have a higher potential for having active antibacterial materials [58]. Water-soluble compounds such as polysaccharides and poly peptides, like all types of lectins, play a more effective role in preventing the absorption of pathogens and have no real effect like antimicrobial agents [59]. Water-soluble flavonoids, which are mostly anthocyanins, and water-soluble phenol compounds are only important as antioxidant compounds and do not have a specific antibacterial effect [60]. In this study, the methanol extract of the root as an organic solvent has the highest effect on the bacteria so it can be concluded that active polar compounds in methanol extract act as antibacterial agents.

M. anisodon did not show proper antibacterial properties in consensus with previous reports. Khalil et al. (2009) reported that *Marrubium vulgare* did not have antibacterial properties on *Staphylococcus aureus* and *Pseudomonas aeruginosa* [61]. Masoodi et al. (2008) have noted the antibacterial properties of the methanol extract of *M. vulgare* only in highly concentration of extract (600 mg/ml) [62]. Aerial parts of *M. anisodon* showed 27.7% inhibitory effect on the acetylcholinesterase activity which used for the treatment of Alzheimer's disease. This inhibitory effect was known related to some components that are functionally or structurally similar to the tacrine which can be alpha-pyrrolidone alkaloids in the chemical composition of this plant [30]. This family of the plant used to treat dandruff and hair regrowth [31]. According to the presence of vitamin B7 in this plant, it can be concluded that this is a good candidate for the treatment of hair loss and alopecia.

The extract of *E. khuzistanicum* can be used in food industries as a protective agent due to high antioxidant activity. This plant is widely used in traditional medicine and is a potential source of valuable compounds such as shikonin and unsaturated fatty acids [63]. This plant is a good candidate to replace the synthetic antibiotics due to good antibacterial properties are seen in this study. Screening, identification, and isolation of the active compounds in the plants and examining the toxicity of these compounds are considered as a way for the commercialization of these compounds.

According to the results, the previous reports on the medicinal properties of the examined plants was confirmed by identification of compounds in the extract of these plants. The synthetic pathways of many secondary metabolites and associated genes in medicinal plants have not yet been completely identified. The amount of a particular secondary metabolite can be increased or decreased by identifying these synthetic pathways and genetic engineering of them. In these medicinal plants, there are valuable secondary metabolites such as alkaloids, flavonoids, diterpenes, unsaturated fatty acids, vitamin B and phenolic compounds which can be used in pharmaceutical and cosmetics industries.

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Evaluation of phenolic profile, antioxidant and anticholinesterase effects of *Fuscoporia torulosa*

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Abstract: In this study, we investigated antioxidant and anticholinesterase activities of the hexane, chloroform, acetone, methanol and water extracts of *F. torulosa* mushroom with total phenolic contents. Also, HPLC-DAD was used to identify phenolic profile of *F. torulosa*. The acetone and methanol extracts of *F. torulosa* with the highest total phenolic contents showed the highest antioxidant activity in all assays except metal chelating assay. Furthermore, antioxidant activities of the acetone and methanol extract were found to be higher than α-tocopherol and BHA used as standards in DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and CUPRAC assays. When *F. torulosa* hexane extract (41.34±1.50 %) showed moderate AChE inhibitory activity, the acetone (40.78±0.30 %) and methanol (45.39±0.65 %) extracts of *F. torulosa* indicated moderate BChE inhibitory activity. Major phenolic compounds were identified as *trans*-2-hydroxy cinnamic acid (10.05 µg/g), gallic acid (5.01 µg/g) and *p*-coumaric acid (3.04 µg/g). These results suggest that *F. torulosa* mushroom could be used as a valuable natural antioxidant source for pharmaceutical industry.

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1. INTRODUCTION

Free radicals are produced from oxygen during aerobic respiration and excessive amount of formation and accumulation causes oxidative stress [1]. The formation of oxidative stress in living organisms, in particular damages the biomolecules such as DNA, proteins and lipids, resulting in many diseases such as hypertension, ischemia, neurodegenerative diseases and rheumatoid arthritis [2]. Antioxidants prevent or reduce the harmful effects of oxidative stress. The use of synthetic antioxidants is an old practice and the safety of these substances is questioned by consumers. At present, interest in alternative natural compounds with high antioxidant effect is increasing [3].

Alzheimer's disease (AD) eliminates neurons in the cortex and limbic structure in the brain, causing learning and memory loss and behavioral disorders in humans. AD is characterized by a reduction of acetylcholine (ACh) due to damage to cholinergic neurons in some specific parts of the brain, such as the hippocampus and cortex (cholinergic hypothesis) [4]. One effective approach in the treatment of AD is the inhibition of acetylcholinesterase

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(AChE), which is responsible for the hydrolysis of ACh [5,6]. During aging, a gradual decrease in antioxidant defense mechanism and increased oxidative stress cause neuronal injury and death, which is another neurotoxic pathway causing AD [7]. Previous studies have demonstrated that antioxidant therapy is successful in improving cognitive function and behavioral deficits in patients with mild to moderate AD [8].

It is well known that mushrooms have therapeutic properties since ancient times. Up to know, many bioactive compounds such as lectins, polysaccharides, terpenoids, alkaloids, sterols and phenolics having anticancer, antioxidant, antitumor, antiinflammatory, antifungal, antibacterial, antiviral, anti-immunomodulatory activities have been isolated from mushrooms. When literature studies are examined, it is seen that mushrooms are used especially due to anticancer activity [9-15]. Mushrooms show beneficial effects on cancer, either directly as antioxidants or preventing genetic factors that cause cancer [16].

Recently, studies on the discovery of bioactive compounds from mushrooms have become more important because of their functional and therapeutic properties. Therefore, in this report, we focused to evaluate antioxidant and anticholinesterase activities of the hexane, chloroform, acetone, methanol and water extracts of *F. torulosa* mushroom with total phenolic contents. Also, phenolic profile of *F. torulosa* were determined by HPLC-DAD.

2. MATERIAL and METHODS

2.1. Mushroom Material

F. torulosa (Pers.) T. Wagner & M. Fisch. was collected from Muğla, Turkey, in November-December, 2014 and January, 2015. The voucher specimen has been deposited at the herbarium of Natural Products Laboratory of Muğla Sıtkı Koçman University with Fungarium No AT-2436.

2.2. Instruments and Chemicals

Bioactivity measurements were carried out on a 96-well microplate reader, SpectraMax 340PC384 (Molecular Devices, Silicon Valley, CA). The measurements and calculations of the activity results were evaluated by using Softmax PRO v5.2 software (Molecular Devices, Silicon Valley, CA).

Pyrocatechol, ethylenediaminetetraacetic acid (EDTA), ethanol, methanol, chloroform, acetone, hexane, ferrous chloride, copper (II) chloride and ammonium acetate were obtained from E. Merck (Darmstadt, Germany). Polyoxyethylene sorbitan monopalmitate (Tween-40), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin–Ciocalteu's reagent (FCR), 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt (ferene), β-carotene, linoleic acid, 2,2′-azino bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), α-tocopherol, neocuproine, butylated hydroxyl anisole (BHA), acetylcholinesterase from electric eels (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg, Sigma), acetylthiocholine iodide, butyrylcholinesterase from horse serum (BChE, EC 3.1.1.8, 11.4 U/mg, Sigma), butyrylthiocholine chloride, 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), galantamine, fumaric acid, gallic acid, protocatechuic acid, *p*-hydroxy benzoic acid, catechin hydrate, 6,7-dihydroxy coumarin, 2,4-dihydroxy benzoic acid, caffeic acid, vanillin, *p*-coumaric acid, ferulic acid, coumarin, *trans*-2-hydroxy cinnamic acid, ellagic acid, rosmarinic acid, *trans*-cinnamic acid were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were in analytical grade.

2.3. Extraction

The aerial parts of *F. torulosa* (2.8 kg) were extracted separately with different solvents according to their increasing polarity: hexane, chloroform, acetone, methanol at room

temperature for 24 h and four times. Solvents were evaporated on a rotary evaporator at 40°C. The hexane (10.5 g), chloroform (29.8 g), acetone (38.4 g) and methanol (55.6 g) extracts were obtained. The remaining mushroom part was allowed to stand for one day with water at 80°C. The water extract of (17.6 g) were obtained by lyophilisation using a freeze-drier. All extracts were stored at +4°C until analysis.

2.4. Determination of Total Phenolic Content

The phenolic content of extracts was stated as microgram of pyrocatechol equivalents (PEs) [17]. The phenolic contents were calculated according to the following equation that was obtained from standard pyrocatechol graph:

$$\text{Absorbance}=0.0176[\text{pyrocatechol } (\mu g)] - 0.355 \ (r^2, 0.9992)$$

2.5. Analysis of Phenolic Profile

The phenolic compounds analysis was performed by our recent publication [18] with slight modification. The mushroom sample (3 g) was extracted with acetone: water (80:20 v/v; 30 mL) at –18 °C for 24 h. After ultrasonic bath for 15 min, the mushroom extract was centrifuged at 4000 rpm for 10 min and filtered through Whatman no. 4 paper. The residue was then re-extracted by two additional 30 mL of the acetone: water. The combined extracts were evaporated at 40 °C under reduced pressure to remove acetone. The obtained extract was solved in water: methanol (80:20) and filtered through a 0.20 µm disposable LC filter disk for HPLC-DAD. Separation was achieved on an Intertsil ODS-3 reverse phase C18 column (5 µm, 150 mm×4.6 mm i.d) thermostatted at 40 °C. The solvent flow rate was 1.5 mL/ min. The sample volume injection was 20 µL. The mobile phases used were: (A) 0.5 % acetic acid in water, (B) 0.5 % acetic acid in methanol. The elution gradient was as follows: 0–20 % B (0–0.01 min); 20–60 % B (0.01–2 min); 60–80 % B (2–15 min); 100 % B (15–30 min); 100–10 % B (3–35 min); 10–0 % B (35–40 min). Detection was carried out photodiode array detector (PDA) using 280 nm as the preferred wavelength. The phenolic compounds were characterized according to their retention times, and UV data were compared with commercial standards. Three parallel analyses were performed. For the quantitative analysis of phenolic compounds, calibration curves were obtained via the injection of known concentrations (0.0, 0.00782, 0.01563, 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0 ppm) of different standards compounds i.e. gallic acid, fumaric acid, protocatechuic acid, catechin hydrate, *p*-hydroxy benzoic acid, 6,7-dihydroxy coumarin, caffeic acid, vanilin, 2,4-dihydroxy benzoic acid, *p*-coumaric acid, ferulic acid, coumarin, *trans*-2-hydroxy cinnamic acid, ellagic acid, rosmarinic acid, *trans*-cinnamic acid. The results were expressed as µg per g of dry weight (dw).

2.6. Determination of Antioxidant Activity

Total antioxidant activity by β-carotene-linoleic acid test, DPPH free radical scavenging assay, ABTS cation radical scavenging assay, cupric reducing antioxidant capacity (CUPRAC) assay and metal chelating activity on Fe^{2+} assays were carried out according to our earlier publication [19]. BHA, α-tocopherol and EDTA were used as antioxidant standards for comparison of the activities. The antioxidant activity results are expressed as 50 % inhibition concentration (IC_{50}) and inhibition percentage (%) at 200 µg/mL and $A_{0.50}$ which corresponds to the concentration producing 0.500 absorbance for CUPRAC assay.

2.7. Determination of Anticholinesterase Activity

Acetylcholinesterase and butyrylcholinesterase inhibitory activity were determined the spectrophotometric method developed by Ellman *et al.* [20]. AChE from electric eel and BChE from horse serum were used, acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. DTNB was used for the measurement of the

cholinesterase activity. Briefly, 150 µL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of the sample solution dissolved in ethanol at different concentrations and 20 µL AChE or BChE solution in buffer were mixed and incubated for 15 min at 25 °C, and 10 µL of 0.5 mM DTNB was added. The reaction was then initiated by the addition of 0.71 mM, 10 µL of acetylthiocholine iodide or 0.2 mM, 10 µL of butyrylthiocholine chloride. The hydrolysis of these substrates was monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, respectively, at a wavelength of 412 nm utilizing a 96-well microplate reader. Galantamine was used as reference compounds. The results were given as inhibition percentage (%) of the enzyme at 100 µg/mL concentration of the extracts.

2.8. Statistical Analysis

All data on antioxidant and anticholinesterase tests were the average of three parallel sample measurements. Data were recorded as mean ± S.E.M. Significant differences between means were determined by student's test, p values <0.05 were regarded as significant.

3. RESULTS and DISCUSSION

3.1. Total Phenolic Content

The calibration curve of pyrocatechol (0.0176[pyrocatechol (µg)] – 0.355; r^2, 0.9992) was used to determine the total phenolic content. Table 1 presents the total phenolic contents of the hexane, chloroform, acetone, methanol and water extracts of *F. torulosa.*

The methanol extract (131.35±0.29 µg PEs/mg) of *F. torulosa* has the highest level of the phenolic compounds among the other extracts. The total phenolic contents of the extracts were decreased in the order of methanol> acetone> water> hexane> chloroform. Total phenolic content of *Inonotus obliquus (Fuscoporia obliqua)* ethanol extract was found as 55.94±1.08 mg GAE/g extract by Zhang *et al.* [21]. The content of total phenols of 80 % ethanol, 80 % methanol and 95 % ethanol extracts of *Inonotus obliquus (Fuscoporia obliqua)* expressed as µg of gallic acid equivalents extracted from 100 mg extracts were found as 1388.505, 1404.907 and 662.184 respectively [22]. In the report of Seephonkai *et al.* [23], total phenolic contents of 50 % EtOH, 80 % EtOH, EtOH, EtOAc extracts of *F. torulosa* were studied and found as 43.80±0.78, 54.86±0.21, 62.51±0.65, 16.56±0.29 mg GA/100 mg of extract, respectively. The results obtained are consistent with the literature. Total phenolic contents of hexane, chloroform, acetone, methanol and water extracts of *F. torulosa* were studied for the first time in this research.

Table 1. Total phenolic contents of the extracts of *F. torulosa*[a]

		Total phenolic content (µg PEs/mg extract)
Extracts	Hexane	42.67±0.36
	Chloroform	19.38±0.18
	Acetone	73.54±0.08
	Methanol	131.35±0.29
	Water	55.50±0.71

[a]Values expressed are means ± S.E.M. of three parallel measurements ($p < 0.05$).

3.2. Phenolic Profile

Phenolic profile of *F. torulosa* mushroom was determined by HPLC-DAD and results are expressed as µg per g of dry weight (dw) in Table 2. HPLC-DAD chromatograms of standards and *F. torulosa* were seen in Figs. 1-2. Totally 16 phenolic and organic acid compounds namely

fumaric acid, gallic acid, protocatechuic acid, *p* hydroxybenzoic acid, catechin hydrate, 6,7-dihydroxy coumarin, 2,4 dihydroxybenzoic acid, caffeic acid, vanillin, *p*-coumaric acid, ferulic acid, coumarins, *trans*-2-hydroxycinnamic acid, ellagic acid, rosmarinic acid and *trans*-cinnamic acid were identified in the mushroom.

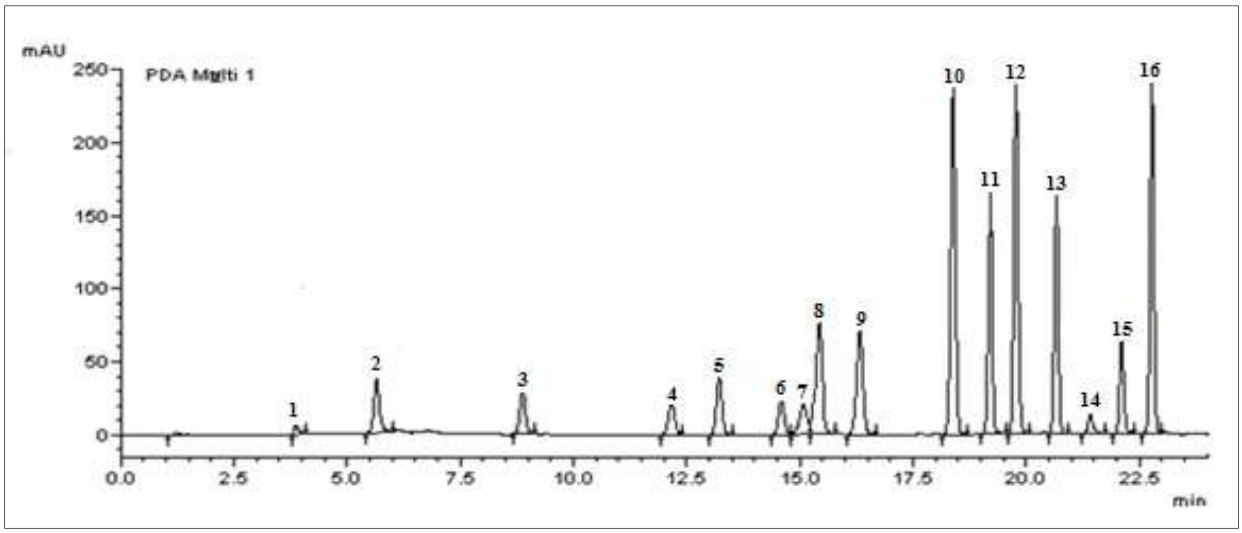

Fig. 1. The HPLC-DAD chromatogram of standards

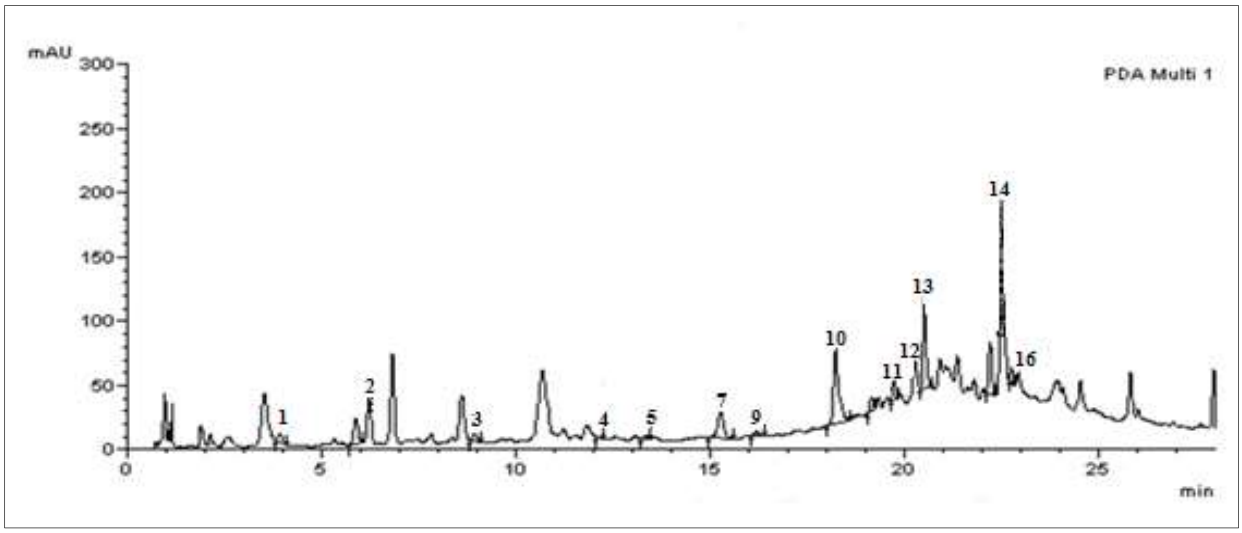

Fig. 2. The HPLC-DAD chromatogram of *F. torulosa*

As it seen Table 2, *trans*-2-hydroxy cinnamic acid (10.05 µg/g), gallic acid (5.01 µg/g) and *p*-coumaric acid (3.04 µg/g) were identified as major phenolic compounds in *F. torulosa*. Cinnamic acid derivatives are known to exhibit a range of bioactivities including antibacterial, antiviral, antifungal and antioxidant properties [24]. Gallic acid, mostly found in plants, have been reported to have antioxidant, antimicrobial, anti-inflammatory, and anticancer effects [25]. *p*-Coumaric acid is widespread in plants and mushrooms, in free or bound form. It is an important phenolic compound because of its antioxidant, anticancer, antimicrobial, antivirus, anti-inflammatory, antiplatelet aggregation, anxiolytic, antipyretic, analgesic, and anti-arthritis activities [26]. The high antioxidant activity exhibited by *F. torulosa* mushroom can be also influenced by the phenolic compounds it contains. Phenolic compounds of *Inonotus obliquus (Fuscoporia obliqua)* were analyzed using high performance liquid chromatography by Kim *et*

al. [27] and *p*-hydroxybenzoic acid (263 µg/g), kaempferol (53 µg/g), quercetin (52 µg/g), homogentisic acid (51 µg/g) and protocatechuic acid (50 µg/g) were identified as major phenolic compounds. In the literature, there is only one study about phenolic compounds of *F. torulosa.* In the report of Bal *et al.* [28], benzoic acid (170.6), chlorogenic acid (42.7), gallic acid (9.8) and catechin (2.7) were identified in *F. torulosa* ethanol extract.

Table 2. Phenolic compounds of *F. torulosa*

No	Compounds	Retention time (min)	Composition (µg/g)
1	Fumaric acid	3.86	0.33
2	Gallic acid	5.66	5.01
3	Protocatechuic acid	8.88	0.17
4	*p*-Hydroxybenzoic acid	12.18	0.01
5	Catechin hydrate	13.22	0.91
6	6,7-Dihydroxy coumarin	14.60	nd
7	2,4-Dihydroxybenzoic acid	15.08	0.48
8	Caffeic acid	15.42	nd
9	Vanillin	16.33	0.07
10	*p*-Coumaric acid	18.39	3.04
11	Ferulic acid	19.21	0.46
12	Coumarin	19.78	0.05
13	*trans*-2-Hydroxy cinnamic acid	20.67	10.05
14	Ellagic acid	21.41	0.39
15	Rosmarinic acid	22.11	nd
16	*trans*-Cinnamic acid	22.78	0.51

nd: Not detected.

3.3. Antioxidant Activity

β-carotene-linoleic acid, DPPH free radical scavenging, ABTS cation radical scavenging, cupric-reducing antioxidant capacity (CUPRAC) and metal chelating activity assays were used to determine antioxidant activities of the hexane, chloroform, acetone, methanol and water extracts of *F. torulosa.* All of the extracts showed antioxidant activities in a dose-dependent manner. Table 3 shows the IC_{50} values and inhibition percentage (%) at 200 µg/mL concentration of the extracts and standard compounds (BHA, α-tocopherol, and EDTA).

The methanol extract of *F. torulosa* showed the highest antioxidant activity in all assays except metal chelating assay and followed by the acetone extract. Antioxidant activity of the methanol extract of *F. torulosa* was found to be higher than α-tocopherol and BHA used as standards in $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and CUPRAC assays with IC_{50} value of 15.03±0.25, 10.06±0.87 and 17.43±0.29 µg/mL, respectively. When the acetone extract of *F. torulosa* showed higher antioxidant activity than α-tocopherol in $DPPH^{\bullet}$ assay (IC_{50}: 25.66±0.38 µg/mL), it showed higher antioxidant activity than α-tocopherol and BHA in $ABTS^{\bullet+}$ (IC_{50}: 11.53±0.41 µg/mL) and CUPRAC ($A_{0.50}$: 17.93±0.06 µg/mL) assays. The methanol extract with higher concentrations of phenolic contents showed the highest activity in all antioxidant activity assays except metal chelating assay. Szychowski *et al.* [22] investigated antiradical activity against $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and inhibition of xanthine oxidase of 80 % ethanol, 80 % methanol, 95 % ethanol and 95 % methanol extracts of *Inonotus obliquus* (*Fuscoporia obliqua*). For 80 % ethanol, 80 % methanol, 95 % ethanol and 95 % methanol IC_{50} values were calculated as 279.60±81.17, 447.70±16.18, 412.30±52.12, 404.10±84.14 µg/mL for $DPPH^{\bullet}$ assay; 4.17±0.76, 4.60±2.52, 4.83±1.07, 4.73±0.13 µg/mL for $ABTS^{\bullet+}$ assay; 34.37±6.08, 44.36±5.44, 62.80±7.32, 58.89±6.03 µg/mL for inhibition of xanthine oxidase assay. Previously, Khadhri *et al.* [29] studied antioxidant activity of the ethanol extract of *F. torulosa* by using $DPPH^{\bullet}$ radical

scavenging, the reducing power of iron and the iron-chelating power assays and the ethanol extract showed high antioxidant activity in DPPH• and iron-chelating assays. In a different study, DPPH• radical scavenging activities of the hexane, chloroform, 50% methanol and water extracts of *F. torulosa* were determined and the water extract indicated higher antioxidant activity among the other extracts [30]. Bal *et al.* [28] investigated DPPH• radical scavenging activity of the ethanol extract of *F. torulosa* and the extract was found to have high activity. Antioxidant activity of crude extracts (water, 50 % EtOH, 80 % EtOH, EtOH, EtOAc) of *F. torulosa* was tested by using DPPH• assays and IC_{50} values were calculated as 134.27±1.41, 18.88±0.38, 7.30±0.34, 19.23±0.42, 49.57±0.96 µg/mL, respectively [23]. Our results are in accordance with the literature. In this report, antioxidant activities of various extracts obtained from *F. torulosa* were investigated in details for the first time.

Table 3. Antioxidant activity of the extracts of *F. torulosa* by β-Carotene-linoleic acid, DPPH•, ABTS•+, CUPRAC and metal chelating assays[a]

		Antioxidant activity								
		β-Carotene-linoleic acid assay		DPPH• assay		ABTS•+ assay		CUPRAC assay		Metal chelating assay
		Inhibition (%) (at 200 µg/mL)	IC_{50} (µg/mL)	Inhibition (%) (at 200 µg/mL)	IC_{50} (µg/mL)	Inhibition (%) (at 200 µg/mL)	IC_{50} (µg/mL)	Absorbance (at 200 µg/mL)	$A_{0.50}$ (µg/mL)	Inhibition (%) (at 200 µg/mL)
Extracts	Hexane	36.84±0.97	>200	4.39±0.48	>200	9.19±0.45	>200	0.44±0.01	>200	39.81±1.68
	Chloroform	82.09±0.76	23.42±0.23	5.70±0.39	>200	15.66±0.71	>200	0.45±0.01	>200	3.51±0.20
	Acetone	95.68±0.03	3.59±0.27	78.92±0.92	25.66±0.38	90.52±0.07	11.53±0.41	3.25±0.04	17.93±0.06	15.21±0.35
	Methanol	97.60±0.70	2.57±0.01	79.20±0.13	15.03±0.25	91.62±0.15	10.06±0.87	3.74±0.04	17.43±0.29	33.67±0.37
	Water	93.94±0.79	8.23±0.31	39.07±0.33	>200	37.58±0.71	>200	0.58±0.01	151.41±0.12	27.16±0.74
Std	α-Tocopherol	90.51±0.18	2.10±0.08	87.14±0.28	37.20±0.41	85.83±0.12	38.51±0.54	0.85±0.02	66.72±0.81	NT[b]
	BHA	92.80±0.02	1.34±0.04	88.36±0.29	19.80±0.36	86.70±0.10	11.82±0.09	2.47±0.01	24.40±0.69	NT[b]
	EDTA	NT[b]	NT[b]	NT[b]	NT[b]	NT[b]	NT[b]	NT[b]	NT[b]	94.70±0.60

[a] Values represent the means ± SEM of three parallel sample measurements ($p < 0.05$).
[b] NT: not tested.

3.4. Anticholinesterase Activity

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of the hexane, chloroform, acetone, methanol and water extracts of *F. torulosa* were screened by using Ellman method. All of the extracts showed anticholinesterase activities in a dose-dependent manner. Inhibition percentage (%) at 100 µg/mL concentration of the extracts and standard compound (galantamine) are given in Table 4.

Among the extracts, the hexane extract (41.34±1.50 %) of *F. torulosa* was found as the most active against AChE enzyme. The chloroform, acetone and methanol extracts indicated moderate activity against BChE enzyme with inhibition value of 35.18±0.55, 40.78±0.30 and 45.39±0.65 %, respectively, when the hexane and water extracts were found to be inactive. According to our knowledge, there is no study about anticholinesterase activities of *Fuscoporia* species in the literature.

Table 4. Cholinesterase inhibitory activities of the extracts of *F. torulosa*[a]

		Cholinesterase Inhibitory Activity	
		AChE assay	BChE assay
Extracts	Hexane	41.34±1.50	NA[b]
	Chloroform	8.42±0.28	35.18±0.55
	Acetone	22.50±0.28	40.78±0.30
	Methanol	14.80±0.06	45.39±0.65
	Water	NA[b]	NA[b]
Standard	Galantamine	78.76±0.52	79.27±0.56

[a] Inhibition % of 100 µg/mL concentration of the extracts and compounds. Values represent the means ± S.E.M. of three parallel measurements ($p < 0.05$).
[b] NA: not active

4. CONCLUSION

In this research, antioxidant and anticholinesterase activities of various extracts of *F. torulosa* were determined with the total phenolic contents. Also, phenolic profile of the mushroom analyzed by HPLC-DAD. The acetone and methanol extracts with the highest content of total phenolic contents displayed higher antioxidant activity than standards in DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and CUPRAC assays. Totally, thirteen phenolic compounds were identified by using HPLC-DAD and *trans*-2-hydroxy cinnamic acid, gallic acid, *p*-coumaric acid were found as major phenolic compounds. In conclusion, this study reveals that extracts obtained from *F. torulosa* mushroom could be used as promising antioxidant and anticholinesterase agents. However, it is necessary to carry out isolation studies to discover the compounds responsible for these bioactivities.

Conflict of Interest

The authors declare that there is no conflict of interests in this current study.

Acknowledgement

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Published at http://ijate.net/index.php/ijsm http://dergipark.gov.tr/ijsm **Research Article**

Temperature Effect of the Theobromine's Electronic and Antioxidant Properties

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Abstract: Theobromine exists in cocoa, which has an antioxidant ingredient. It is also affect our nervous system. For this reason, it's very important to know the properties of the theobromine. Theobromine is an experimentally studied molecule in the health and pharmaceutical fields. However, there are not many studies on theobromine properties in the theoretical field. Here, we show how theobromine electronic and antioxidant properties change with temperature theoretically. The calculations, were done by using Density Functional Theory (DFT), at B3LYP/6-31G(d,p) level. Six different temperature values (263.15 K, 273.15 K, 288.15 K, 298.15 K, 318.15 K, 328.15 K) were taken into account. Our results presented that the electronic structure of the theobromine didn't change while the antioxidant properties were changed. Theobromine indicated the most antioxidant property at 263.15 K. Therefore, this situation should be taken into consideration in order to benefit more from the antioxidant properties of theobromine in the field of health and pharmaceuticals.

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Theobromine,
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Electronic Properties

1. INTRODUCTION

Theobromine is a bitter and inhalant compound got from cacoa seeds with the chemical formula $C_7H_8N_4O_2$. When someone see or hear word theobromine there is a bromine atom in the structure of it but there is no bromine atom. The root of the word theobromine is Greek, theo means '*God*' and bromine means '*food*', so *theobromine* means *'food of God'*. In the sixteenth century the consumption of cocoa drink (chocolate) expanded in Europe especially in Spain which started in America. Because of the rich lasting aroma, chocolate was considered as a good nutrition. Conservatively, high antioxidant ingredient of theobromine found in cocoa beans, is being used in medicine and pharmacology. Especially, the methylxanthines in cocoa are remarkable because of their psychoactive effect. Theobromine and caffeine exist in cocoa, affect our psychology and our state of alertness. Theobromine has much more desirable effects than caffeine so that it is a remarkable molecule in cocoa. To understand the physiological effects of the theobromine in cocoa, characteristics of the ingredients need to be examined

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carefully. Besides, the high ingredients of carbohydrates in cocoa goods may take into account further [1].

The amount of adenosine effect on receptors in neurons is related to brain physiology. The impact of theobromine on adenosine in the brain by using blockers of its specific receptors is begin the daily activities quickly. The blockers of adenosine receptors are caffeine and theobromine. The studies in the recent years represented that theobromine has psychoactive actions in humans are much more effective than caffeine [2,3]. Also, the effect of theobromine on blood pressure distinctive than that of caffeine but the reasons for these differs are not retained [2,4].

The basics of experimental and theoretical studies in the literature for the antioxidant activity of compounds are to give a hydrogen atom to free radicals. Especially, quantum chemical calculations for the quantitative structure-antioxidant activity relationship (QSAR) are more economical process than experimental studies [5–14].

The aim of this study was to investigate the electronic properties antioxidant effect of theobromine changing by temperature using quantum chemical calculations. There are five antioxidant mechanisms that explain antioxidant reactions we used are [15-23]:

1) HAT (Hydrogen Atom Transfer) mechanism:

$$ArOH + X^{\bullet} \rightarrow ArO^{\bullet} + XH \quad (1.1)$$

2) SET (Single Electron Transfer) mechanism:

$$ArOH + X^{\bullet} \rightarrow ArOH^{\bullet+} + X^{-} \quad (1.2)$$

3) SET-PT (Single-Electron Transfer followed by Proton Transfer):

$$ArOH + X^{\bullet} \rightarrow ArOH^{\bullet+} + X^{-} \quad (1.3)$$

$$ArOH^{\bullet+} \rightarrow ArO^{\bullet} + H^{+} \quad (1.4)$$

The SET-PT procedure has two steps. The initial step is an antioxidant molecule reacts with the free radical, and a cationic radical form of the antioxidant and an ionic form of the radical occur. In the continuing step the cationic radical form of the antioxidant separates into a radical and proton.

4) SPLET (Sequential Proton Loss Electron Transfer):

$$ArOH \rightarrow ArO^{-} + H^{+} \quad (1.5)$$

$$ArO^{-} + X^{\bullet} + H^{+} \rightarrow ArO^{\bullet} + XH \quad (1.6)$$

This procedure has also two steps. The initial step the antioxidant separates into an anionic form and proton, and then ions occurred in the first reaction react with the free radical. In this reaction a radical form of the antioxidant and a neutral molecule occur.

5) TMC (Transition Metals Chelation): Metals in their low oxidation state may generate free radicals according to the Fenton reaction:

$$H_2O_2 + Mn^{+} \rightarrow HO^{-} + HO^{\bullet} + M^{(n+1)+} \quad (1.7)$$

$$ArOH \rightarrow ArO^{-} + H^{+} \quad (1.8)$$

In this procedure the ability of a molecule to produce the proton is taken into consideration. One of the significant elucidatory of the electronic and antioxidant properties are dispersion of HOMO and LUMO orbitals. The higher HOMO energy is responsible for the rich

abilities of a molecule to donate a proton. Therefore, the HOMO dispersion exposes which chemical groups in a molecule are freely attacked by free radicals. From the difference between LUMO and HOMO energy we can accomplish about chemical activity of the molecule. The higher ΔE (LUMO – HOMO) is bounded with higher activity of the molecule [24].

2. MATERIAL and METHODS

The electronic and thermal properties of the theobromine in gas phase are theoretically examined by means of Density Functional Theory (DFT) using B3LYP method with 6-31G(d,p) basis set. All the calculations (optimization of the geometry of mono- and dianions, optimization of the geometry of the radicals and cation radicals presented in Figure 1) were performed by using Gaussian 16. Revision B.01 program [25] and GaussView 6.0.16 [26] was used for the visualization of the structure and simulation.

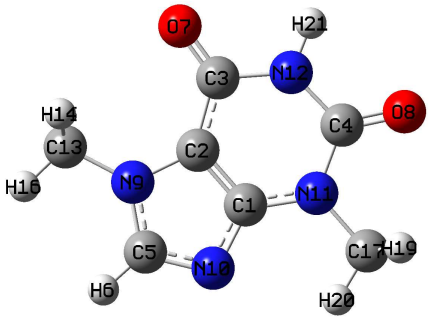

Figure 1. Optimize geometry of Theobromine

Numerical descriptors of the antioxidant mechanism such as Bond Dissociation Enthalpy (BDE), Adiabatic Ionization Potential (AIP), Proton Dissociation Enthalpy (PDE), Proton Affinity (PA), Electron Transfer Enthalpy (ETE), gas phase acidity ($\Delta H_{acidity}$) defined below have been calculated for the theobromine [15-23].

$$BDE = H_{ArO}^{\bullet} + H_{H}^{\bullet} - H_{ArOH} \quad (2.1)$$

in which $H_{ArO}^{\bullet}$ is the enthalpy of the radical, $H_{H}^{\bullet}$ is the enthalpy of the H atom, H_{ArOH} is the enthalpy of the compound.

$$AIP = H_{ArOH}^{\bullet+} - H_{ArOH} \quad (2.2)$$

here $H_{ArOH}^{\bullet+}$ is the enthalpy of cationic radical, H_{ArOH} is the enthalpy of the compound.

$$PDE = H_{ArO}^{\bullet} + H_{H}^{+} - H_{ArOH}^{\bullet+} \quad (2.3)$$

in which $H_{ArO}^{\bullet}$ is the enthalpy of the radical, H_{H}^{+} is the enthalpy of the proton, $H_{ArOH}^{\bullet+}$ is the enthalpy of cationic radical.

$$PA = H_{ArO}^{-} + H_{H}^{+} - H_{ArOH} \quad (2.4)$$

here H_{ArO}^{-} is the enthalpy of the anion, H_{H}^{+} is the enthalpy of the proton, H_{ArOH} is the enthalpy of the compound.

$$ETE = H_{ArO}^{\bullet} - H_{ArO} \quad (2.5)$$

Here $H_{ArO}^{\bullet}$ is the enthalpy of the radical, H_{ArO^-} is the enthalpy of the anion.

$$\Delta H_{acidity} = H_{ArO^-} - H_{ArOH} \quad (2.6)$$

in which H_{ArO^-} is the enthalpy of the anion, H_{ArOH} is the enthalpy of the compound.

3. RESULTS and DISCUSSION

3.1. Antioxidant Properties

For the HAT mechanism BDE is a significant numerical parameter so it presents the stability of the N-H bond in the theobromine. The lower BDE value identifies better antioxidant property. Our calculations presented in the Table 1 indicated and exhibited Figure 2 that theobromine in the 263.15 K we selected randomly is the better antioxidant activity selected.

Table 1. Antioxidant Parameters Changing by Temperature.

Temperature (K)	BDE (Hartree)	AIP (Hartree)	PDE (Hartree)	PA (Hartree.)	ETE (Hartree.)	ΔH (Hartree.)
263.15	0.176603	0.283914	0.392961	0.568707	0.108168	0.566624
273.15	0.176715	0.283920	0.393068	0.568805	0.108183	0.566642
288.15	0.176883	0.283929	0.393226	0.568949	0.108206	0.566668
298.15	0.176995	0.283936	0.393331	0.569441	0.107826	0.567081
318.15	0.177214	0.283950	0.393537	0.569236	0.108251	0.566717
328.15	0.177324	0.283958	0.393639	0.569330	0.108267	0.566732

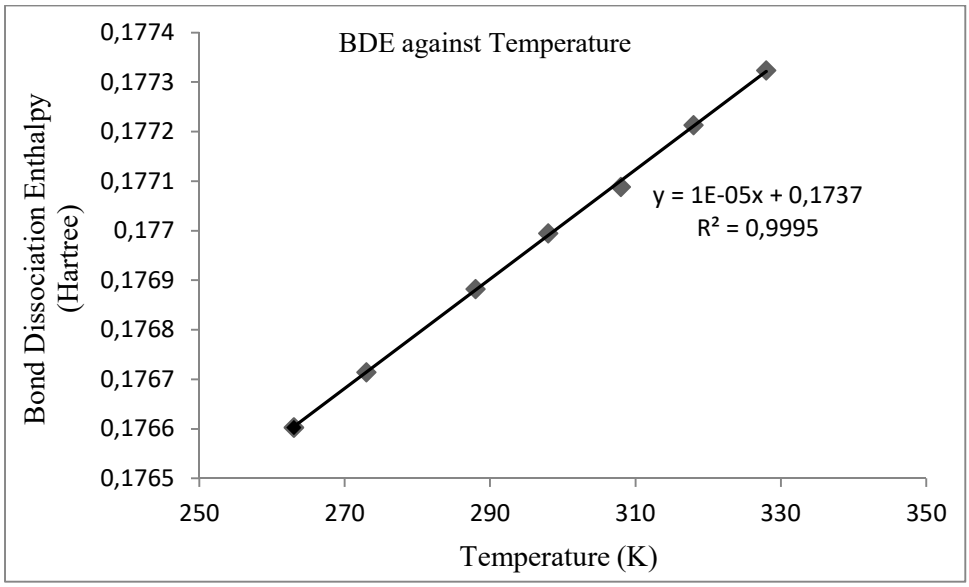

Figure 2. Bond dissociation enthalpy of the theobromine changing by the temperature.

For the SET-PT mechanism AIP is an important parameter because it defines electron forgiving by the antioxidant molecule. The molecule has low AIP parameter exhibited is more sufficient to ionization and shows strong antioxidant property. So from the Table 1 and Figure 3 that theobromine in the 263.15 K also better antioxidant activity.

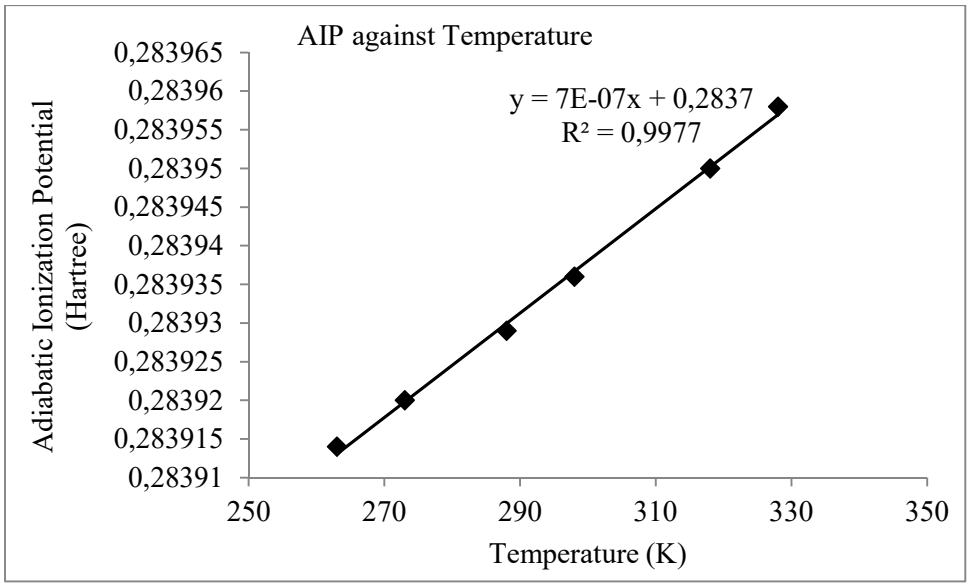

Figure 3. Adiabatic ionization potential theobromine changing by the temperature.

The second step of the SET-PT mechanism the PDE is a significant parameter. The low value of the PDE parameter indicates that SET-PT mechanism is energetically preferred. For the SPLET mechanism PA and ETE are very important parameters. Because of the PA values are higher than BDE and AIP, theobromine molecule doesn't favored SPLET mechanism.

3.2. Electronic Properties

For the electronic properties the energies of Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) are very important parameters. These parameters not explain the antioxidant property directly but describing the electron donating properties they can be connected to antioxidant activity. HOMO-LUMO energy difference represents the chemical reactivity of a molecule. The low HOMO-LUMO gap levels aren't beneficial to hole-injection [27] and evidence of the high probability of the charge transfer in the molecule [28].

In our calculations electronic parameters of the Theobromine didn't turn by the changing the selected randomly temperature. So, here in Figure 4, we use the only 298.15 K parameters for calculating the electronic parameters and presenting the HOMO-LUMO orbitals. E_{HOMO} is an energy of HOMO orbital, E_{LUMO} is an energy of LUMO orbitals, I represents ionization potential, A represents electron affinity, χ represents electronegativity, η represents global hardness, S represents global softness, ω represents global electrophilicity index and μ represents electronic chemical potential in the Table 2. The energy value between HOMO and LUMO orbitals is 5.13 eV (ΔE) as given Table 2. This value is high for the transition of the electron from the HOMO orbital to LUMO orbital. As seen from the Table 2 theobromine is a hard and electronegative molecule.

Table 2. Electronic Parameters of the Theobromine.

E_{HOMO} (eV)	E_{LUMO} (eV)	ΔE (eV)	I (eV)	A (eV)	X (eV)	H (eV)	S (eV)	M (eV)	Ω (eV)
-6.04	-0.91	5.13	6.04	0.91	3.48	2.57	0.39	-3.48	2.35

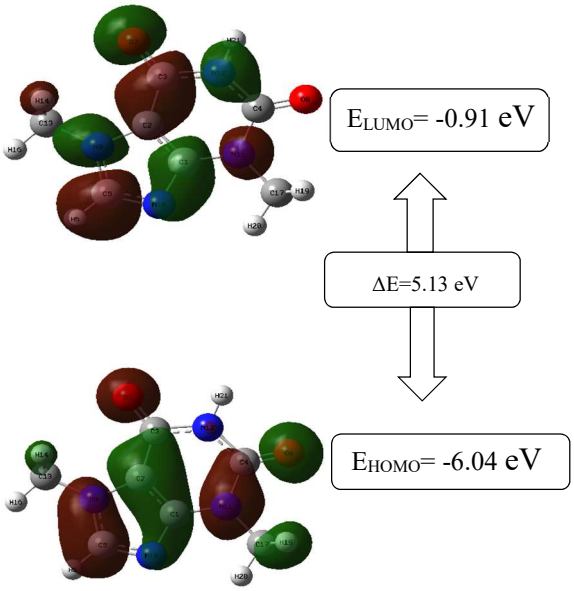

Figure 4. The atomic orbital compositions of the Theobromine.

4. CONCLUSION

In this paper, antioxidant and electronic properties of theobromine depending on the temperature were investigated theoretically using density functional theory calculations based on a B3LYP and 6-31G(d,p) basis set quality. Our calculations represented that theobromine showed best antioxidant activity in the 263 K since the bond dissociation enthalpy and adiabatic ionization potential values are lower in that temperature. However, the electronic properties of the theobromine didn't vary depending on temperature. Antioxidant parameters of the theobromine molecule presented that molecule prefer SET-PT reaction instead of SPLET. The energy difference between HOMO and LUMO orbitals of the theobromine is 5.13 eV. This large value is pointed out the proof of the poor probability of the charge transfer in the molecule and also the theobromine is a soft molecule. As a result, methods used in this article can applied to a variety of biomolecules and can enhance our understanding biomaterials nature and are shed light on how we will use such molecules.

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Published at http://ijate.net/index.php/ijsm http://dergipark.gov.tr/ijsm **Research Article**

The lipid-soluble vitamins contents of some *Vicia* L. species by using HPLC

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Abstract: In the present study, lipid-soluble vitamin compositions in the seeds of the *Vicia* L. taxa (*V. ervilia* (L.) Willd., *V. cuspidata* Boiss., *V. peregrina* L., *V. cracca* L. subsp. *stenophylla* Gaudin, *V. mollis* Boiss.& Hausskn., *V. hybrida* L., *V. sativa* L. subsp. *nigra* (L.) Ehrh. var. *nigra* L., *V. sativa* L. subsp. *sativa* (Ser.) Gaudin var. *sativa, V. crocea* (Desf.) B. Fedstch., *V. noeona* Reuter ex Boiss. var. *noeona, V. narbonensis* L. var. *narbonensis*) were determined by using HPLC. It was found that studied *Vicia* species apart from *V. ervilia* and *V. cuspidata* have highest ß-carotene contents, 1523,7±6,4 µg/g and 236,62±1,8 µg/g, respectively. Also, this study showed that *V. ervilia* and *V. cuspidata* have highest γ-tocopherol content. On the other hand, current study indicated that *Vicia* species have D3 vitamin contents between 13,8±0,62 µg/g and 50,5±2,13 µg/g. However, the α-tocopherol, α-tocopherol acetate, D2, K1, retinol and retinol acetate contents of studied *Vicia* L. species were lowest.

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1. INTRODUCTION

Fabaceae, is the third major family of higher plants, contains about 730 genera and 19400 species and are second to Poaceae in agricultural and economic priority [1-4]. The members of family are harvested as yield and used in many areas such as medicines, fuel and chemistry [5]. They are oldest crops consumed as important source of protein in the various areas of world including Europe, Middle East, Africa, and South Asia [6-8]. These are rich in carbohydrates, proteins, minerals, polyunsaturated fatty acids, fibers, carotenoids and vitamins [9-11].

Vicia L., is located tribe *Viciaeae* of the Papilionoideae, is includes about 210 species and divided into two subgenera, Vicia and Vicilla (Schur) Rouy and also 22 sections [12-14]. The species of *Vicia* are widely distributed Europe, Asia and America [15]. It was suggested that

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the Mediterranean region is the main centre of diversification of *Vicia* and is represented by 64 species, 22 subspecies and 18 varieties in Turkey [16-21]. *Vicia* L. is most significant sources for human diet in the many regions of world [22-25].

Not only plants consumed as food they can also used as the therapeutic effects against different diseases [26,27]. Although some studies performed about determining phenolics, proteins and fatty acid compositions of legumes including *Vicia* there were no enough information about the lipid-soluble vitamins contents [28-31]. Vitamins are important both human metabolism and also redox reactions in the plants [32]. Lipid-soluble vitamins including tocopherols and carotenoids, are non-enzymatic components of antioxidant system, have important role to protect cell membranes against free radical damage by working together [33-35]. Vitamin E, is a most powerful interrupting chain breaking antioxidant, defends cell membrane fatty acids agaist lipid peroxidation [36]. Also, carotenoids supply antioxidative preservation against lipid rich tissues [37-38]. It has been demonstrated that there is an inverse linking between the dietary intake of antioxidant-rich food and apparance of human diseases [39]. Studies showed that α-tocopherol, γ-tocopherol, ß-carotene, lycopene and lutein inhibit cancer, coronary heart disease, neurological diseases, diabetes [40].

The major goal of present study is to determine the lipid-soluble vitamin contents of some *Vicia* L. taxa. Hereby, it was intended to contribute the biochemical studies of legumes which not enough study about vitamin contents. It is thought that such biochemical studies will provide support to researchers working legumes and also antioxidants.

2. MATERIAL and METHODS

2.1. Collection of Plant Materials

Sample plants were gathered from the natural habitats and were stored in the Firat University Herbarium (FUH;4001-4011). The species were identified by Dr. Ahmet Şahin and Dr. Hasan Genç. Details about the materials are explained in Table 1.

Table 1. Localities of studied *Vicia* L. taxa

Taxa	Section	Locality
Vicia ervilia	Ervum	Elazig, Fırat University campus, 1060 m
Vicia cuspidata	Vicia	Elazig –Sivrice, Firat University Camp, 1100 m
Vicia peregrina	Vicia	Elazig, Fırat University campus, 1060 m
Vicia mollis	Vicia	Elazig, Hankendi Tilek hill, 1050 m
Vicia hybrida	Vicia	Malatya-Poturge, Gunduzkoy, 1250 m.
Vicia sativa subsp. *nigra* var. *nigra*	Vicia	Elazig, Fırat University campus, 1060 m
Vicia sativa subsp. *sativa* var. *sativa*	Vicia	Elazig, Fırat University campus, 1060 m
Vicia noeona var. *noeona*	Vicia	Elazig-around Cip dam, 1100 m
Vicia crocea	Cracca	Trabzon-Araklı, 1900 m
Vicia cracca subsp. *stenophylla*	Cracca	Elazig, Degirmenonu village, 1100 m
Vicia narbonensis var. *narbonensis*	Faba	Elazig-Sivrice, Firat University camp, 1100 m.

2.2.Extraction of plant materials

1 g seed used to analyse the lipid-soluble vitamins contents. The seeds are homogenised with hexane/isopropanol (3:2 v/v) [41]. The solvent was removed on a rotary evaporator at 40°C after samples were centrifuged at 10.000 rpm for 5 minutes. After that lipid-soluble vitamins were extracted according to the method of Sánchez-Machado [42]. Laboratory works were repeated three times.

2.3. Chromatographic analysis and quantification of lipid-soluble vitamins

Samples were vortexed and kept at 85 ºC for 15 min. After 5% KOH added to samples to determine the vitamin contents. Following, the tubes were cooled to room temperature and pure water was added and stirred. The non-soaped lipophilic molecules were extracted with 2x5 ml hexane. Later, hexane phase was evaporated using nitrogen gas. Finally, residue was solved 1 ml methanol/acetonitrile (25/75 v/v) and taken to autosampler tubes.

The column used was a HPLC Column A Supelcosil ^{TM} LC18 column (250 x 4.6 mm, 5 mm, Sigma, USA). The elution was performed at the flow rate of 1 ml/min. The analytical column was worked at 40 °C. One ml extract gained by saponification and it is subtilized by using 500 µl of the mobile phase. It was used LC-10 ADVP UV-visible as pump, SPD-10AVP as detector, CTO-10ASVP as column furnace, SIL-10ADVP as otosampler, DGU-14A as degasser unite. Detection was conducted at 320 nm for retinol acetate, retinol, 215 nm for vitamin D2, D3, γ-tocopherol, α-tocopherol acetate, α-tocopherol, 235 nm for vitamin K1 and 450 nm for ß-carotene. Identification of the vitamins were performed by external standard mixture. Class Vp 6.1 software assisted at workup of the data [43]. The results of analysis were uttered as µg/g for samples.

3. RESULTS

The lipid-soluble vitamins contents of *Vicia* L. taxa were given in Table 2. It was found that *V. peregrina* has highest ß-carotene content (1523,7±6,4 µg/g) in this study (Table 2). *V. sativa* subsp. *nigra* var. *nigra* (614,6±4,5 µg/g) and *V. sativa* subsp. *sativa* var. *sativa* (746,2±3,3 µg/g) have high ß-carotene content among studied 11 *Vicia* species. The other species have between 236,62±1,8 µg/g and 452,8±3,5 µg/g except for *V. ervilia* and *V. cuspidata.* However, *V. ervilia* and *V. cuspidata* have quit high the γ-tocopherol content (1326±3,21 µg/g; 2148±4,12 µg/g, respectively). Also, *V. narbonensis* have 86,3±2,11 µg/g γ-tocopherol content. The other studied species have low amounts or don't have γ-tocopherol content. It was found that r-tocopherol and D2 contents were quit poor. The D3 vitamin contents of studied *Vicia* species found between 50,5±2,13 µg/g (*V. naeona* var. *naeona*) and 13,8±0,62 µg/g (*V. hybrida*). It was found that *Vicia mollis* have high α-tocopherol content (14,3±1,19 µg/g) while *V. cuspidata* have low amount (0,7±0,1 µg/g). *V. ervilia* don't have α-tocopherol acetate content. *V. cracca* subsp. *stenophylla* (8,8±0,9 µg/g), *V. cuspidata* (5,7±1,1 µg/g), *V. mollis* (2,5±0,2 µg/g), *V. sativa* subsp. *sativa* var. *sativa* (2,2±0,2 µg/g) and *V. peregrina* (0,3±0,01 µg/g) have K1 vitamin content among studied *Vicia* species. The other studied lipid-soluble vitamins (α-tocopherol, α-tocopherol acetate, D2, K1, retinol, retinol acetate) were low amounts or absent (Table 2).

Table 2. The lipid-soluble vitamins contents of studied *Vicia* L. taxa

	Lipid-soluble vitamins (µg/g)									
Taxa	ß-carotene	γ tocopherol	R-tocopherol	D2	D3	α -tocopherol	α -tocopherol acetate	K1	Retinol	Retinol acetate
Vicia ervilia	-	1326±3,21	-	-	18,8±,0,97	-	-	-	0,2±0,02	0,9±0,06
Vicia cuspidata	-	2148±4,12	-	-	33,9±1,49	0,3±0,03	0,7±0,1	5,7±1,1	0,2±0,03	0,9±0,04
Vicia peregrina	1523,7±6,4	-	-	-	34,4±1,49	0,8±0,02	2,2±0,4	0,3±0,01	0,4±0,01	0,6±0,03
Vicia mollis	331,6±3,1	-	-	-	18,2±0,95	-	14,3±1,19	2,5±0,2	0,1±0,01	0,2±0,01
Vicia hybrida	452,8±3,5	-	-	-	13,8±0,62	0,3±0,01	5,4±1,1	-	0,1±0,01	0,2±0,03
Vicia sativa subsp. *nigra* var. *nigra*	614,6 ±4,5	-	-	-	33,8±1,12	0,2±0,04	1,7±0,3	-	0,3±0,01	0,7±0,02
Vicia sativa subsp. *sativa* var. *sativa*	746,2±3,3	7,9±0,98	-	-	32,9±0,94	1,1±0,1	1,5±0,4	2,2±0,2	0,2±0,02	0,4±0,03
Vicia naeona var. *naeona*	350,94±2,7	-	-	0,9±	50,5±2,13	0,1±0,01	2,2±0,1	-	0,4±0,02	0,7±0,05
Vicia crocea	439,4±1,6	0,6±0,04	-	-	12,5±0,85	1,9±0,7	7,1±0,12	-	-	0,2±0,01
Vicia cracca subsp. *stenophylla*	347,8±3,8	-	-	-	14,1±0,94	0,6±0,01	2,9±0,6	8,8±0,9	-	0,2±0,01
Vicia narbonensis var. *narbonensis*	236,62±1,8	86,3±2,11	0,3±0,01	-	27,6±1,31	0,6±0,03	3,1±0,1	-	0,2±0,01	0,5±0,03

4. DISCUSSION

Legumes are low in calories and significant source of protein, vitamin, starch, mineral, fiber and pyhonutrient content [11,44]. They have protective role against various diseases such as cardiovascular, cancer and diabet [45,46]. Present study showed that studied *Vicia* species apart from *V. ervilia* and *V. cuspidata* have highest ß-caroten content between 1523,7±6,4 µg/g and 236,62±1,8 µg/g. Carotenoids including ß-carotene and α-carotene have important role due to provitamin A activity [47]. They play vital role the protection against cancer, cardiovascular diseases [48-49]. However, Mamatha et al. [47] found that studied legumes including *Phaseolus, Vigna, Lens* and *Cicer* have lowest α-and β-carotene contents. Similarly, Sahin et al. [50] found that *Vicia* species have lowest β-carotene content between 0,96 µg/g and 0,3 µg/g. And also it was found that legumes don't have β-carotene content a study done by Yao et al [51].

Vitamin E, the other studied lipid-soluble vitamins, which has several health benefits such as hypoglisemic, antihypertensive, neuroprotective and anti-inflammatory [52-54]. In this study, it was found that some species from *Vicia* (*V. ervilia* and *V. cuspidata;* 1326±3,21 µg/g; 2148±4,12 µg/g, respectively) have high the γ-tocopherol content while the other species have low amounts or don't have γ-tocopherol content except for *V. narbonensis*. However, Bağcı et al. [45] indicated that α and γ–tocopherol were detected as the most abundant tocopherol components in all of the studied taxa except *Lathyrus inconspicuus, Onobrychis luetiana* and *Onobrychis hypargyrea.* It was determined that α-tocopherol acetate was the major compound in all the botanical species except for *R. bulbosus* in which free α-tocopherol was the major compound work done by Valdivielsa et al. [56]. Present study determined that *Vicia* species have D3 vitamin contents (13,8±0,62 µg/g-50,5±2,13 µg/g). It was showed that *Lathyrus*, the other genus from legume, has vitamin D3 content between 8,27±0,04 µg/g and 45,07±2,23 µg/g (Sahin et al., 2009). On the other hand, the other studied lipide-soluble vitamins (α-tocopherol, α-tocopherol acetate, D2, K1, retinol, retinol acetate) were low amounts or absent based on present results. Similarly, Sahin et al. [57] found that retinol acetate and vitamin D2 contents of *Lathyrus* low or absent. Another study showed that *Vicia* species have vitamin A content between 0,397 µg/g and 0,922 µg/g [57]. Also, it was determined that *Vicia* species have high vitamin E content (2,778-19,19 µg/g) study done by Sahin et al. [50]. However, Sahin et al. [50] indicated that *Lathyrus* has vitamin K1 content between 2,1±0,06 µg/g and 11,15±0,08 µg/g. Also, they found that *Lathyrus* has α-tocopherol content between 11,51±0,2 µg/g and 67,22±0,14 µg/g.

5. CONCLUSION

In conclusion, this study presented that studied *Vicia* taxa have highest β-carotene contents except for *V. ervilia* and *V. cuspidata* which have highest γ-tocopherol content. Also, studied *Vicia* taxa have high D3 vitamins. It was fouınd that the other studied lipid-soluble vitamins low amounts or absent in this study.

Conflicts of Interest

Authors declare no conflict of interest.

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