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Preface

Dear Academicians, Readers and Educators,

We are pleased to present the issue of the *International Journal of Secondary Metabolite* as a special issue entitled '*I. International Congress on Medicinal and Aromatic Plants - "Natural And Healthy Life"*'. This special issue contains some of scientific studies presented in the congress.

Hosting the *I. International Medical and Aromatic Plant Congress*, held in Konya on 9-12 May 2017, by the coorperation *T.R. Ministry of Forestry and Water Affairs*, *General Directorate of Forestry* and *Necmettin Erbakan University* was a great honor for us.

The total number of abstract submission for the congress was 1923. After the scientific evaluation, 85 abstracts were rejected and 244 abstracts were withdrawn. As a result, a total of 1594 abstracts were accepted for presentation: 280 of them as oral presentation and 1314 as poster presentation. 2604 authors were contributed and 1543 participants were participated to the congress. The studies presented in the congress was electronically shared in terms of accessibility.

The authors of 220 papers, presented in the congress, submitted to the International Journal of Secondary Metabolite for publication. 70 of them were published and 150 full papers were rejected due to revision deadline, reviewing process etc. after reviewing process.

I would like to special thank to the Journal founder for publishing and also to the editor, editorial board and authors for contributing this issue.
Best regards.

Dr. Muzaffer ŞEKER
Rector of Necmettin Erbakan University

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Effect of *Ocimum basilicum* on mesenchymal stem cell proliferation and differentiation: Does the effect change according to niches?

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Abstract: It is a big issue that reduced bone density and large fractıres in dentistry and orthopedics. Side effects caused by synthetic drugs lead to medical and ethical problems. Thus, plants and medicinal plant research take attention. Aim of this preliminary in vitro study is to investigate the effect of Ocimum basilicum extract on dental pulp (DP) and bone marrow (BM) derived mesenchymal stem cell (MSC) proliferation, osteogenic differentiation and immunological response to TNF-α. Human dental pulp tissue was obtained from patients (15-20 years of age) who were undergoing extraction of third molars for orthodontic reasons at the Department of Oral and Maxillofacial Surgery, University of Gazi University*. xCELLigence system was used to determine prolfieration of DP- and BM-MSCs. Adipogenic and osteogenic differentiation was shown and calcium concentration, osteocalcin and osteonectin levels were examined. Inflammatory environment was mimiced through TNF-α stimulation and IL-6 and IL-10 levels were defined by ELISA. Doubling time mwith O. basilicum was found in DP- MSCs (38 h) and BM-MSCs (76 h). IC50 value was shown as 148 µg/mL in DP-MSCs and 178 µg/mL in BM-MSCs. Calcium concentration of BM-MSCs was found decreased in O. basilicum treated groups. Level of ostoenectin was reduced in O. basilicum treated cells suggesting that the Extract accelerated the osteogenic differentiation. We suggest that O. basilicum could be a smart ostoeinductive agent where BM-MSCs should be investigated further. Rich flora of Turkey is an opportunity for us and encourangement can easily give inside to medicinal plant investigations. *B.30.2.GÜN.0.20-122 Ethics Committee Report

Keywords: mesenchymal stem cell, osteogenic differentiation, dentistry, plant extract, Ocimum basiliicum

1. INTRODUCTION

Dental pulp mesenchymal stem cells (DP-MSCs) are a type of mesenchymal stem cell (MSCs) found in the cell-rich zone of the pulp tissue of teeth [1]. DP-MSCs have a strong self-renewal ability and the potential for multi-directional differentiation, which gives them great therapeutic potential for repairing damaged and/or defective tissue [2].

The presence and maintenance of alveolar bone is tooth dependent. After tooth extraction, the alveolar bone is slowly resorbed down to the body of the jaw bones. In cases of complete tooth loss, there is progressive bone resorption, which can result in extensive atrophy of the jaw

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bones and lead to major clinical challenges for implant placement and the construction of dental prostheses [3]. Even without therapeutic intervention, the periodontium can exhibit a significant capacity for regeneration. However, such endogenous activity has limited capacity for periodontal regeneration. In addition, the impairment of bone formation increases in patients with osteoporosis and diabetes mellitus and related conditions. We hpothesize that a natural agent that maintains MSCs viability, promotes osteogenic differentiation while modulating the immunological response could achieve success in regeneration during healing and may also prevent bone resorption and improve regeneration.

Ocimum basilicum belonging to the Lamiaceae family, is a pleasent by smelling perennial shrub which grows in several regions all over the World [4, 5]. O. basilicum is one of the species used for the commercial seasoning. O. basilicum is a condimental plant cultivared in some parts of Turkey, and used frequently in soups, desserts, pickles, pizza, spagetti sauce, egg, cheese dishes, tımato juice, dressings, meat products etc. Also used in pharmacy for diuretic [5]; and its oil has been found to be beneficial for the alleviation of mental fatigue, colds, spasms, rhinitis, and as a first aid treatment for wasp stings and snake bites. The essential oil has antifungal, physicochemical and inset repelling activiity [6-9]. It is also regarded as highly antiseptic and has been applied in boths to prevent postpartum infections.

Ocimum group of species have been shown to possess a wide range of chemopreventive and medicinal activities [10-13]. In addition, extracts of the leaves displayed powerful antioxidant activity in various assay models [14, 15]. O. basilicum had been found to contain linalool, eugenol, methyl chavicol, methyl cinnamate, ferulate, methyl eugenol, triterpenoids and steroidal glycoside known to exhibit antioxidant activities [16-18]. It is therefore possible that the extracts may serve as a remedy by blocking or intercepting the activity of environmentally acquired toxins such as mycotoxins, insecticides and pesticides.

Although various physiological activities of O. basilicum have been demonstrated, its link to osteogenic differentiation of mesenchymal stem cells has never been explored. In the present study, we hypothesized that O. basilicum could maintain the viability of DP-MSCs, induce promote their osteogenic differentiation, which may enable the successful regeneration of hard tissues. We also used bone marrow (BM) derived MSCs as control.

2. MATERIAL and METHODS

2.1. Extraction of Plant Samples

O. basilicum flower buds were purchased from the local market in Mugla, Turkey. The air-dried plant samples were extracted with ethanol (Merck, Taufkirchen Germany) using a Soxhlet apparatus. The extracts were evaporated and stored in sterile opaque glass bottles under refrigerated conditions until use. The dried extract was prepared in DMEM-LG with 10% foetal bovine serum (FBS) (Invitrogen, Carlsbad, Calif., USA), 1% L-glutamine (Sigma, Taufkirchen Germany) and 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, Calif., USA) for the studies.

2.2. Isolation and Culture of Dental Pulp Mesenchymal Stem Cells

Human dental pulp tissue was obtained from patients (15-20 years of age) who were undergoing extraction of their third molars for orthodontic reasons at the Department of Oral and Maxillofacial Surgery, University of Gazi, Ankara. All patients provided informed consent (Ethics Commit. Rep. No: G.Ü. B30.2. GÜN 0.21.71.00). After the tooth surfaces were disinfected, the teeth were mechanically fractured, and the dental pulp was gently isolated with forceps. The pulp tissue was rinsed in α-MEM supplemented with 2 nM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS, Invitrogen) (hereafter referred to as the MSC culture medium), after which it was minced into fragments of 1 to 2 mm3. The tissue fragments were cultured in T75 Nunc plates in the MSC culture medium at

37°C in a humidified atmosphere containing 5% CO2. Human BM-MSCs were a kind gift from Hacettepe University Center for Pediatric Stem Cell and Research and Development. BM-MSCs were suspended at a concentration of 1x106 cells/mL in MSC culture medium. The culture media was changed every 2 to 3 days, and the cell cultures were monitored regularly with an inverted microscope (Olympos CKX41, Tokyo, Japan). Upon reaching 70-80% confluence, the cells were harvested with 0.05% Trypsin/EDTA (Sigma, Taufkirchen Germany) and sub-cultured for further experiments.

2.3. Immunophenotypic Analysis

The culture-expanded adherent cells were analysed by flow cytometry (BD FACSAria, USA). The antibody panel included CD29- FITC (e-bioscience, USA); CD73-PE (BD, USA), CD 90-PE (BD, USA), CD44-PE (e-bioscience, USA) as mesenchymal stromal markers, as well as their isotype controls. CD45-FITC (BD, USA); CD14-PE (BD, USA); and CD34-FITC (BD USA) were used as haematopoietic markers to exclude cells of haematopoietic origin. The relative frequencies of the cells that expressed the respective surface markers were analysed using FACS Diva software 6.0.0 (BD) by acquiring 10,000 events for each sample.

2.4. Effect of O. basilicum on Proliferation of the MSCs Using the xCELLigence System

Initially we examined the proliferation of DP-MSCs in a 24 well culture microplate seeded at a density of 5000 cell/cm2. DP-MSCs were cultured with different concentrations (1, 3, 5, 10, 25, 50, 75, and 100 µg/mL) of O. basilicum up to the control group had 90% confluency. Cells were counted by trypan blue method and the three concentrations which induced the cell number was selected for xCELLigence analysis. The xCELLigence system was used according to the manufacturer's instructions [19]. Briefly, the E-plate 96 was connected to the xCELLigence system and verified in the cell culture incubator to ensure that proper electrical contacts were established, and the background impedance was measured. Subsequently 100 µl of MSCs culture media containing 5, 10 and 25 µg/mL O. basilicum extract and standart culture media as control were added into each well of E-plate 96. Meanwhile, the cells were resuspended (5000 cells/cm2) in MSC culture media for their concentration. 100 µl of each cell suspension was added to each well, in order to determine effect of O. basilicum extract on cell proliferation. Cell growth and proliferation were monitored every 30 min for up to 290 h. The growth curve, cell index, and doubling time (DT) values were determined.

2.5. Effect of O. basilicum on MSCs Differentiation

The concentration that decreased the doubling time and increased the proliferation was selected based on the results from the xCELLigence system analysis. The selected concentration was added to the osteogenic and adipogenic differentiation media [20]. The images were obtained with a CKX41 digital imaging microscope (Olympus, Tokyo, Japan). The secreted Osteocalcin (OCN) and Osteonectin (ON) levels in the supernatants were assessed using an ELISA kit according to the manufacturer's instructions (R&D Systems, Inc. Minneapolis). The limits of detection for the ELISA were 1.2 to 75 ng/mL for OCN and 1.56 to 50 ng/mL for ON.

2.6. Determining the Immunomodulatory Activities

DP- and BM-MSCs were plated at a density of 5000 cell/cm2 in 96-well culture plates and allowed to attach overnight. The cells were pretreated with 10 µg/mL O. basilicum extract for 1 h, and 10 ng/mL TNF-α were then added. After 24 h, the cell culture supernatants were collected and stored at -80°C for use in the IL-6, IL-10 ELISAs, according to the manufacturer's instructions. The ELISA limits were 0,052-0,118 pg/mL for IL-6 and 0,39-25 pg/mL for IL-10. Media alone, with TNF-α, and with T. spicata var. intricata were included as controls.

2.7. Statiscal Analysis

All calculations were performed using the RTCA integrated software of the xCELLigence system. The RTCA software fits the curve of the selected sigmoidal dose response equations to the experimental data points. The data are presented as the mean (µg/mL) ± SD (n=4). For the proliferation experiments, the statistical analysis was performed using one-way analysis of variance (ANOVA) ($p<0.05$).

3. RESULTS and DISCUSSIONS

3.1. Identification of MSCs

The common MSC markers (CD29, CD73, CD44, and CD90) were constitutively positive (>95%) and the hematopoietic markers (CD14, CD34, and CD45) were negative (>95) in all samples tested, indicating a mesenchymal origin of the cells (Figure 1).

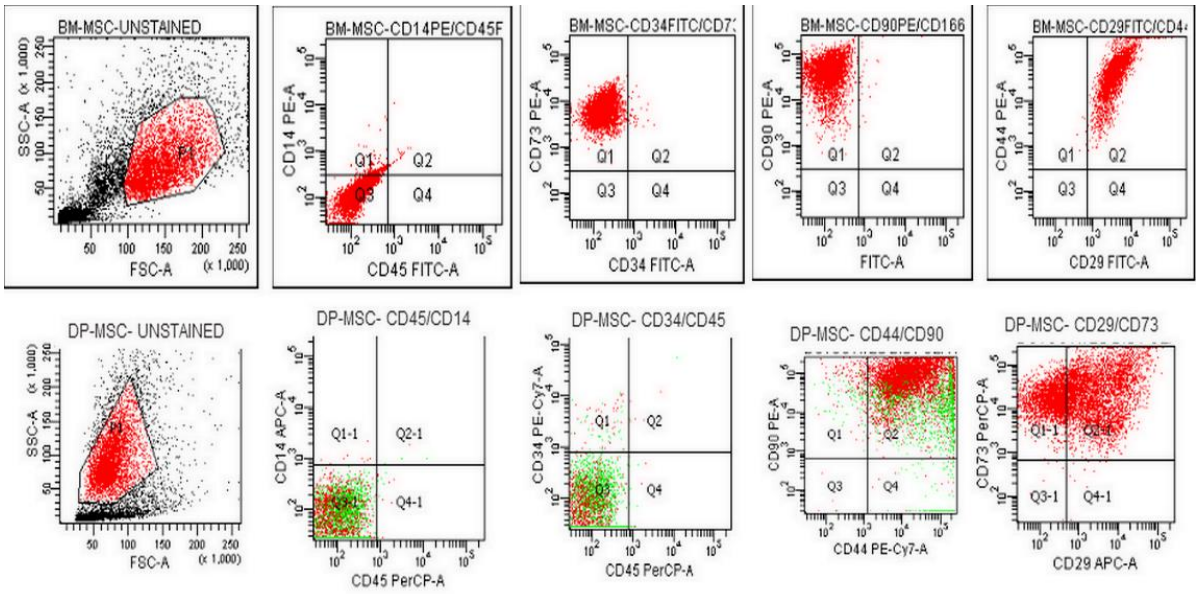


Figure 1. Surface markers of DP-MSCs and BM-MSCs

3.2. xCELLigence Assays

Trypan blue assay exhibited an interesting result that cell number at 1 µg/mL was higher than the Control (Figure 2). However at 3 µg/mL the cell number decreased at a ration of 21%, and at the subsequent concentration 5 µg/mL cell number was found increased to the 50 µg/mL concentration. At 75 and µg/mL the cell number was found reduced afor a 42% ratio. We estimated at 100 µg/mL the cell number would decrease, however it increased again .

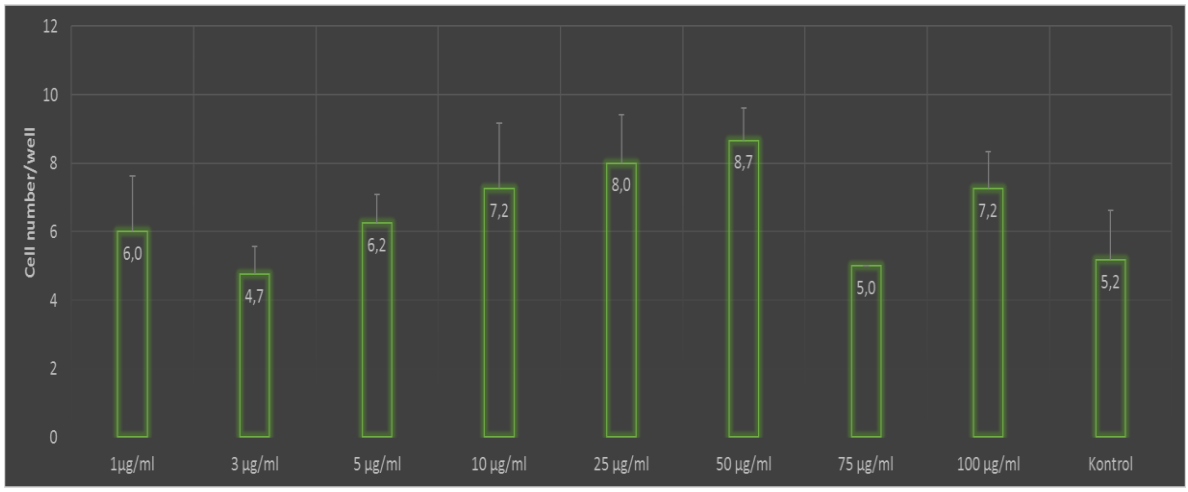
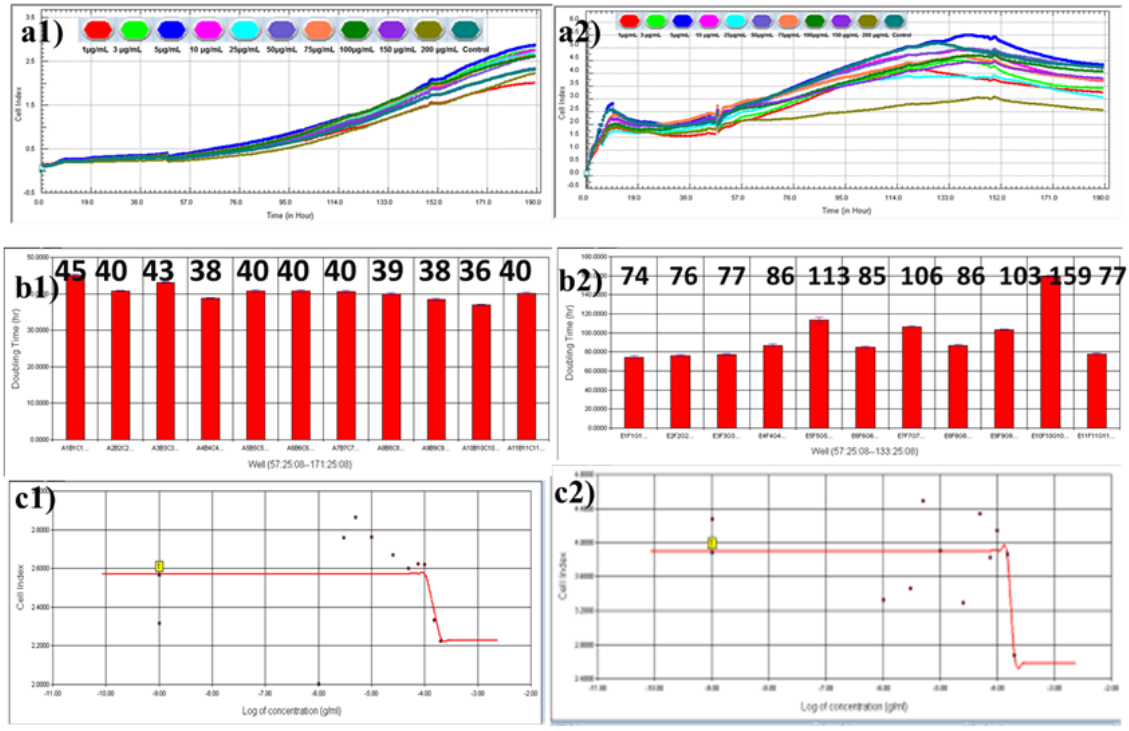


Figure 2. Trypan Blue Assay results.

Subsequently we conducted the xCELLigence assay for the same concentrations and found different values. of course, we could examine more parameters than the Trypan Blue Assay. We obtained the growth curves of DP-MSCs and BM-MSCs. Using the xCELLigence system, we obtained a growth curve for MSCs which provides information on three parameters: the lag phase before cell proliferation is initiated after subculture, the DT in the middle of the exponential growth phase, and the terminal density [2]. The lag phase is the time period that the cells are recovering from trypsinization, synthesizing new extracellular matrix and cytoskeleton, and adapting for attachment, spreading and re-entering the cell cycle. Cells do not divide in the lag phase [21]. We could conclude that *O. basilicum* showed similar adhesive effect on DP-MSCs till 98h whilst BM-MSCs were adhered and proliferated more than the Control group (Figure 3). 200 µg/mL showed lethal effect in BM-MSCs while 1 µg/mL but not 200 µg/mL for DP-MSCs. Ideally, towards the end of the log phase, the culture becomes confluent and its growth rate reduces, and in some cases, cell proliferation ceases. At this stage, the culture enters the plateau, or stationary phase, in which cell division is balanced by cell loss. DP-MSCs were still alive where BM-MSCs began to reduced their cell number. We determined the cell indexes for the time intervals of the lag, log, and plateau phases using the growth curve. xCelligence assay showed that cells treated with *O. basilicium* had lower adhesion, but similar viability when compared with control cells. The doubling time (DT) was reduced at 10 µg/mL in DP-and BM-MSCs. Thus, *O. basilicum* could be a good proliferation inducer. The IC50 value was found 7,7 µg/mL for DP-MSCs and 17 µg/mL for BM-MSCs at 290 h. Oriental medicine practices are primarily based on personal experience, which often result in unknown mechanisms and difficulties in dose specification. We used a real-time monitored system, xCELLigence, to determine the effective concentration. The xCELLigence system is much more sophisticated than other conventional endpoint, cell-based assays. Real-time and continuous monitoring enable the label-free assessment of cell proliferation, viability, and cytotoxicity by showing the physiologic state of the cells and eliminating expensive reagents that are used in conventional cell analyses [21].



	57 h	171 h	189 h
1µg/ml	0,35	1,83	2
3 µg/ml	0,36	2,46	2,75
5 µg/ml	0,4	2,49	2,77
10 µg/ml	0,33	2,54	2,95
25 µg/ml	0,36	2,4	2,66
50 µg/ml	0,34	2,28	2,59
75 µg/ml	0,36	2,37	2,62
100 µg/ml	0,36	2,37	2,62
150 µg/ml	0,29	2,06	2,33
200 µg/ml	0,24	1,87	2,22
Kontrol	0,32	2,06	2,31

Figure 3. xCELLigence analysis of DP-MSCs and BM-MSCs. Growth curve of the cells. a1) DP-MSCs, a2) BM-MSCs; doubling time of the DP-MSCs (b1) and BM-MSCs (b2); IC50 values for DP-MSCs (c1) and BM-MSCs (c2); the cell indexes at lag mid log, and stationary phases (d1) of the cells were determined.

3.3. Differentiation Assays of MSCs

The differentiation characteristics features of the cells were studied. (Figure 4). Adipogenic differentiation exhibited a quite difference in DP-MSCs treated with *O. basilicium*. Approximately 20% of the cells became rounder; however, no lipid droplets were observed in DP-MSCs. In contrast to adipogenic differentiation, the DP-MSCs underwent rapid osteogenic differentiation. Calcium granules similar to bone nodules were seen in BM-MSCs. The osteogenic differentiation potentials of DP-MSCs in vitro and in vivo have been well documented in a variety of studies [2, 22]. Our findings were agree with those of Gronthos et

al (2000) who expanded DP-MSCs from single-cell clones and demonstrated that they exhibited osteogenic differentiation but did not form lipid-laden adipocytes [2]. We found well deposited calcium granules in *O. basilicum* treated Group in DP-MSCs. Surprisingly in BM-MSCs osteogenic differentiation did not found on *O. basilicum* Group. To determine the osteogenic markers in culture supernatant we examined osteonectin and osteocalcin levels by ELISA. During osteogenic cell differentiation, the markers of the undifferentiated cells are gradually turned off, and the differentiation markers are sequentially expressed. We observed the sequential secretion of proteins at the end of the assay, in which the ON levels decreased in the *O. basilicum*-treated group compared to the control group. ON is an early marker of osteogenesis that is synthesized by preosteoblasts and has less affinity to collagen. The ON transcript is quite stable, with a half-life of >24 hours under conditions of transcription arrest [23].

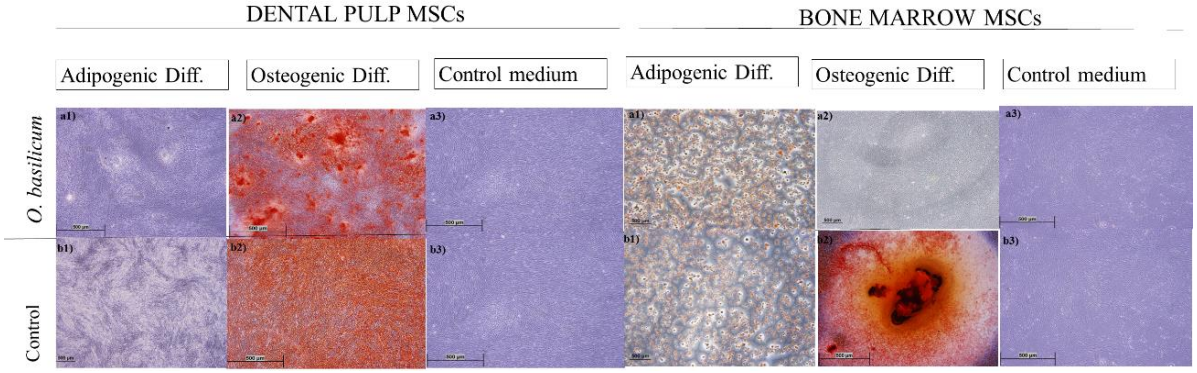


Figure 4. Differentiation potential of DP-MSCS and BM-MSCS. adipogenic differentiation was not shown in DP-MSCs while BM-MSCs were well differentiated.

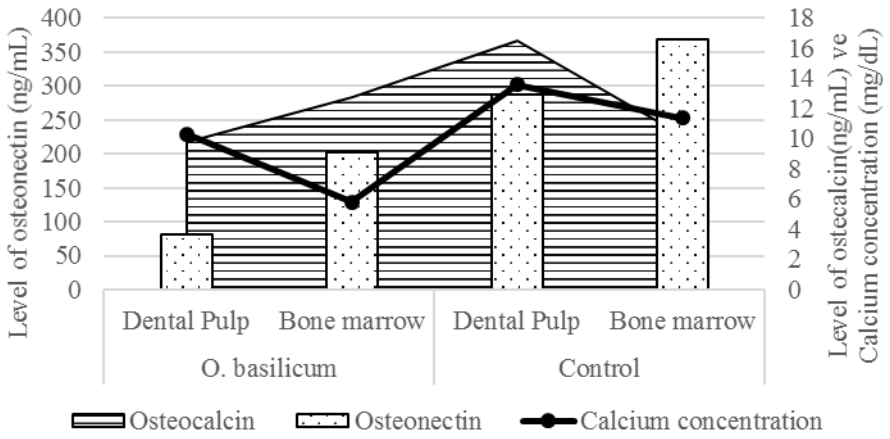


Figure 5. Osteonectin, osteocalcin and calcium concnetration of DP-MSCs and BM-MSCs with *O. basilicum*

So we suggested that *O. basilicum* may accelarate the ostegenic differentiation in both DP- and BM-MSCs since the ON level was reduced signed to loose the stability being secreted early. O. basilicum-treated BM-MSCs exhibited higher OCN levels than the untreated BM-MSCs while DP-MSCs did not. OCN is the represent of calcium granules, however we could not see the calcium graules in BM-MSCs and the calcium concetrations were too low either.

Therefore, *O. basilicum* could be a safe inducer for both healthy and medically compromised patients. In a study of essential oils of different geographical origins, Lawrence (1988) found that the main constitutents of the essential oil of basil are methylchavicol, eugenol,

methyleugenol and methyl cinnamate [24]. Eugenol and methyl eugenol remarked our previous study that eugenol containing *S. aromaticum* inhibited the osteogenic differentiation [25]. Similarly Anpo et al. (2011) provided evidence that eugenol/*S. aromaticum* reduces collagen synthesis, which play a critical role in osteogenesis [26]. From this point we suggest that *O. basilicum* may inhibit the collagen synthesis that leads to inhibit calcium granulation in BM-MSCs.

3.4. Determining the Preventive Effect of *O. basilicum* on the Inflammatory Response of MSCs Following TNF-α Stimulation

Figure 6 shows the anti-inflammatory activities of *O. basilicum*. Obtained data showed an antinnflammatory and immunomodulatory effect of the extract. Both IL-6 and IL-10 were present in the DP- and BM-MSCs cell culture supernatants [27]. Our results showed that when the extract used alone the IL-6 level was increased in BM-MSCs while it was decreased in DP-MSCs. On the other hand in contrast with alone used, the BM-MSCs were pretreated with *O. basilicum*, before TNF-α stimulation the IL-6 level were found decreased. We asked the literature if any relation was observed between increased IL-6 and inhibition of ostoegenic differentiation in BM-MSCs. It was clear that osteoblasts express low levels of IL-6 R [28]. Numerous reports indicate that IL-6 + IL-6R enhance in vitro differentiation on osteoblasts or osteoblast precursor [29]. On the other hand, there are reports showing the inhibitory effect of IL-6 type cytokines on bone formation [30-33]. It is possible that on precursor cells, IL-6 type cytokines would stimulate the first stages of differentiation but when stage progresses to maturation they would prevent stimulation by reduced ostoeblastic marker expression and enhanced apoptosis. So the study should be forther analysed for whole ostegenic markers and IL-6 secretion.

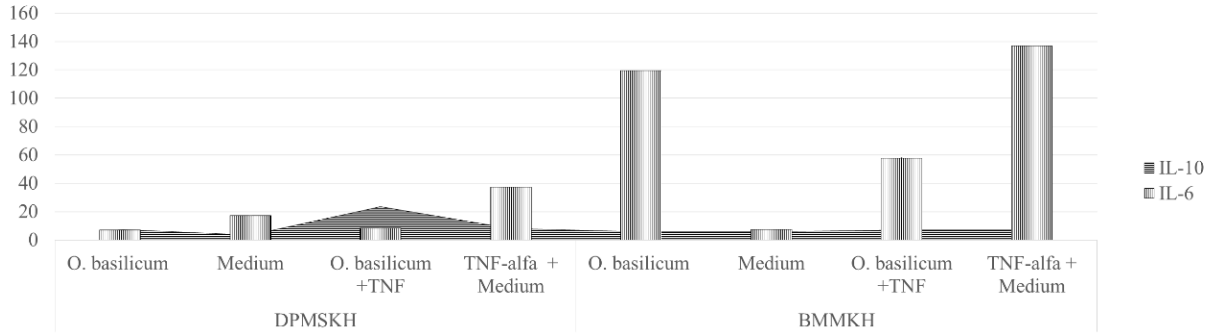


Figure 6. IL-6 (a) and IL-10 (b) level determined in the culture supernatant of DP and BM-MSCs.

Here we demonstrated that *O. basilicum* effect differently on BM- and DP- mesenchymal stem cells. It is important to determine the differentiation, and proliferation of cells with promising inductive agents. Studies should avoid to suggest any plant extract as an agent without evidence of safe.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Essential Oil Composition of *Clinopodium vulgare* L. subsp. *arundanum* (Boiss.) Nyman from Bingöl (Turkey)

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Abstract: Many of the medicinal and aromatic plants from Lamiaceae taxa are uses presumed to be connected to the terpenic constituents of the essential oils. In this study aerial parts essential oil of *Clinopodium vulgare* L. subsp. *arundanum* was analyzed by HS-SPME. As a result thirty seven components were identified. β-caryopyllene (16.1%), γ-terpinene (15.4%), germacrene D (10.3%), *p*-cymene (8.6%) and thymol (6.4%) were detected the major constituents. With this study, chemotypes of studied sample were detected β-caryopyllene, γ-terpinene, germacrene D, *p*-cymene and thymol. In addition studied plant sample was found to be rich in respect to essential oils and the results discussed natural product, renewable resources and chemotaxonomy

Keywords: *Clinopodium*, essential oil, HS-SPME, Apiaceae

1. INTRODUCTION

Clinopodium vulgare L. is one of the two *Clinopodium* spp. (Lamiaceae) growing wild in Anatolia. In Flora of Turkey, two subspecies are defined: vulgare and arundanum. The latter is widespread in Anatolia [1]. Until recently, essential oils have been studied most from the point of view their flavour and fragrance chemistry for flavouring foods, drinks and other goods. Actually, however, essential oils and their components are gaining increasing interest because of their relatively safe status, wide acceptance by consumers, and exploitation for potential multi-purpose functional uses [2]. Many authors, in fact, have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties by spices and essential oils and, in some cases, a direct food-related application has been tested [3]. *Clinopodium vulgare* is one of the curative plants used in Turkish folk medicine, mainly during wars for the purposes of healing wounds; this plant also showed a very strong action on bacteria [4]. Aqueous extract of C. vulgare showed strong antitumor activity [5]. A literature survey has shown that there is one report on the volatile constituents of *C. vulgare* subsp. *arundanum* Boiss. growing in Turkey [6].

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In our study, essential oil composition of *C. vulgare* subsp. *arundanum* which was collected from Bingöl (Turkey) was undertaken for the first time.

2. MATERIAL and METHODS

2.1. Plant Material

C. vulgare subsp. *arundanum* was collected from Bingöl-Solhan, vicinity of Hazarşah village, dry slopes, 1700-1750 m, July 2015, O.Kilic. The taxonomic description of the plant sample was made according to volume 4 of Flora of Turkey [1]. Voucher specimen was deposited in the Bingöl University, Department of Park and Garden Plants.

2.2. HS-SPME Procedure

Dried aerial part powder of five grams plant samples were carried out by a head space solid phase microextraction method using a divinyl benzene/carboxen/polydimethylsiloxane fiber, with 50/30 um film thickness; before the analysis the fiber was conditioned in the injection port of the gas chromatography (GC) as indicated by the manufacturer. For each sample, 5 g of plant samples, previously homogenized, were weighed into a 40 ml vial; the vial was equipped with a ‘‘mininert’’ valve. The vial was kept at 35°C with continuous internal stirring and the sample was left to equilibratefor 30 min; then, the SPME fiber was exposed for 40 min to the headspace while maintaining the sample at 35°C. After sampling, the SPME fiber was introduced into the GC injector, and was left for 3 min to allow the analyzes thermal desorption. In order to optimize the technique, the effects of various parameters, such as sample volume, sample headspace volume, sample heating temperature and extraction time were studied on the extraction efficiency as previously reported by Verzera et al., [7].

2.3. GC-MS Analysis

A Varian 3800 gas chromatograph directly inter faced with a Varian 2000 ion trap mass spectrometer was used with injector temperature, 260°C; injection mode, splitless; column, 60 m, CP-Wax 52 CB 0.25 mm i.d., 0.25 mm film thickness. The oven temperature was programmed as follows: 45°C held for 5 min, then increased to 80°C at a rate of 10°C/min, and to 240°C. at 2°C/min. The carrier gas was helium, used at a constant pressure of 10 psi; the transfer line temperature, 250°C; the ionisation mode, electron impact (EI); acquisit ion range, 40 to 200 m/z; scan rate, 1 us^{-1}. The compounds were identified using the NIST library, mass spectral library and verified by the retention indices which were calculated as described by Van den Dool and Kratz [8]. The relative amounts were calculated on the basis of peak-area ratios. The identified constituents of *C. vulgare* subsp. *arundanum* is listed in Table 1.

Table 1. Essential oil composition of *Clinopodium vulgare* subsp. *arundanum* (%)

Compounds	RRI*	%
α-thujene	1016	1.5
α-pinene	1022	0.8
Camphene	1034	0.4
Sabinene	1052	0.1
β-pinene	1056	2.1
β-mrycene	1064	2.5
α-terpinene	1085	1.8

α-phelladrene	1090	2.2
Limonene	1096	0.1
***p*-cymene**	1098	**8.6**
β-ocimene	1100	0.2
1,3,6- octatriene	1108	0.1
γ-terpinene	1117	**15.4**
Trans-sabinene hydrate	1126	0.1
Linalool	1148	0.2
Terpineol-4-ol	1166	0.4
Camphor	1182	0.1
Borneol	1200	1.3
Pulegone	1205	0.1
α-terpinolene	1220	2.5
Thymol	1295	**6.4**
Carvacrol	1300	3.3
α-copaene	1350	0.2
β-Caryophyllene	1382	**16.1**
β-cubebene	1400	0.3
Aromadendrene	1406	0.1
α-humulene	1418	3.4
Germacrene D	1435	**10.3**
Piperitenone	1441	1.2
β-bisabolene	1450	0.6
Bicyclogermacrene	1455	0.1
δ-cadinene	1462	0.1
Spathulenol	1495	0.2
Caryophyllene oxide	1497	4.1
α-muurolene	1520	1.1
Spathulenol	1620	0.1
Hexadecanoic acide	1702	0.4
RRI*: Relative Retention Index	**Total**	**88.5**

3. RESULTS and DISCUSSIONS

In this study, β-caryopyllene (16.1%), γ-terpinene (15.4%), germacrene D (10.3%), p-cymene (8.6%) and thymol (6.4%) were detected the major constituents of the essential oil plant. In a study was designed to examine the chemical composition essential oil of *Clinopodium vulgare* by GC-MS analysis of the oil resulted in the identification of 40 compounds, representing 99.4% of the oil; thymol (38.9%), γ-terpinene (29.6%) and *p*-cymene (9.1%) were the main components [9]; similarly in our study γ-terpinene (15.4%), thymol (16.4%), p-cymene (8.6%) and thymol (6.4%) were detected high percentages (Table 1).

In another research, a total of 34 components of the essential oil of *Clinopodium gracile* were identified and principal compounds of the essential oil were germacrene D (20.59%), nootkatone (8.22%), morillol (7.74%), β-elemene (7.38%), α-bergamotene (6.08%), cis-β-farnesene (5.47%) and caryophyllene (5.17%) [10]. It is noteworthy that, nootkatone and morillol were not detected in the essential oil of C. vulgare subsp. arundanum (Table 1). In another investigation, *C. vulgare* contains piperitone oxide (11.4%) [11]; this compound was not found in the oil of *C. vulgare* subsp. *arundanum* as main component (Table 1).

The differences in chemical composition of oil of present study and previous research may be because of the collection time, chemotypes, drying conditions, mode of distillation, geographic and climatic factors. The essential oil composition of C. vulgare subsp. arundanum collected from two different localities in Turkey, was analysed by means of GC and GC-MS; thirtyseven compounds were identifed, representing 89.6-90.5% of the samples; and the main constituents of the oils were germacrene-D, β-caryophyllene and β-caryophyllene oxide [6]. Germacrene-D and β-caryophyllene also were found to be the main constituents of studied sample (Table 1).

4. CONCLUSION

In conclusion, the present work is the first report on the composition of essential oils obtained from the aerial parts of *C. vulgare* subsp. *arundanum* from Bingöl (Turkey). Sample extracts from C. vulgare subsp. arundanum was rich in essential oil compounds. The biologic activity of Clinopodium taxa may be related to its richness in secondary metabolites, especially essential oils. β-caryopyllene, γ-terpinene, germacrene D, p-cymene and thymol were found to be the reason of chemotypes of *C. vulgare* subsp. *arundanum*.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Effect of Salt and pH Stress of Bioactive Metabolite Production in *Geitlerinema carotinosum*

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Abstract: Cyanobacterial metabolites are natural products that have an important features in pharmaceutical and medicinal industries. In this study, the presence of the secondary metabolite norharmane in the indole structure was determined in *Geitlerinema carotinosum* (Geitler) Anagnostidis isolated from Tokat Yesilirmak River and its production in salt stress and pH stress was investigated. In salt stress experiments, cyanobacterium was cultured for two weeks by adding NaCl to BG11 medium in erlenmayers of 0.5, 1.0, 3.0, 5.0 M. pH stress was executed at 5 and 9. Norharmane amount was determined by HPLC using C18 reverse phase column at a temperature of 40 °C and a flow rate of 1 ml / min. The amount of norharman metabolite (µg/g) was calculated according to the Gauss method by drawing a calibration curve over the absorbance value of the standard 247 nm wavelength. According to the analysis results, metabolite production was 0.612, 1.299, 0.011 at 0.5 M, 1.0 M, 3.0 M respectively. At 5 M, there was no norharmane production. The norharmane production is higher at pH 5 (1.293 µg /g) than that of the pH 9 (0.448 µg /g).

Keywords: *Geitlerinema carotinosum*, stress conditions, norharmane, HPLC

1. INTRODUCTION

Cyanobacteria produce many important secondary metabolites which are promising compounds for drug discovery and development process. Among the cyanobacteria, *Spirulina* Turpin ex Gomont, *Anabaena* Bory ex Bornet & Flahault, *Nostoc* Vaucher ex Bornet & Flahault and *Oscillatoria* Vaucher ex Gomont contain a large variety of substantial secondary metabolites [1] which reveal the toxins, antitumor, antifungal, antiinflammatory, siderophores, phytohormones, photoprotective effects and protease inhibitors [2]. Some of these secondary metabolites have been produced from cyanobacterial biomass extraction with solvents (intracellular). In addition, cyanobacteria are able to secrete various organic compounds in their environment as exo-metabolites (extracellular) [3]. Norharmane (9H-pyrido (3,4-b) indole) (Figure 1) is an exo-metabolite that can be produced in some cyanobacteria species and is released into the growth environment [4]. Due to the importance of norharmane for

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pharmaceutically and biologically, we investigated the production of norharmane in salt and pH stress conditions on *G. carotinosum*.

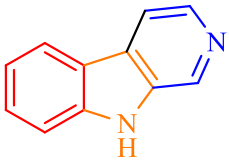

Figure 1. Chemical structure of norharmane.

2. MATERIAL and METHODS

2.1. Collection and growth conditions of *G. carotinosum*

Cyanobacterium was collected from Yesilirmak river, Tokat, Turkey (49° 17.40' 19" N, 36° 23' 4.69" E). *G. carotinosum* was isolated by micro piped and micro injector under the inverted microscope. Then it was streaked onto agarised Blue Green Algae (BG-11) medium *G. carotinosum* was grown under controlled conditions at 26 ± 2 °C with white fluorescent lamp of 2465 lux [5].

2.2. Salt and pH experiments

In salt stress experiments, cyanobacterium was cultured for two weeks by adding NaCl to BG11 medium in erlenmayers the concentration of 0.5, 1.0, 3.0, 5.0 M. pH stress was executed at 5 and 9. The control cultures were kept in the BG11 medium without sodium chloride (pH 7). Each inoculation was carried out by adding from 25 ml of stock culture at logarithmic phase of growth to 250 ml erlenmayer [6].

2.3. Biomass

Culture samples (14 ml) were centrifuged at 4000 g for 15 min. The pellets were washed twice with distilled water (pH 4), dried at 60 °C for 6 h, and weighed with a precision balance [7].

2.4. Statistical analysis

The statistical analysis were analysed by ANOVA and using the SPSS software (SPSS Inc., version 20). Tests of significance were carried out using Duncan's multiple range tests.

3. RESULTS and DISCUSSIONS

3.1. Morphological analyses

G. carotinosum was identified under the light microscope with a micrometer. The trichomes are smooth or slightly wrapped. Fill it up towards the ends. The cells were arranged in bundles in the form of fascicles. Cells are 1.5-3 μm wide; It is 3-9 μm long [9, 10].

3.2. Effects of salt stress on the growth and production of norharmane

When the stress conditions were applied to *G. carotinosum*, the control cell biomass was 0.260 g/l. While the salt concentrations increased, biomass of cells decreased. Therefore, this cyanobacterium was not halotolerant. In 1.0 M salt stress, the amount of norharmane was observed to have been 1.299 μg / g, which was more than the control. On the other hand, norharmane production decreased to 0.011 μg/g at 3.0 M concentration. Norharmane secretion was not took place at 5.0 M. As a consequence, the optimum norharmane production was observed at 1.0 M concentration (Table 1).

Table 1. Biomass and norharmane production under salt stress

Salt (M)	Biomass (g l^{-1})	Norharmane (µg g^{-1})
Control	0.260 ± 0.000^{e}	0.830 ± 0.000^{d}
0.5 M	0.248 ± 0.003^{d}	0.612 ± 0.007^{c}
1.0 M	0.106 ± 0.005^{c}	1.299 ± 0.005^{e}
3.0 M	0.052 ± 0.005^{b}	0.011 ± 0.004^{d}
5.0 M	0.023 ± 0.002^{a}	0.000 ± 0.000^{a}

Values are means ± Standard deviation (n= 3)
Means followed by different letters are significantly different at $p < 0.001$

3.3. Effects of pH stress on the growth and production of norharmane

At pH 5 and 9, the biomass of cells was lower than that of the control cells. It was found out that the acidic and basic media were not suitable for growth. The Natural medium (pH 7) has been determined the best growth condition of *G. carotinosum*. Hovewer, the production of norharmane at pH 5 was 1.293 µg/g, which is better than that of pH 7 and 9 (Table 2).

Table 2. Biomass and norharmane production under pH stres

pH	**Biomass (g l^{-1})**	**Total norharmane (µg g^{-1})**
5	0.068 ± 0.002^{a}	1.293 ± 0.006^{c}
9	0.115 ± 0.007^{b}	0.448 ± 0.002^{a}
Control	0.260 ± 0.000^{c}	0.830 ± 0.00^{b}

Values are means ± Standard deviation (n= 3)
Means followed by different letters are significantly different at $p < 0.001$

The needs for the survive of cyanobacteria are mainly water, light, carbon dioxide and simple inorganic compounds [11]. In addition, cyanobacteria can grow rapidly under certain environmental conditions [12]. Cyanobacteria can adapt to a wide range of environmental factors. For example, *Synechococcus* sp., *Microcystis* sp., *Arthrospira* sp. are thermotolerant, alkalitolerant, halotorant respectively [13].

Cyanobacteria contain numerous pharmacologically important secondary metabolites. Metabolites retained within the cell may not be released unless cell integrity is impaired. However, they become free under stress conditions or when the cells are disintegrated [14].

The secretion of significant compounds by algae caused by the protection of algal cells against stressful conditions like ultraviolet radiation, temperature change, fluctuation in nutrient and salinity level [15]. In this study, cyanobacterium *G. carotinosum* was exposed to salt and pH stress, resulted in higher norhamane production than controls.

4. CONCLUSION

Cyanobacteria contain numerous pharmacologically important secondary metabolites. *G.carotinosum* could be an important source of norharmane which is valuabe compound for pharmaceutical.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Effects of Different Salt Concentrations on Quinoa Seedling Quality

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Abstract: The experiment designed a completely randomized experimental design was carried out Adnan Menderes University, Agriculture Faculties greenhouse. Quinoa variety candidate named "Saponinsiz" is used experimental material. The seeds were sowed in plastic pots filled with soil and perlite (%50+%50) at the greenhouse with six replicates. Five different salt concentrations were determined as 0 (control), 4 ds m^{-1}, 8 ds m^{-1}, 16 ds m^{-1} and 30 ds m^{-1} and were applied with NaCl solution which was prepared before sowing. Leaf number, leaf length, leaf width, leaf thickness, stem thickness and green biomass weight values were measured when the quinoa plant reached 6 leaf stage. As a result of the study, it was observed that the differences between the salt concentrations in leaf number, leaf length, leaf width and green biomass weight were significant. The maximum leaf length (11.53 mm) was measured with 8 ds m^{-1} salt concentration applied plants, whereas the maximum leaf width (4.99 mm) and green biomass (1019.5 mg) were measured with 4 ds m^{-1}. The control plot only showed the highest values for the leaf number value. These results confirmed that the quinoa plant was facultative halophytic species (salt-resistant). It was determined that 16 ds m^{-1} dose gave the lowest values in all measurements. And any plant wasn't growing at the 30 ds m^{-1} applied pots. The values of the experiment measured of 4 ds m^{-1} pots and 8 ds m^{-1} pots, which is considered the limit values for the field crops, were approximately equal or greater than control pots. Moreover, there was a rapid decline of plant on the 16 ds m^{-1} values.

Keywords: Salinity, Quinoa, Seedling Quality, Green biomass

1. INTRODUCTION

Salinity is one of the significant problems commonly observed among irrigated agricultural lands. Nowadays it is showed that salinity soils affect between 20 and 50% of irrigated arable lands worldwide [1]. Because of natural causes such as Irrigation water, soil structure, regional factors and some human activities, growth retardation and yield loss owing to salinity of soils is a common problem all over the world as most crop plants [2]. One option is the use of halophytic crop species, which can tolerate high levels of soil salinity. Many members of the family Chenopodiaceae are classified as salt tolerant one member of this family, quinoa (*Chenopodium quinoa* Willd.) is able to grow on salinity soils [3]. Therefore, quinoa plant for new approaches is necessary to cope with the problem.

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The progressive salinization of irrigated land is a major environmental threat for crop production. Therefore, the selection and characterization of salt-resistant species are important to ensure future productivity of the arid and semi-arid agricultural regions [4]. Although quinoa (Chenopodium quinoa Willd) has been cultivated in the South America for at least 7000 years [5], it has garnered much attention around the world in recent years [6], [7]. This interest based on not only its stress tolerance especially salinity soil condition, but also its nutritional quality especially high essential amino acid rates [8]. The main use of quinoa is for people such as cooking, baking, and various products for allergic to gluten and for animals such as feed, green fodder and for regulate plant growth with contains some phytohormones [6]. Besides, it has been considered an oil crop which has proportion of omega-6 and a notable vitamin E content [9]. Moreover, the seed has an outstanding rich in vitamins (A, B_2, C) and many of minerals such as calcium, magnesium, iron, copper, zinc and lithium, starch and essential fatty acids for human nutrition [10]. For these reasons, the year 2013 was declared "International Year of Quinoa" by the UN Food and Agriculture Organization (FAO) to focus attention on this crop worldwide [11]. Plants develop defense strategies against salt stress based on the activation of the ion transport system, osmotic adjustment and induction of antioxidant enzymes [12]. Seedling period is a critical process during plant life, particularly in the presence of adverse environmental factors such as salinity [13]. The capability of seedling establishment under saline conditions is dependent on cultivar [14]. Even though halophytic crop species are resistant against salinity levels into the soil, it has been shown in several studies that even halophytes are relatively sensitive to salinity during the stages of germination and seedling period [15, 16].

The study was conducted in Adnan Menderes University, Agriculture Faculties greenhouse to prove of different salinity concentration effects on quinoa leaf number, leaf length, leaf width, leaf thickness, stem thickness and green biomass weight. Moreover, we planned to determination of quinoa seedling quality onto different salinity levels certainly.

2. MATERIAL and METHODS

2.1. Plant material and salinity treatments

Quinoa variety candidate named "Saponinsiz" is used experimental material. The seeds of quinoa (Chenopodium quinoa Willd) were obtained from the local producer in Denizli and brought they into Adnan Menderes University, Agriculture Faculties greenhouse in 2016. Prior to experiment they were selected for uniformity of size and twenty-five seeds germinated on filter paper in petri dishes with six replicates for viability test during 7 days. After the period number of seeds germinated on filter paper in petri dishes was determined and the numbers which measured six replicates were Multiplied by 4 to find % value. 6 replicates were averaged.

The soil used at the study is contents 1.2% of organic matter, 2978 mg kg^{-1} P_2O_5, 101 mg kg^{-1} exchangeable K, 19 mg kg^{-1} Ca and 5,6 mg kg^{-1} Na. Fe and Mn were determined as 594 mg kg^{-1} and 21 mg kg^{-1} respectively. 50% the soil and 50% perlite mixtures were put in medium pots (15 L.). Twenty quinoa seeds were sowed the pots. Percent of field capacity calculated randomly selected 3 pots. Afterwards 0 (control), 4 ds m^{-1}, 8 ds m^{-1}, 16 ds m^{-1} and 30 ds m^{-1} NACl concentration were threatened with calculated water. Infiltration cases were placed under the pots against the possibility of infiltration. These proses were repeated again to 6 pots each salt concentrations. Thus, the study was conducted with six replicates. Leaf number, leaf length, leaf width, leaf thickness, stem thickness and green biomass weight values were measured 50 days after sowing (45 days after the germination) of quinoa when the seedlings reached 6 leaf stage.

The traits studied in this research were determined in the following ways: Leaf number: 5 plants randomly selected from each pot and were separated from others. All leaves of the plants were counted. The average was taken to obtain the replicate value of salt concentrations.

Leaf length: Leaf length was measured all leaves of the plants from the tip to bottom of the leaf blade by electronic compass. The average was taken to obtain the replicate value of salt concentrations.

Leaf width: Leaf width was measured all leaves of the plants at the widest part of the leaf by electronic compass. And the average was taken to obtain the replicate value of salt concentrations**.**

Leaf thickness: Leaf thickness were measured all leaves of the plants by Electronic compass. And the average was taken to obtain the replicate value of salt concentrations.

Stem thickness: Stem thickness were measured all stems of the plants by Electronic compass. And the average was taken to obtain the replicate value of salt concentrations.

Green Biomass: Randomly selected 5 plants from each pot were weighed. The weight of this named fresh, or "green" biomass [17, 18]. The average was taken to obtain the replicate value of salt concentrations.

Statistical Analysis

Data of growth and seedling quality were analyzed by TARİST [19]. Means were compared between treatments by LSD (least significant difference) at the 0.05 confidence level.

3. RESULTS and DISCUSSIONS

The calculated mean squares with variance analyses for salt concentration levels are presented in Table 1.

Table 1. The calculated mean squares with variance analyses for all components measured of quinoa seed

Variance Source	Leaf Length	Leaf width	Leaf thickness	Leaf number	Stem thickness	Green biomass
Salt concentration levels	12,95*	7,52**	0,03ns	7,49**	0,03ns	444867,38**
Error	2,38	0,26	0,01	0,91	0,01	19518,50

The effect of different salt concentrations during germination and seedling period on Leaf number, leaf length, leaf width, leaf thickness, stem thickness and green biomass values were determined. Salt concentration level was found to be significant in some parameters measured such as leaf number, leaf length, leaf width and green biomass, but leaf thickness and stem thickness values were found to be no significant.

Table 2 were edited some seedling quality characteristics as leaf number, leaf length, leaf width, leaf thickness, stem thickness and green biomass values under different salt concentrations. 30 ds m^{-1} pots were ignored. Because germination of the quinoa seeds in the pots were realized either no one exited or few were exited than died for over salt level. Quinoa optimal plant growth was obtained between 10 ds m^{-1} and 20 ds m^{-1} NaCl. Only some variety of quinoa such as Titicaca observed a significant inhibitory effect on seed germination for concentrations higher than 40 ds m^{-1} NaCl. Therefore, this is said that quinoa plant was tolerated under moderately saline conditions (10 –20 ds m^{-1}) [6].

Leaf area is an important trait for the yield, because biomass was dictated the amount of light which can be absorbed. As the one of the main criteria that determine the leaf area, maximum leaf length was obtained from 8 ds m^{-1} (11.52 cm) salt concentration. The average of 4 ds m^{-1} (11.15 cm) were followed. Minimum average of leaf length value was given from 16 ds m^{-1} (8,46 cm). As another of the main criteria that determine the leaf area, maximum leaf width was obtained from 4 ds m^{-1} (4,99 cm) salt concentration. The average of 8 ds m^{-1} (4,82 cm) were followed. Minimum average of leaf width value was given from 16 ds m^{-1} (2,54 cm). When measured leaf size values were evaluated, 16 ds m^{-1} salt concentration caused a decrease of both leaf length and leaf width approximately 37% and 49% respectively as compared with maximum leaf length (8 ds m^{-1}) and maximum leaf width (4 ds m^{-1}). The results of these two measurements, we can say that quinoa is salt-resistant plant up to 8 ds m^{-1} level and the yield could not be significantly affected to these salt levels due to the significant correlations between leaf area and dry matter of quinoa [20].

Leaf thickening is considered as a mechanism to increase the water retention by mesophyll tissues in order to counteract salt toxicity [21]. Because leaf thickness was observed the relationship between stomatal conductance, mesophyll conductance, and mesophyll thickness [22]. Maximum leaf thickness was obtained from 16 ds m^{-1} (0,58 cm) salt concentration. The average of 8 ds m^{-1} (0,57 cm) were followed. The control group was given minimum average of leaf thickness value (0,42 cm). Our results are consistent with the other investigations [16, 23]. The leaf thickness significantly increased as the percentage of salinity increased. Increasing of leaf thickness can be referred to the water storage in the hypodermal tissue, which affected of leaf thickness values especially under high salinity. Therefore, salinity stimulates the increasing of leaf thickness and fresh weight owing to water storage perhaps contributes to decreased toxicity of salinity [24]. Leaf thickness were considerably higher in the leaves of high salt treated plants [25, 26]. Increased leaf thickness has been reported as a successful trait for plant species growing under saline conditions. There was a positive correlation between the stomatal conductance and mesophyll conductance that affect diffusion of CO2 and rate of photosynthesis inside leaf cells. Thus, mesophyll thickness was inversely correlated with mesophyll conductance and leaf porosity. Further reduction in K+ content under the condition of increased salinity may damage the photosynthetic machinery [27], [28].

Contrary to leaf thickness, stem thickness decreased during the increase in salinity doses. Maximum stem thickness was obtained from control (0,76 cm) salt concentration. The average of 4 ds m-1 (0,73 cm) were followed. Minimum average of stem thickness value was given from 16 ds m-1 (0,61 cm).

Leaf number can be used to characterize plant assimilation capacity [29]. Maximum leaf number was obtained from control parcels (6,00) salt concentration. The average of 4 ds m-1 (5,17) and 8 ds m-1 (5,00) were followed. These three levels different from the other level (16 ds m-1) statistically. Minimum average of leaf number value was given from 16 ds m-1 (3,33). High salt concentration (16 ds m-1) caused a decrease of leaf number approximately 45% as compared with control parcels which obtained maximum leaf number average. The leaf number increased in the control and decreased as NaCl dose was increased [30, 31].

There are many studies which have been determined to correlation between biomass [32], [33] and grain yield [20]. Maximum green biomass was obtained from 4 ds m-1 (1019,50 mg) salt concentration. The average of 8 ds m-1 (852,17 mg) and control (727,67 mg) were followed. Minimum average of green biomass value was given from 16 ds m-1 (377,17 mg). Biomass reduction into 16 ds m-1 level was determined as nearly by half of control level. Moreover 16 ds m-1 salt concentration caused a decrease of biomass approximately 73% as compared with biomass of 4 ds m-1 given maximum green biomass. The really depressive effects of salinity on seedling was showed into the 16 ds m-1 salinity level. Thus, it is expected

that the quinoa will decrease in biomass up to 16 ds m-1. Quinoa has demonstrated the ability to accumulate salt ions in its tissues in order to control and adjust leaf water potential. This enables the plants to maintain cell turgor and limit transpiration under saline conditions, avoiding physiological damage [34]. But there were very different results among to quinoa cultivars. Nevertheless, some varieties can withstand very high salt doses, generally average of salt level 11 ds m-1 for an increase in both leaf area and biomass of plants grown at compared to those grown at control level.

Table 2. Trypan Blue Assay results.

SCL	LL (cm)	LW (cm)	LT (cm)	ST (cm)	LN	GB (mg)
Control	9,30 bc	4,32 b	0,42	0,76	6,00 a	727,67 b
4 dsm	11,15 ab	4,99 a	0,51	0,73	5,17 a	1019,50 a
8 dsm	11,52 a	4,82 ab	0,57	0,72	5,00 a	852,17 ab
16 dsm	8,46 c	2,54 c	0,58	0,61	3,33 b	377,17 c
LSD	1,86	0,61	ns	Ns	1,15	168,37

SCL: salt concentration levels, LL: leaf length, LW: leaf width, LT: leaf thickness, ST: stem thickness, GB: green biomass

4. CONCLUSIONS

Many regions of the world especially in arid and semi-arid regions are affected by salt problems. Few crops can be grown in these marginal areas, as plants grown on saline soils must be species tolerant to salinity, such as quinoa because of both stress tolerances to salinity soil condition and its nutritional quality especially high essential amino acid rates. Maximum values of leaf length, leaf width and green biomass of the experiment were measured 4 ds m^{-1} pots and 8 ds m^{-1} pots, which is considered the limit values for the field crops. We can say that quinoa is salt-resistant plant up to 8 ds m^{-1} level. Moreover, there was a rapid decline of plant on the 16 ds m^{-1} which minimum values were measured of all the traits. Therefore, new salt doses between 8 ds m^{-1} and 16 ds m^{-1} (10 ds m^{-1}, 12 ds m^{-1} and 14 ds m^{-1}) may be determined because of be able to see that the certain limit of salt concentration which is quinoa seedling resistant.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Investigation of Relationship Between Chemical Stress Factors and Certain Metabolites Including Cardenolides in Callus Cultures of Endemic Turkish *Digitalis* L. Species

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Abstract: The aim of the present research is to obtain relationship between different stress treatments [Cu (copper) and Hg (mercury)] and content of cardiac glycosides (digoxigenin, gitoxigenin, lanatoside C, digoxin and digitoxin) as secondary metabolites of commercial value for the pharmaceutical industry and to determine the antioxidant metabolites against stress conditions in callus cultures of endemic Turkish *Digitalis* species. The effects of different stress treatments on cardiotonic glycoside accumulation in *D. lamarckii* Ivanina, *D. trojana* Ivanina, *D. davisiana* Heywood and *D. cariensis* Boiss. ex Jaub. et Spach were investigated using HPLC. HPLC analysis revealed that all stress conditions were significantly effective at 5% significance level according to their control groups. The predominant cardiac glycoside was lanatoside C (Lan C) followed by digitoxin, digoxigenin, gitoxigenin and digoxin. No digoxin was detected in all treatments as well as in control groups. For the calibration curves, concentrations of 5, 10, 20, 30 and 40 mg/l digoxigenin, gitoxigenin, lanatoside C, digoxin and digitoxin were used (R^2= 0.99). Cardenolides were eluted with acetonitrile (A) and water (B) gradients as follows: 0 to 20 min 20% (A), 80% (B); 20 to 23.40 min 30% (A), 70% (B); 23.40 to 30 min 25% (A), 75% (B) and 30 to 40 min 40% (A), 60%(B). Average peak area of the glycoside in samples was automatically calculated and monitored by ChemStation LC/MS software against that of standards. Enhanced production of cardenolides was achieved from callus cultures elicited with 50 µm $CuSO_4$ and $HgCl_2$. Higher amounts of cardenolides were obtained when callus of four *Digitalis* species were elicited with CuSO4. Results demonstrated that catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1) activities, the total contents of phenolics and proline were markedly stimulated under stress conditions. All these results indicated that treatments have induced changes in the redox state of callus cells and suggest that this alteration change cardenolides accumulation and antioxidative status in *Digitalis* L. callus cultures.

Keywords: Antioxidant, cardiac glycosides, *Digitalis* L., heavy metal stress

1. INTRODUCTION

Digitalis L. produces various cardiac glycosides which have potential to treat many diseases such as edema, myocardial infarction, arterial hypertension, cardiac dysfunction, angina and hyertropy [1]. Besides their cardiotonic effects, these compounds are also effective chemotherapeutic agents, especially in breast and prostate cancer treatments [2]. *Digitalis* species is distributed in the Mediterranean region, Western Asia and Europe. *Digitalis davisiana* Heywood, *Digitalis lamarckii* Ivanina, *Digitalis cariensis* Boiss. ex Jaub. et Spach

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and *Digitalis trojana* Ivanina are endemic to Turkey [3]. Traditionally, cell and organ cultures have been used for production of secondary metabolites, but the yield of cardiac glycosides have been low. Attempts have been made to increase concentration of metabolites in shorter period of time. Exogenous addition of biotic and abiotic elicitors is considered to be one of the most promising strategies for the induction of secondary metabolites production [4]. Zhao et al. [5] proposed that these elicitor molecules act as extracellular or intracellular signals and initiate a signal transduction network that is required for the activation of transcription factors, which organize the expression of genes included in plant secondary metabolism [6]. Among the various abiotic elicitors, heavy metal stresses have been considered as effective elicitors for the increased production of secondary metabolites in *in vitro* cultures. Heavy metal stress has become a headmost environmental threat to crop production. Being a potential hazardous factor, toxic metals decrease the plant growth, yield and sustainability of production, thus can cause the alarming situation for food availability. Plants under the stress environment facing the alterations of cellular protein functions, lipid and thylakoid structures. Disturbance or breakage of these structures is directly linked with plant photosystem that can affect the senescence process [7,8]. Copper (Cu), microelement, has important physiological functions in plants. At higher concentrations, it leads to physiological and morphological disturbances, as a consequence decrease the yield [9]. Mercury (Hg) is a toxic metal that can be absorbed from the atmosphere and soil. It can be accumulate in the plant organs and cause to phytotoxic effects. The toxic metal threshold level in the tissue is defined by the 'stress point'. Beyond the stress point, the cell will be irreversibly damaged [10]. These metabolic changes can directly trigger the plant defense system, including enzymatic and non-enzymatic antioxidants to cope with overproduce of reactive oxygen species (ROS) in the cell [11]. As a consequence of unfavorable conditions, activation of genes related to the enzymatic defense system, including catalase (CAT), superoxide dismutase (SOD), guaicol peroxidase (POD), glutathione reductase (GR) and the non-enzymatic antioxidants such as glutathione, ascorbate (vitamin C), carotenoids, α-tocopherol (vitamin E), proline and various phenylpropanoid derivatives (phenolic compounds) were observed [12]. On the basis of background information, the aim of this work is to investigate the induction of cardenolide compounds (lanatoside C, digitoxin, digoxigenin, gitoxigenin and digoxin) in *D. davisiana* Heywood, *D. lamarckii* Ivanina, *D. trojana* Ivanina and *D. cariensis* Boiss. ex Jaub. et Spach, in response to application of heavy metal toxicity and to determine the correlation between the cardiotonic glycosides accumulation and stress responsive antioxidant defense system.

2. MATERIAL and METHODS

2.1. Plant materials

Seeds of four endemic Digitalis species (*D. davisiana* Heywood, *D. lamarckii* Ivanina, *D. trojana* Ivanina and *D. cariensis* Boiss. ex Jaub. et Spach) were collected in August to September, 2010. Seeds of *D. davisiana* and *D. cariensis* from Alanya-Mahmutlar (N360 31.916', E032014.402') (N360 30.767', E032012.695', 03.09.2010), seeds of *D. lamarckii* around the Ankara-Kızılcahamam (N40037.709', E032026.265') and *D. trojona* were collected from the National Park of Ida Mountains (N390 38.885', E0260 57.402').

2.2. Experimental Design

The seeds were cultured on MS medium including containing 3% sucrose and 0.8% agar (pH 5.7-5.8) for germination. Hypocotyl explants were cultured on MS medium including vitamins (0.5 ppm TDZ and 0.25 ppm IAA) (Sahin et al., 2013) for callus induction. After 30 days of culture 50 µm $HgCl_2$ and 50 µm $CuSO_4$ were used in cultures in order to expose the callus cultures to chemical stress for 10 days [13].

2.3. Extraction and HPLC analysis of cardenolides

Cardenolide extraction was determined according to the modified method of Wiegrebe and Witchtl [14]. Qualitative and quantitative analysis of cardenolides were detected and calculated as previously described HPLC protocol [15].

2.4. Enzyme extraction, protein determination and assays of enzymes

Callus material was homogenized as previously described protocol [15], then tissue extracts were kept at -80 ^{o}C for determination of superoxide dismutase (SOD, EC 1.15.1.1) [16] and catalase (CAT, EC 1.11.1.6) activity [17]. The protein content was determined according to Lowry method [18].

2.5. Total phenolic assay

The total phenolic content were determined using the Folin-Ciocalteau [19] method. The total phenolic content of extracts was expressed as mg gallic acid equivalents (GAE)/ g dw.

2.6. Proline analysis

Proline was determined according to the method of Bates et al., [20]. Proline content was calculated as µmol proline /g dw.

2.7. Statiscal Analysis

Data were statistically analyzed using SPSS Version 15.0 (SPSS Inc., Chicago, IL, USA) and Duncan's multiple range test at $P \leq 0.05$. Each treatment was made in triplicate.

3. RESULTS

Callus was initiated from hypocotyl explants cultured on MS medium supplemented with 0.25 mg L^{-1} IAA and 0.5 mg L^{-1} TDZ. After 30 days, callus was transferred to MS medium containing 50 µm $HgCl_2$ and 50 µm $CuSO_4$ for 10 days. Here, we examined the effects of $HgCl_2$ and $CuSO_4$ as an heavy metal stress factors on cultures which induced cardiotonic glycoside accumulation and antioxidant activities in four *Digitalis* species (*D. cariensis, D. davisiana, D. trojana, D. lamarckii*). Application of $HgCl_2$ and $CuSO_4$ to the medium were not affected cell viability. However, the colour of the callus changed from green (control group) to brownish with $HgCl_2$ and $CuSO_4$ applications (Figure 1). Results related to cardenolides accumulation of treated and untreated plants of four *Digitalis* species (*D. davisiana Heywood, D. lamarckii Ivanina, D. trojana Ivanina* and *D. cariensis* Boiss. ex Jaub. et Spach) are shown in Table 1.

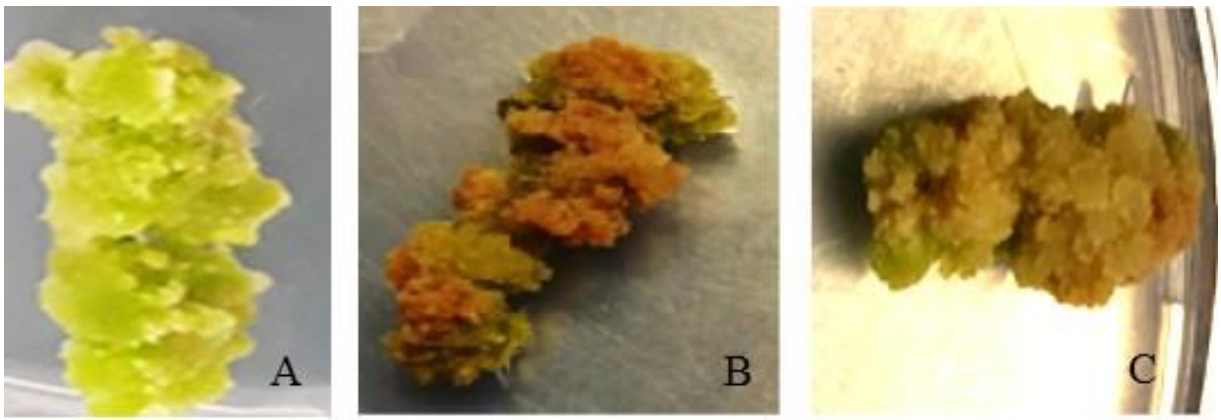

Figure 1. Effects of culture media and stress factors on morphological characters of induced callus: (A-C): (A): Callus growth in MS medium+0.25 mg L^{-1} IAA and 0.5 mg L^{-1} TDZ (control group), (B): Callus growth in MS+50 µm $HgCl_2$, (C): Callus growth in MS+50 µm $CuSO_4$

Table 1. Results of cardenolides accumulation in the control and elicited callus tissues of *Digitalis* species. Means followed by different letters in the same column are significantly different ($P < 0.05$).

Species	Treatments	Amount of cardenolides (μg g^{-1}, dw)				
		Digoxigenin	Gitoxigenin	Lan C	Digoxin	Digitoxin
D. lamarckii	Control	9.04^{h}±0.62	6.46^{def}±0.36	302.04^{i}±7.49	<LOD	8.67^{d}±0.49
	$CuSO_4$	11.41^{ef}±1.96	6.41^{ef}±0.51	721.80^{c}±8.78	<LOD	11.83^{bc}±3.78
	$HgCl_2$	10.37^{fgh}±0.41	6.49^{ef}±0.57	377.23^{g}±5.20	<LOD	9.27^{cd}±2.08
D. trojana	Control	12.96^{de}±0.56	6.12^{fg}±0.13	285.63^{j}±2.57	<LOD	7.48^{d}±0.50
	$CuSO_4$	17.13^{b}±1.31	7.62^{cd}±1.13	619.83^{e}±7.23	<LOD	9.66^{cd}±0.77
	$HgCl_2$	12.49^{de}±1.10	6.61^{def}±0.83	346.37^{h}±9.47	<LOD	8.68^{d}±0.77
D. davisiana	Control	9.56^{gh}±0.10	5.16^{g}±0.20	259.85^{k}±8.17	<LOD	7.55^{d}±0.50
	$CuSO_4$	18.99^{a}±0.17	7.75^{c}±0.21	844.097^{b}±7.89	<LOD	13.52^{b}±0.93
	$HgCl_2$	15.44^{c}±0.77	7.34^{cde}±0.33	461.13^{f}±6.08	<LOD	9.33^{cd}±0.31
D. cariensis	Control	10.73^{fg}±0.17	8.33^{c}±0.47	280.71^{j}±7.14	<LOD	9.06^{d}±0.27
	$CuSO_4$	14.09^{cd}±0.59	12.16^{a}±0.63	939.21^{a}±9.09	<LOD	16.13^{a}±0.54
	$HgCl_2$	13.07^{d}±0.43	10.01^{b}±0.67	673.23^{d}±6.58	<LOD	13.92^{ab}±0.16

Note: LOD; limit of detection.

Addition of $HgCl_2$ and $CuSO_4$ (50 μm) into media significantly affected the cardenolides accumulation as compared to control. Especially, $CuSO_4$ treatments played a pivotal role to the accumulation of cardenolides. Lanatoside C was the predominant cardiac glycoside followed by digoxigenin, digitoxin, gitoxigenin and digoxin. On the other hand, the content of digoxin was below the limit of detection in all treatments. The use of $CuSO_4$ and $HgCl_2$ led to a drastic increase in the accumulation of Lan C in all *Digitalis* species. The control (non-treated) callus produced 302.04±7.49 μg/g dw Lan C while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 721.80±8.78 μg/g dw and 377.23±5.20 μg/g dw Lan C respectively in *D. lamarckii* callus cultures. Similar to *D. lamarckii*, the positive correlation between the accumulation of Lan C and $CuSO_4$ - $HgCl_2$ applications was detected in *D. trojana* callus cultures. 285.63±2.57 μg/g dw Lan C was found in the control (non-treated) callus while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 619.83±7.23 μg/g dw and 346.37±9.47 μg/g dw Lan C, respectively. In *D. davisiana* cultures, the accumulation of Lan C was significantly induced as a concequence of $CuSO_4$ and $HgCl_2$. Lan C of control was 259.85±8.17 μg/g dw while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 844.097±7.89 μg/g dw and 461.13±6.08 μg/g dw Lan C, respectively. CuSO4 as well as $HgCl_2$ stress was followed by a significantly enhanced accumulation of Lan C in *D. cariensis* callus cultures. The control (non-treated) callus produced 280.71±7.14 μg/g dw Lan C while those cultured on under chemical stress by $CuSO_4$ and $HgCl_2$ producing 939.21±9.09 μg/g dw and 673.23±6.58 μg/g dw Lan C respectively. Although a noticeable increase was observed in digitoxin, digoxigenin content under the exposure of $CuSO_4$, there was not any significant increase in gitoxigenin, digitoxin, digoxigenin content under the exposure of $HgCl_2$ compared to non-treated callus in *D. lamarckii*. In *D. trojana* cultures, digoxigenin (17.13±1.31 μg/g dw) and gitoxigenin levels (7.62±1.13 μg/g dw) were significantly increased under $CuSO_4$ stress. But it is apparent that, at 5% significance level, there was not any significant change observed between control groups and digitoxin content under both applied treatments. Addition of $CuSO_4$ and $HgCl_2$ into the medium significantly increased the digoxigenin, gitoxigenin and digitoxin content in *D. davisiana* and *D. cariensis* callus cultures as compared with respective controls. The data regarding the antioxidant and non-enzymatic antioxidant enzymes are presented in

Table 2. Activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) were significantly enhanced in all species under 50 µm $CuSO_4$ and $HgCl_2$ treatments as compared with respective controls.

Table 2. Effect of different treatments of heavy metals on enzymatic and non-enzymatic antioxidant activities in the callus cultures of *Digitalis* species.

Species	Treatments	SOD	CAT	Total phenolic	Proline
D. lamarckii	Control	$0.14^{g}\pm0.04$	$19.30^{d}\pm0.70$	$154.45^{k}\pm0.72$	$0.76^{j}\pm0.006$
	$CuSO_4$	$0.55^{de}\pm0.07$	$28.61^{a}\pm1.20$	$196.12^{i}\pm1.25$	$1.33^{e}\pm0.006$
	$HgCl_2$	$0.31^{f}\pm0.03$	$21.60^{c}\pm0.94$	$174.87^{j}\pm0.72$	$1.23^{g}\pm0.012$
D. trojana	Control	$0.16^{g}\pm0.05$	$15.55^{f}\pm0.98$	$219.46^{g}\pm0.72$	$0.72^{k}\pm0.010$
	$CuSO_4$	$0.46^{e}\pm0.08$	$23.50^{b}\pm1.31$	$276.54^{d}\pm1.90$	$1.29^{f}\pm0.006$
	$HgCl_2$	$0.36^{f}\pm0.09$	$21.85^{c}\pm1.41$	$238.20^{f}\pm1.44$	$1.13^{h}\pm0.006$
D. davisiana	Control	$0.48^{e}\pm0.05$	$11.45^{gh}\pm0.59$	$198.62^{h}\pm1.76$	$0.76^{j}\pm0.006$
	$CuSO_4$	$0.88^{b}\pm0.04$	$17.68^{e}\pm1.05$	$371.95^{b}\pm0.88$	$1.68^{b}\pm0.012$
	$HgCl_2$	$0.78^{c}\pm0.02$	$14.82^{f}\pm0.44$	$277.37^{d}\pm1.76$	$1.46^{d}\pm0.010$
D. cariensis	Control	$0.46^{e}\pm0.03$	$8.24^{i}\pm0.93$	$252.38^{e}\pm1.25$	$0.81^{i}\pm0.006$
	$CuSO_4$	$1.14^{a}\pm0.01$	$12.66^{g}\pm0.63$	$476.95^{a}\pm0.72$	$1.82^{a}\pm0.006$
	$HgCl_2$	$0.64^{d}\pm0.05$	$10.69^{hi}\pm0.63$	$330.29^{c}\pm1.90$	$1.66^{c}\pm0.010$

Note: Values followed by same small letters are not significantly different at $P<0.05$.

The CAT activity in control group ranged from 8.24 µmol H_2O_2/min/mg protein for *D. cariensis* to 19.30 µmol H_2O_2/min/mg protein for *D. lamarckii* followed by 15.55 µmol H_2O_2/min/mg protein for *D. trojana*. When incubating callus with 50 µm $CuSO_4$ induced CAT activity ranged from 12.66 µmol H_2O_2/min/mg protein for *D. cariensis* to 28.61 µmol H_2O_2/min/mg protein for *D. lamarckii* followed by 23.50 µmol H_2O_2/min/mg protein for D. trojana. Addition of 50 µm $HgCl_2$ to the media also enhanced CAT activity ranged from 10.69 µmol H_2O_2/min/mg protein for *D. cariensis* to 28.61 µmol H_2O_2/min/mg protein for *D. lamarckii*. Exposure of heavy metals was followed by a remarkable increase in SOD activity as compared to the control groups. In control, SOD activity ranged from 0.14 U/mg protein for *D. lamarckii* to 0.48 U/mg protein for *D. davisiana* followed by 0.46 U/mg protein for *D. cariensis*. While that cultured expose with $CuSO_4$ induced SOD activity ranged from 0.55 U/mg protein for *D.lamarckii* to 1.14 U/mg protein for *D. cariensis*. Exogenously applied $HgCl_2$ induced SOD activity ranged from 0.31 U/mg protein for *D.lamarckii* to 0.78 U/mg protein for *D. davisiana.* Besides the enzymatic defense machinery, we also studied non-enzymatic antioxidant response under different treatments of heavy metals. Non-enzymatic antioxidant named as total phenolic and proline levels were increased in all species under $CuSO_4$ and $HgCl_2$ stress as compared to control. Maximum total phenolic and proline production were determined as 476.95 µg GA mg^{-1} (1.88 -fold higher than control) and 1.82 µmol g^{-1} (2.24- fold higher than control) with the elicitation of $CuSO_4$ respectively.

4. DISCUSSION

Plant secondary metabolism is the source for many fine chemicals of commercial importance. One group of natural products of major interest in the pharmaceutical industry is cardiac glycosides from *Digitalis* species. Levels of plant carbon-based secondary compounds are partly under genetic control and determined in part by environmental conditions [21] therefore, in order to maximise the production of a specific natural product, it will be necessary to understand the various factors that control and influence its biosynthesis. In the case of

Digitalis plants, previous studies have reported that cardenolide biosynthesis is basically dependent on morphological differentiation [22] and genotype [23], although numerous environmental factors may determine, in a greater or lesser degree, plant productivity. Thus, it is known the influence that mineral nutrients [24, 25], CO_2 and water stress [26], and light conditions [27] exert on cardenolide accumulation. Moreover, in our previous papers, we showed that H_2O_2 pre-treatment [15] and elimination of Ca, Mg or both from the medium [28] resulted in an increase in cardenolides, enzymatic and non-enzymatic antioxidants in callus cultures of *Digitalis davisiana* Heywood, *Digitalis lamarckii* Ivanina, *Digitalis trojana* Ivanina and *Digitalis cariensis* Boiss. ex Jaub. et Spach. Elicitation strategies have been widely used to induce the production of secondary metabolites in *in vitro* cell cultures [29]. In the light of earlier studies, it was concluded that H_2O_2 increase occurred after Cu, Cd [30] and Hg [13] treatment of *A. thaliana* and *S. lycopersicum*, respectively. Similarly, Smith et al. [31] showed that production of umbelliferone, which is a phytoalexin produced in response to stress or infection in whole plants, was stimulated in suspension cultures of *Ipomoea batatas* (L.) Poir. using $HgCl_2$. Korsangruang et al. [32] found that $CuSO_4$ enhanced the accumulation of isoflavonoid compounds in *Pueraria candollei* cell suspension culture. However, there are not any reports of *Digitalis* tissue cultures in which improvements in cardenolide production have been achieved by heavy metal treatments.

The more recent identification and characterization of several enzymes/genes involved in pregnane and cardenolide metabolism, such as 3β-hydroxysteroid dehydrogenase and progesterone 5β-reductase. P5βR is considered to be a key enzyme in cardenolide biosynthesis as: it is the first stereospecific enzyme of the pathway leading to 5β-configured derivatives; it appears to be the initial step in cardenolide biosynthesis. Pérez-Bermúdez et al. [33] indicated that P5βR2 is a critical component for the chemical defense of foxglove plants against herbivores, through cardenolide accumulation, in association with ethylene and H_2O_2 signaling in *Digitalis purpurea*. Available information suggests that H_2O_2 directly regulates the expression of numerous genes involved in plant defense and the related pathways such as antioxidant enzymes, defense proteins and transcription factors [34]. In our studies, heavy metal stress significantly induced cardenolide production in callus cultures of all *Digitalis* species. The increase in secondary metabolite concentrations in the callus cultures under heavy metal stress may also be associated with the alterations in the activity of P5βR2 gene related to cardenolides. This was probably due to the reason that heavy metal-induced ROS generation was responsible for transcriptional activation of genes encoding enzymes involved in cardenolide biosynthesis. Although, the applications of heavy metals significantly increased the cardenolides accumulation in all *Digitalis* species, there was no digoxin detected in all treatments. It is well known that digilanidase enzyme catalyzed deglucosylation and subsequent deacetylation of Lan C to make into digoxin in the leaves [35]. This was probably due to the reason that the amount of digoxin in the callus tissues examined was found to be below the detection limit of the determination used.

In current work, CAT and SOD activity were significantly increased with the $CuSO_4$ and $HgCl_2$. According to Romero-Puertas et al. [36], many heavy metals could result in increased activity of NADPH oxidase partially related with O_2^- formation. Correspondingly, heavy metal-induced O_2^- formation could cause to transcriptional activation of genes responsible for antioxidative enzymes. O_2^- formation could be associated with an increased activity of SOD for conversion and parallel increased activity of CAT. Furthermore, Mittler [37] reported that increased level of antioxidants has a pivotal role in deteriorating the ROS activity, thus plants could be able to maintain their physiological functions under the stress environment. Along with primary defense mechanism, plants also activated their non-enzymatic antioxidant system named as phenolics and proline as a result of biotic and abiotic stresses, including heavy metal

toxicity [38]. Stress induced proline accumulation can reduce photochemical activity losses and the production of free radicals in the thylakoid membrane of the chloroplast [39]. Thus, proline contributed to arrest photo inhibitory damage in plants. Many plants accumulate proline at higher concentration in response to toxic concentrations of heavy metals [40]. Some researchers conclude that proline accumulation is not related to protection against metal stress [41], is just a symptom of injury. On the contrary, it has been suggested that proline might have an adaptive role related to survival of plants against heavy metal toxicity [42]. Zengin and Munzuroglu [43] showed that copper and mercury toxicity increased the proline content of the leaves of bean (*Phaseolus vulgaris* L.) seedlings. A similar observation was also recorded in our experiment that there were a positive correlation between metal toxicity and proline accumulation in callus cultures of *Digitalis* species. Therefore, we may conclude that proline may have a protective an adaptive role against stress conditions.

Studies suggested that H_2O_2 contents increased under both biotic and abiotic stresses induced expression of phenyl ammonia lyase (PAL) accompanied with the de novo synthesis of phenolics [44]. Phenolic compounds have antioxidant action because they are particularly bind iron and copper owing to their high tendency to chelate metals [45]. The content of free phenols was found to increase in two lines of wheat (*T. aestivum*) and root cultures of *Lupinus albus* L. with increasing Cu and Hg concentration in the medium, respectively. [46, 47]. In our studies, the increase in phenolic levels observed in the callus cultures of *Digitalis* species to reduce the oxidative stress caused by Cu and Hg.

5. CONCLUSION

This study demonstrated the role of heavy metals in the stimulated production of cardenolides in the callus cultures of *Digitalis* species. The productivity of cardenolides was found to be dependent on types of species and two elicitors. The present study has established that $CuSO_4$ is a better elicitor than $HgCl_2$ for cardenolide production from *Digitalis* callus cultures. This study would help to intentionally manipulate elicitation strategy to improve the yield of cardenolides profile in the callus cultures as well as to extend this protocol for large scale production of cardenolides in bioreactor utilizing *Digitalis* species in order to cope up with the demand for cardenolides in international pharmaceutical markets in future. Our results also showed that enzymatic and non-enzymatic antioxidative system are sensitive to these stress factors. Different accumulation trends were detected between individual compounds against applied treatments. We expect that further analysis of cardenolides and antioxidant molecules will provide insights into the regulatory relationships among these molecules and the role of these molecules in the establishment of mechanism for cardenolide production. The results presented in this work regarding the analysis of cardenolides under stress conditions are believed to create a useful base for the future studies in understanding the antioxidant mechanism which can be employed for the improvement of a large scale production of cardenolides.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Composition of essential oil and fatty acids of *Centaurea pichleri* ssp. *pichleri*

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Abstract: In this study, the essential oil of *Centaurea pichleri* ssp. *pichleri* was obtained by hydrodistillation using a Clevenger-type apparatus. GC and GC/MS analyzes of the essential oil from *Centaurea pichleri* ssp. *pichleri* were determined the identification of 48 components representing 86.9% of the oil. With this analysis, the major component was found as hexadecanoic acid (31.4%). Subsequent to this component, other major components were caryophyllene oxide (6.4%), spathulenol (6.2%) and dodecanoic acid (4.5%). In addition to this, fatty acid methyl esters (FAMEs) from *Centaurea pichleri* ssp. *pichleri* were prepared for analyzes of fatty acids. By this test the amount of saturated fatty acid (SFA) was found as 47.79% with major fatty acid was stearic acid (18.64%). The amount of monounsaturated fatty acid (MUFA) was found as 16.88% with major fatty acid was oleic acid (14.20%). The amount of polyunsaturated fatty acid (PUFA) was found as 21.29% with major fatty acid was linoleic acid (15.20%). The results from this work were compared with the previous works in terms of essential oils and fatty acids.

Keywords: *Centaurea pichleri ssp. pichleri*; essential oil; GC; GC/MS; fatty acid

1. INTRODUCTION

In Asteraceae family, *Centaurea* genus is one of the largest genus according to having species. *Centaurea* genus is represented with 192 taxa in Turkey, 114 of which are endemic [1]. Many species of the genus *Centaurea* have traditionally been used for their antirheumatic, diuretic, choleretic, stomachic, astringent, cytotoxic, antibacterial, antipyretic and tonic properties [2, 3]. The essential oil compositions of some *Centaurea* species from Turkey have been investigated. Generally, germacrene D, hexadecanoic acid, caryophyllene and caryophyllene oxide were reported to be the major volatile components in the earlier studies. In *Centaurea* genus, *Centaurea pichleri* ssp. *pichleri* is known as "gelin düğmesi, peygamber çiçeği" in Turkey.

Fatty acid, either saturated or unsaturated, is a carboxylic acid with a long aliphatic chain. Most naturally occurring fatty acids have an even numbered chain of carbon atoms ranging from 4 to 28. Fatty acids that have carbon–carbon double bonds are known as unsaturated fatty

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acids whereas acids without double bonds are known as saturated fatty acids. They differ in chain length as well.

To our knowledge, there are no previous studies on the essential oil and fatty acids of *Centaurea pichleri* ssp. *pichleri.* The analysis of essential oil of some plants from *Centaurea* genus have previously been reported [4-10].

2. MATERIAL and METHODS

2.1. Plant Material and Isolation of Essential Oil

The plant *Centaurea pichleri* ssp. *pichleri* was collected on 12 June 2011 at an altitude of 1450 m in Elazığ, Turkey. The plant was identified by Ugur Cakilcioglu (Elazığ Directorate of National Education, Elazığ, Turkey). Voucher speciments of the plant are deposited in Faculty of Pharmacy, Ege University with the number 1470. For obtaining the essential oil of *Centaurea pichleri* ssp. *pichleri* was done by using the method 'hydrodistillation'. For this, the air dried aerial parts of the plant was subjected to distillation by Clevenger apparatus for 3 h. After that the essential oil of the plant was obtained. This essential oil was stored at +4 ºC until using.

2.2. Oil Extraction and Preparation of Fatty Acid Methyl Esters (FAMEs)

For the analysis of fatty acid of *Centaurea pichleri* ssp. *pichleri,* air-dried and powdered aerial parts of the plant was extracted at 60 °C by Soxhlet extractor, using petroleum ether as a solvent. After oil extraction the solvent was removed by a rotary evaporator.

The extracted oil was esterified to determine the fatty acid composition. The fatty acids in the total lipid were esterified into methyl esters by saponification with 0.5 N methanolic NaOH and transesterified with 14% BF3 (v/v) in methanol [11].

2.3. Gas Chromatography (GC)

Gas chromatography analysis was carried out with an Agilent 6890 N GC system. Temperature of FID detector was 300 °C. Simultaneous autoinjection was done on a duplicate of the same column applying the same operational conditions to obtain the same elution order with GC-MS. The relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Fatty acid methyl esters (FAMEs) were analyzed on a HP (Hewlett Packard) Agilent 6890 N model gas chromatograph (GC), equipped with a flame ionization detector (FID) and fitted to a Supelco SP-2380 Fased Silica capillary column (60 m, 0.25 mm i.d. and 0.2 μm). Injector and detector temperatures were set at 250 ºC and 260 ºC, respectively. The oven was programmed at 140 °C for initial temperature and 5 min for initial time. Thereafter the temperature was increased up to 240 °C at a rate of 3°C/min. The total run time was 41.33 min. For the carrier gas helium was used (1 ml/min). Identification of fatty acids was carried out by comparing sample FAME peak relative retention times. The results were expressed as FID response area in the relative percentages. Each reported result was given as the average value of three GC analyzes. The results are offered as means±S.D.

2.4. Gas Chromatography / Mass Spectrometry (GC / MS)

The GC/MS analysis were carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m × 0.25 mm, 0.25 mm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min and kept constant at 220 °C for 10 min and then programmed to 240 °C at a rate of 1 °C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 450.

The essential oil components were identified by comparison of Mass spectra with those in Wiley GC/MS Library, Adams Library, MassFinder Library and in Baser Library of Essential Oil Constituents which was built up by genuine compounds and components of known oils. Identification of the essential oil components were carried out by comparison of their relative retention times and their relative retention indices (RRI). The results of analysis are given in Table 1.

3. RESULTS and DISCUSSIONS

Composition of the essential oil of *Centaurea pichleri* ssp. *pichleri* is listed in Table 1 with relative retantion rates (RRI) and percentages. With this analysis 48 components were identified in the essential oil of the plant. These components were represented 86.9 % of the oil. Fatty acid composition of *Centaurea pichleri* ssp. *pichleri* is listed in Table 2.

Table 1. Composition of the essential oil of *Centaurea pichleri ssp. pichleri*

RRI	Component	Percentage
1360	1-Hexanol	0.3
1391	(*Z*)-3-Hexenol	1.2
1400	Nonanal	0.3
1452	1-Octen-3-ol	0.5
1553	Linalool	0.7
1612	β-Caryophyllene	1.0
1664	Nonanol	0.8
1668	(*Z*)-β-Farnesene	0.7
1706	α-Terpineol	0.4
1726	Germacrene D	1.8
1741	β-Bisabolene	0.8
1766	Decanol	0.4
1773	δ-Cadinene	0.4
1838	(*E*)-β-Damascenone	0.4
1868	(*E*)-Geranyl acetone	0.3
1871	1-Undecanol	0.1
1941	α-Calacorene	0.6
1945	1,5-Epoxy-salvial(4)14-ene	1.5
1958	(*E*)-β-Ionone	1.4
1973	Dodecanol	0.3
2008	Caryophyllene oxide	6.4
2037	Salvial-4(14)-en-1-one	0.6
2071	Humulene epoxide-II	1.1
2080	Junenol (=Eudesm-4(15)-en-6-ol)	0.5
2098	Globulol	0.5
2130	Salviadienol	0.5
2131	Hexahydrofarnesyl acetone	1.2
2144	Spathulenol	6.2
2179	3,4-Dimethyl-5-pentylidene-2(5H)-furanone	0.6
2187	T-Cadinol	0.5
2192	Nonanoic acid	0.9
2209	T-Muurolol	0.6
2247	Trans-α-Bergamotol	0.3
2255	α-Cadinol	1.4
2278	Torilenol	0.8
2298	Decanoic acid	0.7
2324	Caryophylla-2(12),6(13)-dien-5α-ol (=*Caryophylladienol II*)	1.0

2369	Eudesma-4(15),7-dien-4β-ol	1.3
2389	Caryophylla-2(12),6-dien-5α-ol (=*Caryophyllenol I*)	1.4
2392	Caryophylla-2(12),6-dien-5β-ol (=*Caryophyllenol II*)	1.3
2500	Pentacosane	0.5
2503	Dodecanoic acid	4.5
2509	Methyl linoleate	0.8
2622	Phytol	3.0
2670	Tetradecanoic acid	2.7
2700	Heptacosane	1.5
2822	Pentadecanoic acid	0.8
2931	Hexadecanoic acid	31.4
	TOTAL	**86.9**

Table 2. Fatty acid composition of *Centaurea pichleri ssp. pichleri*

Fatty acids	*Centaurea pichleri* ssp. *pichleri*
C 6:0 (Caproic acid)	2.09[a]
C 8:0 (Caprylic acid)	5.22
C 14:0 (Myristic acid)	3.09
C 15:0 (Pentadecanoic acid)	1.18
C 16:0 (Palmitic acid)	9.28
C 17:0 (Heptadecanoic acid)	2.01
C 18:0 (Stearic acid)	18.64
C 21:0 (Heneicosanoic acid)	4.20
C 22:0 (Behenic acid)	2.08
∑SFA[b]	47.79
C 18:1 ω9 (Oleic acid)	14.20
C 20:1 ω9 (Gondoic acid)	2.68
∑MUFA[b]	16.88
C 18:2 ω6 (Linoleic acid)	15.20
C 18:3 ω6 (γ-linolenic acid)	6.09
∑PUFA[b]	21.29

[a] Values reported are means ± SD of 3 lots analysed.
[b] SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids.

4. DISCUSSION

From the analysis of essential oil of the plant, it is seen that hexadecanoic acid is the major acid. Hexadecanoic acid was previously found as the major component of essential oils of *Centaurea aladagensis, C. luschaiana, C. tossiensis, Centaurea aggregata subsp. aggregata, C. balsamita, C. behen, C. wagenitzii, C. iberica, C. hyalolepis* and *C. polyclada* from Turkey [9, 10, 12, 13]. Hexadecanoic acid, the most common saturated fatty acid which is found in animals, plants and microorganisms was known to raise plasma cholesterol concentrations and also dietary intakes of saturated fatty acids were shown to increase the possibility of coronary heart diseases [14].

With the fatty acid analysis, totally 13 fatty acids were identified in the oil of the plant. For the saturated fatty acids, the major acid was stearic acid as 18.64%. For the monounsaturated fatty acids the major acid was oleic acid as 14.20%. for polyunsaturated fatty acids the major acid was linoleic acid as 15.20%. Saturated fatty acids amounted to 47.79% of the total fatty acids, while the unsaturated fatty acids were 38.17%. There have been previous

studies on fatty acids of some *Centaurea* species [5, 15]. When these results are compared with the previous studies, our results are shown meaningful.

Linoleic acid, for the major polyunsaturated acid, is necessary in adequate amounts for health. Lack of dietary essential fatty acids such as linoleic acid has been implicated in aetiology of diseases including cardiovascular disease and its progression [16]. And also oleic acid, the major monounsaturated fatty acid for the plant, has the capability to lower blood cholesterol levels like linoleic acid. Intake of these fatty acids (oleic and linoleic acids) are promoted by nutritionists and the health professionals [17]. Oleic acid, with the ability of reducing low-density lipoprotein (LDL) levels and possibly increasing high-density lipoprotein (HDL) levels, is known as a monounsaturated fatty acid in normal diet [16,18].

In conclusion, this is the first report on the essential oil and fatty acid composition of *Centaurea pichleri* ssp. *pichleri*.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Effect of Magnetic Field Treatments on Seed Germination of *Melissa officinalis* L.

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Abstract: *Melissa officinalis* L., commonly known as lemon balm, is a perennial herb belonging to Lamiaceae family. It has therapeutic properties, such as sedative, carminative, antispasmodic, anti-viral, wound healing, digestive, diuretic, diaphoretic, anti-septic and anti-thyroid. Lemon balm has been used for the treatment of headache, indigestion, colic, nervousness, cardiac problems, depression, rheumatism, indigestion, hypersensitivities, anxiety and depression traditionally. Beneficial effects of lemon balm are ascribed to the phenolic compounds such as rosmarinic acid, tannins and flavonoids. Studies showed that magnetic field applications in agriculture can be used to improve the quality and quantity of the product. Positive effects of the stationary magnetic field on the plant seed germination have been recorded with some plant species. In this study, effects of magnetic field on *M. officinalis* seeds were investigated. Seeds were sterilized in 0.1 % $HgCl_2$ for 10 min and 70 % Ethanol for 1-2 min. After surface sterilization of the seeds, they were placed in petri dishes containing Murashige and Skoog's medium with sucrose and agar. Neodymium block magnets (100 X 50 X 5 mm) were used to create magnetic fields (50 mT and 100 mT). Ten seeds were placed in each petri plates and 10 petri plates were used for each treatment. Petri dishes containing surface sterilized seeds were placed in 3 different conditions [without magnetic field application (control) and magnetic field applications (low-50 mT and high-100 mT)] and the germination (radicle protrusion) was assessed. Seeds were exposed to magnetic fields for 1, 3, 6, 12, 24, 48, 72, 144 and 240 hours. The number of germinated seeds was recorded for 20 days. Best seed germination was obtained with 100 mT magnetic field application for 1 hour (52 %). Seed germination rate was rather low (28 %) without magnetic field application (control). In consistent with 100 mT magnetic field application, 1 hour exposure to 50 mT magnetic field gave better germination rate (36 %) than control. The lowest seed germination was observed with 240 hours exposure to both magnetic fields (27 % for 100 mT and 16 % for 50 mT). Magnetic field applications also decreased the seed germination time. Although seed germination was observed in 11. day with control, it was obtained in 7. day with both magnetic field applications. Magnetic field application enhanced the percentage of germinated seed and shortened the period of seed germination in *M. officinalis*.

Keywords: Magnetic field, *Melissa officinalis*, seed germination

1. INTRODUCTION

Melissa officinalis L. is a perennial bushy plant belonging to Lamiaceae family [1]. It is commonly called as 'lemon balm' because of its lemon-like flavor and fragrance [2]. Native to Europe, lemon balm is grown all over the world. It is grown not only in herb gardens or to attract bees, but also in crops for medicine, cosmetics, and furniture polish manufacturing [3].

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It is an aromatic plant with useful applications in medicine, cookery and perfumery [4]. Lemon balm has been used traditionally for centuries as sedative, relaxant, stomachic, antispasmodic, carminative, diaphoretic, febrifuge, antiviral, antiseptic and nerve tonic in folk medicine. Traditional uses include healing wounds, relieving palpitations and relaxing the hearth, and treating toothache [5-10]. Preparations from leaves are used to treat feverish colds and headaches, to relieve menstrual cramps, and to calm nervous stomachs [11]. All of these actions of *M. officinalis* may be related to the high levels of phenolic acids especially rosmarinic acid [12]. Citral, citronellal and geraniol are the main components of essential oil of lemon balm in various climates [13].

In recent decades, physical techniques based on the application of magnetic fields are being developed in the agriculture sector [14]. Studies showed that low magnetic field applications in agriculture can be used to improve the quality and quantity of the product [15]. Numerous authors have reported the positive influence of the stationary magnetic field on the plant seeds. The treatment fastens plants development, improves germination and seedling growth, and activates protein formation and enzymes activity [16]. The investigations have shown that the treatment of the seeds with magnetic field increases the germination of nonstandard seeds and improves their quality [17]. Positive effects of the stationary magnetic field on the plant seed germination have been recorded with several plant species [14, 18-23]. The objective of this study was to investigate the effect of magnetic field on *M. officinalis* seed germination.

2. MATERIAL and METHODS

2.1. Magnetic Field Establishment

Neodymium block magnets (100 x 50 x 5 mm) were used to create a magnetic field. The magnets were fixed on the aluminum stand. Magnetic field ranging between 50 ± 5 mT and 100 ± 5 mT was adjusted putting magnets side by side (magnet set) and magnetic canal was created between two magnet set. Magnetic field strength was measured using Teslameter. Petri dishes with a diameter of 90 mm were placed between magnet sets.

2.2. Germination

Seeds of *M. officinalis* were collected from Bolu, Turkey. Identification of the plant was made by using "Flora of Turkey and the East Aegean Islands" [1]. Seeds were washed with an anti-bacterial soap, rinsed with distilled water, surface sterilized by shaking for 15 min in 0.1 % $HgCl_2$, then washed with sterilized water three times and sterilized in 70 % Ethanol for 1-2 min., and finally washed again with sterile water for three times. After surface sterilization of the seeds, seeds were placed in sterile, disposable petri dishes containing Murashige and Skoog's minimal organics (MSMO) medium [24] with 30 g/l sucrose, 8 g/l Difco Bacto-agar (pH 5.7, autoclaved for 20 minutes at 121°C and 105 kPa). Ten seeds were placed in each petri plates and 10 petri plates were used for each treatment. Petri dishes containing surface sterilized seeds were placed in 3 different conditions [without magnetic field application (control) and magnetic field applications (low-50 mT and high-100 mT)] and the germination (radicle protrusion) was assessed. Seeds were exposed to magnetic fields for 1, 3, 6, 12, 24, 48, 72, 144 and 240 hours. The number of germinated seeds was recorded for 20 days.

3. RESULTS and DISCUSSION

M. officinalis seeds were magnetically exposed to static magnetic field intensity of 50 mT and 100 mT for different time of exposure, 1, 3, 6, 12, 24, 48, 72, 144 and 240 hours, respectively. It was found that magnetic field intensity and exposure time were a very significant factor on the germination process of lemon balm seeds comparing with non-treated

seeds (Figure 1, 2 and 3). Seeds were considered germinated when the protrusion of the radicle became evident. The highest seed germination was observed with 100 mT magnetic field application for 1 hour (52 %) (Figure 3). Germination rate of control group (non-exposed seeds) was 28 % that is nearly half of the treatment group with application of 100 mT magnetic field for 1hr. One hour exposure to 50 mT magnetic field also gave better result than control (36 % and 28 %, respectively) (Figure 1, 2 and 3). Exposure time was a determinant factor for the germination of lemon balm seeds and best germination rate was obtained with low exposure time (1 hour). Increasing exposure time lowered germination rate in both magnetic field applications (100 mT and 50 mT) (Figure 1, 2 and 3) and the lowest seed germination was obtained with 240 hours in both of them (27 % for 100 mT and 16 % for 50 mT). Furthermore, the onset of the germination occurred earlier with magnetically treated seeds. The length of time for seed germination was shortened with magnetic field applications observing seed germination in 7. day with both magnetic field applications and 11. day with non-treated seeds (Figure 2 and 3).

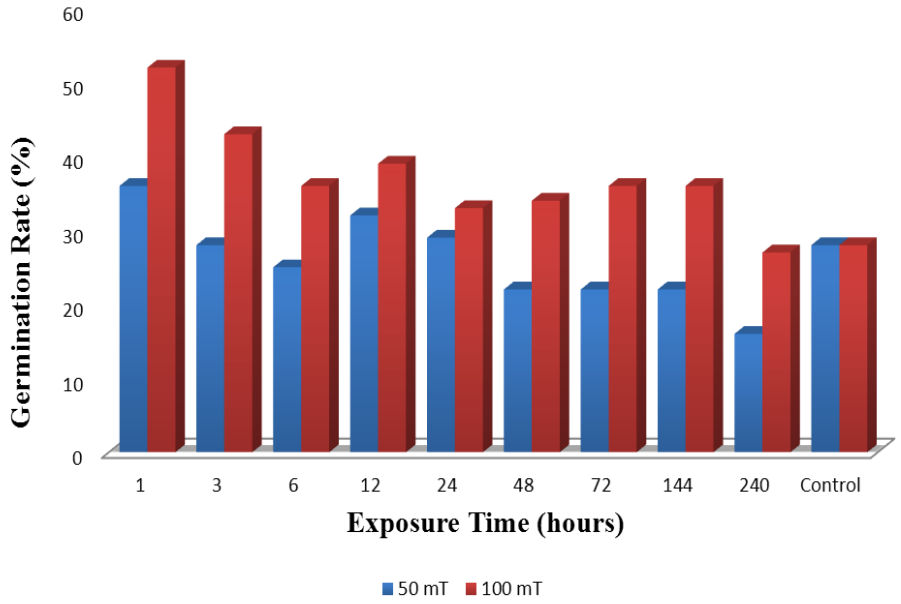

Figure 1. Effect of magnetic fields (50 mT and 100 MT) with different exposure times on germination rates of lemon balm seeds.

Yalçın and Tayyar [25] reported the effects of magnetic field on *M. officinalis* seeds. They were exposed the magnetic field strengths (1, 3, 9 and 15 times) in the range of 3.8-4.8 mT on the movable ground with the velocity of 1m/sec. Germination rates were higher with magnetic field applications than control group. Many studies showed that 125 mT and 250 mT magnetic treatment produced a biostimulation on the initial growth stages and an increased the germination rate of several seeds such as rice, wheat and barley [18, 19, 21-23]. Magnetic field application enhanced the percentage of germinated seed on the treated group compared to non-exposed in both *Salvia officinalis* and *Calendula officinalis* [14]. Maize seeds exposed to magnetic field of 125 mT or 250 mT for varying periods of time germinated faster and the treated seedlings grew higher and heavier [23]. In wheat, Pittman and Ormrod [26] reported that the seedlings grown from magnetically treated seed (180 mT) absorbed more moisture, respired more slowly, released less heat energy and grew faster than the untreated controls. In soybean, Kavi [27] observed that the seeds exposed to magnetic field of 300 mT had increased capacity to absorb moisture. It was postulated that the ion-cyclotron resonance might interfere

with the Ca^{2+} ion sequestering and thereby enable the increase in free Ca^{2+} concentration in the system. The increased Ca^{2+} concentration might signal the cell to enter into early mitotic cycle [28]. Garcia et al. [29] carried out an experiment on water absorption by lettuce seeds previously treated in a stationary magnetic field of 1 to 10 mT. They reported an increase in water uptake rate due to the applied magnetic field, which might be the explanation for the increase in the germination of treated lettuce seeds. They concluded that changes in intracellular levels of Ca^{2+} and in other ionic current density across cellular membrane caused alteration in osmotic pressure and changed in the capacity of cellular tissues to absorb water. Reina and Pascual [20] investigated the influence of a stationary magnetic field on water relations in lettuce seeds and observed an increase in water uptake rate due to the applied magnetic field. They deduced that magnetic field interacting with ionic current in the cell membrane changed the ionic conductivity of the membrane and therefore, the concentration and the osmotic pressure in both sides of the membrane, and this change in the osmotic pressure altered the mechanism of water imbibition of seeds [20].

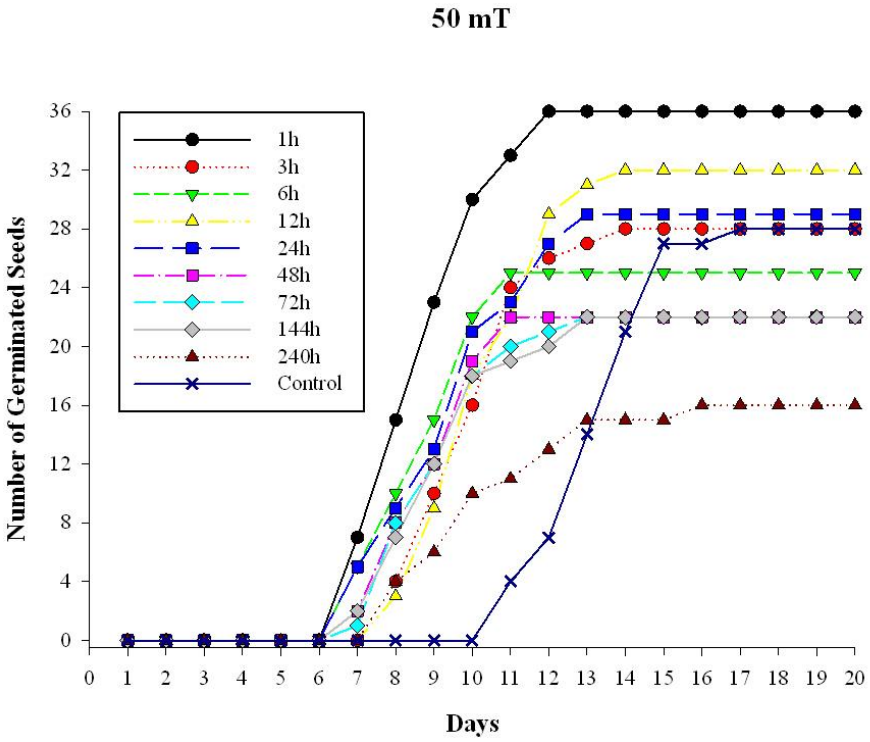

Figure 2. Effect of 50 mT magnetic field on germination of lemon balm seeds.

In recent decades, physical techniques based on the application of magnetic fields are being developed in the agriculture sector [14]. Studies showed that low magnetic field applications in agriculture can be used to improve the quality and quantity of the product [15]. Numerous authors have established the positive influence of the stationary magnetic field on the plant seeds. The treatment fastens plants development, improves germination and seedling growth, and activates protein formation and enzymes activity [16]. The investigations have shown that the treatment of the seeds with magnetic field increases the germination of nonstandard seeds and improves their quality [17].

In consistent with other studies, our results showed that germination percentages were higher for magnetically treated lemon balm seeds. Also, germination time was significantly reduced when seeds were exposed to magnetic fields. Our results suggest that magnetic treatments can be used as a physical technique to improve the germination of *M. officinalis* seeds.

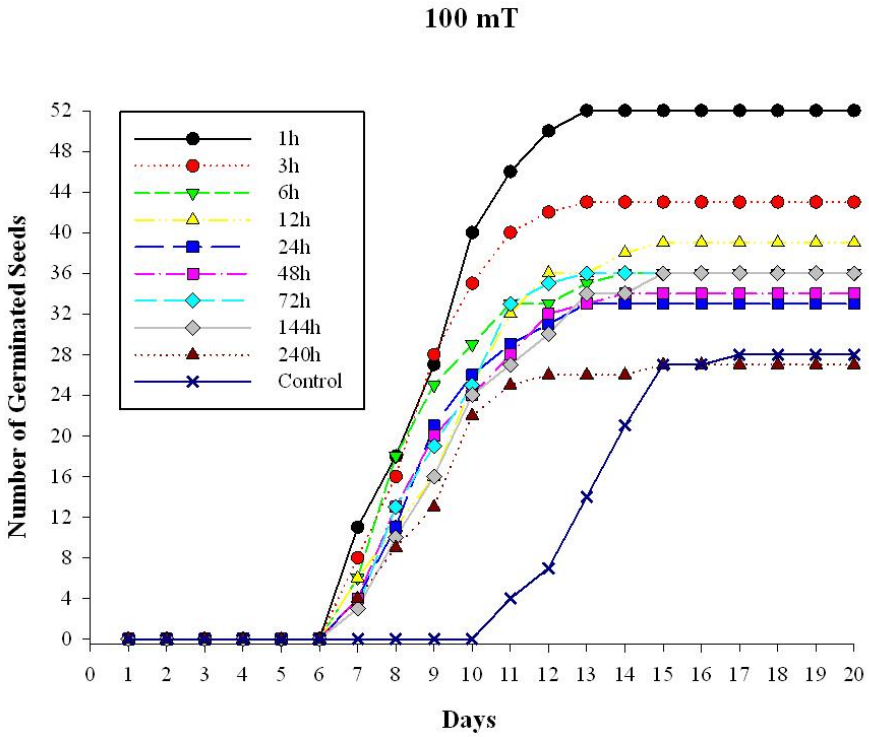

Figure 3. Effect of 100 mT magnetic field on germination of lemon balm seeds.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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In Vitro Bulb Regeneration from Stem Explants of Endemic Geophyt *Muscari aucheri* (Boiss.) Baker

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Abstract: *Muscari aucheri* (Boiss.) Baker bears beautiful and attractive purplish blue flowers bloom between May and June; it grows stony slopes, mountain pastures an altitude of 1000-3000 meters. The natural populations of *Muscari aucheri*, is seriously affected by increased environmental pollution, urbanization and grazing. Therefore, this study aimed to accelerate multiplication by devising a strategy for an efficient *in vitro* bulblet regeneration system of *Muscari aucheri* using stem explants on MS medium containing 1, 3, 5 mg/l TDZ plus 0, 0.1, 0.2, 0.4 mg/l NAA (12 combinations). The stem explants induced direct bulblet regeneration on explants. Maximum mean number of bulblets per explant was noted on MS medium containing 3 mg/l TDZ + 0.4 mg/l NAA and maximum mean number of bulb diameter was noted on MS medium containing 5 mg/l TDZ + 0.2 mg/l NAA. The regenerated bulblets were isolated from stem explants and cultured on MS medium containing 40 g/l sucrose; where they gained diameter and rooted

Keywords: *Muscari aucheri*, *in vitro*, bulb regeneration

1. INTRODUCTION

Because of important location of Turkey has created a diversity of climates, habitats and ecosystems that has resulted in extraordinary plant diversity of above 13.000 native vascular plants. At least 3025 (33%) taxa among them are endemic, about 2222 endangered and 585 as vulnerable or critically endangered. Most of the vulnerable geophythes belong to Liliaceae, Amaryllidaceae and Iridaceae families [1]. Bingol also has a very rich flora and many endemic species and the main reasons of this richness are its geomorphologic features, microclimate and habitat diversities. The genus *Muscari* Mill. includes nearly 61 taxa distributed in the world and its members mostly occur in the whole Mediterranean basin as far as the Caucasus, temperate Europe, North of Africa and South West of Asia [2]. In Flora of Turkey Muscari has about 30 taxa and the endemism rate in this genus is consequently very high, about 60% [3]. Endemic *Muscari aucheri* is most fragrant plant among *Muscari* taxa and has high ornamental potential [3]. Ten species of *Muscari* including ornamental *M. aucheri* are endemic or vulnerable and has concolorous flowers [4]. The natural populations of *M. aucheri*, is seriously affected by

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increased environmental pollution, urbanization and grazing. *M. aucheri* bears beautiful and attractive purplish blue flowers bloom between May and June; it grows stony slopes, mountain pastures an altitude of 1000-3000 meters [5]. A number of *Muscari* species are used as ornamental garden plants. However, commercial propagation especially micropropagation of *M. aucheri* has to be advanced. Nowadays, due to number of abiotic and biotic stresses M. aucheri populations are under great pressure at its habitat. For example, some Muscari species has been included in the list of export prohibited plant species [4]. So there is urgent need to production with micropropagation method that should serve effectively for commercial propagation and conservation of geophytes, especially endemic *M. aucheri*.

The present investigation is a preliminary study. Extension of this study purposely may help in multiplication of this plant with unrestricted and safe availability of *M. aucheri* throughout the year. Therefore, this study aimed to develop and optimise a bulblet regeneration protocol using stem explants.

2. MATERIAL and METHODS

2.1. Plant Material and Sterilization

M. aucheri were collected from Bingol-Center, above Alatepe village, vicinity of Fırın river, plateau, stony slopes, 1800-1900 m. The plants were identified based on "The Flora of Turkey and East Aegean Islands, volume 8"[6]. The voucher specimens are deposited at the herbarium of the Department of Biology, Hacettepe University Ankara and the Department of Park and Garden Plants of Bingol University, Turkey. Before starting the experiment, the stems from the plants were washed under running tap water for 30 min to remove all adhering contaminants. Thereafter, stems of these plantlets were treated with 35% H_2O_2 solution for 30 min under aseptic conditions followed by 5×5 min rinsing with sterilized distilled water. Thereafter, ~0.5 cm long stems explants were excised aseptically.

2.2. Culture Conditions

All explants were cultured in petri dishes on 0.65% (w/v) plant agar (Duchefa) solidified MS medium [7] containing 1, 3, 5 mg/l TDZ plus 0, 0.1, 0.2, 0.4 mg/l NAA (12 combinations) supplemented with 3% sucrose to regenerate bulblets. A control was also planted on MS medium supplemented with 3% sucrose without growth regulators. The pH of all media was adjusted to 5.6 with 1M NaOH or 1 M HCl and media were autoclaved for 20 min at 120 °C, 1.4 kg cm^{-2}. Unless otherwise stated, all cultures were maintained under 16 h light/ 8 h dark photoperiod (35 μmol m^{-2} s^{-1}) in versatile growth chamber at 24±1°C.

3. RESULTS and DISCUSSIONS

The present investigation was carried out to optimize micropropagation and fast multiplication protocol from stems explants of *M. aucheri*. All stems explants regenerated bulblet on MS medium five months after culture initiation. The results showed that the combinations and rates of TDZ and NAA sharply affected bulblet regeneration percentage. Bulblets regeneration percentage ranged between 100.0±0.3 to 36.4±0.7l % (Table 1). Furthermore, equal to or over 60.3±0.5% bulblet regeneration was noted on 6 treatments. The highest bulblet regeneration percentage was obtained on MS medium containing 3 mg/l TDZ + 0.4 mg/l 2 NAA (Fig 1 a) followed very closely by MS medium containing 5 mg/l TDZ + 0.4 mg/l NAA. The results further showed that combinations and rates of plant growth regulators used in the study also affected the mean number of bulblets per explant. The stems explants were found very suitable for bulblet regeneration. Bulblets were induced directly on the stems explants (Fig 1 b, c, d). The mean number of bulblets per explant ranged between 37.3±0.8 to 9.7±0.4. The maximum mean number of bulblets per explants (37.3±0.8) were recorded on MS

medium containing 5 mg/l TDZ + 0.20 mg/l NAA. It was followed by a significantly reduced number of 22.7±0.6 bulblets per explant on MS medium containing 5 mg/l TDZ + 0.1 mg/l NAA (Table 1).

Table 1. Effect of different concentrations of TDZ + NAA on MS medium on bulblet regeneration from stems explants of *M. aucheri*.

Growth Regulators		Bulblet regeneration	Mean number of bulblets	Mean bulblet
TDZ(mg/l)	NAA(mg/l)	percentage (%)	per explant	diameter (cm)
MS medium (control)		0.0±0.0	0.0±0.0	0.0±0.0
1	0	36.4±0.7l	11.6±0.2j	0.1±0.0c
3	0	38.3±0.4k	9.7±0.4l	0.1±0.0c
5	0	42.6±1.2j	12.3±0.7i	0.1±0.0c
1	0.1	46.7±0.5i	17.6±0.5e	0.2±0.1b
3	0.1	50.3±0.1h	10.3±0.3k	0.2±0.2b
5	0.1	52.7±0.1g	22.7±0.6b	0.3±0.1a
1	0.2	60.3±0.5f	14.6±0.8f	0.2±0.1b
3	0.2	66.6±1.0e	18.7±0.3d	0.1±0.1c
5	0.2	68.3±0.4d	37.3±0.8a	0.1±0.2c
1	0.4	72.7±0.7c	14.6±0.5g	0.1±0.1c
3	0.4	100.0±0.3a	13.3±0.3h	0.2±0.1b
5	0.4	84.6±0.4b	21.7±0.7c	0.2±0.2b

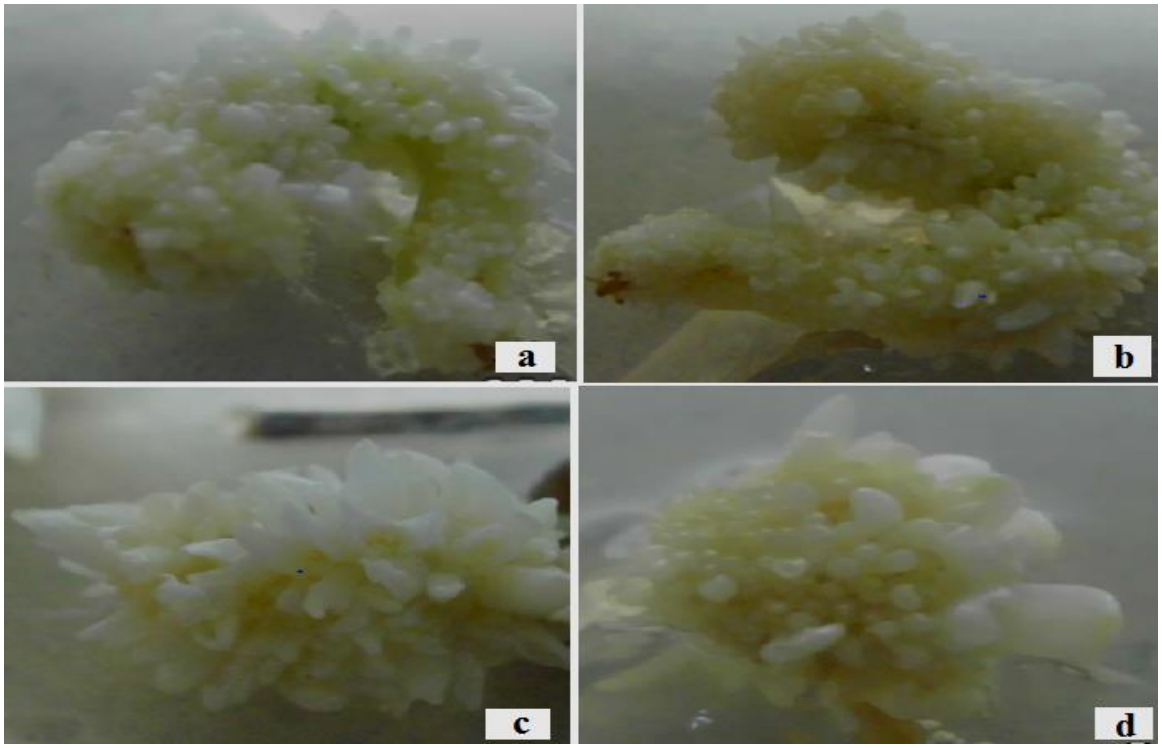

Figure 1. Bulblet regeneration on stems explant of *M. aucheri*. (a) using MS medium containing 3 mg/l TDZ + 0.4 mg/l NAA (b), developing and growing bulblets on MS medium (Bar Fig 1 a, b, c, d =0.3 cm).

Mean bulblet diameter varied in the range of 0.3±0.1 to 0.1±0.0 (Table 1). The maximum mean bulblet diameter (0.3±0.1 cm) was recorded on MS medium containing 5 mg/l TDZ + 0.1 mg/l NAA. Bulblet diameter equal to 0.10 cm or above was recorded for 6 treatments. It was noted that the bulblets induced a positive increase in bulblet diameter on culturing on MS

medium containing 40 g/l sucrose, where they induced a bulblet diameter. Plant tissue culture techniques provide possibility to introduce new approaches for direct regeneration depending on strong [8]. The major problem to regeneration from any plant could be right choice of the explants and plant growth regulator combinations. In vitro micropropagation of *M. aucheri* is very important and needs attention for development of micropropagation system for commercial use. MS medium supplemented with 12 different combinations of TDZ plus NAA provided profuse and high percentage shoot regeneration on stems explant used in this study. The results of this study showed that use of stems explants for the micropropagation of M. aucheri may have great value. Previous studies on other bulbous plants [9, 10] recorded that the media containing TDZ induce positive effects on regeneration capacity.

Results of the present study agreement with these sudies. Also TDZ did not effectively promote bulblet regeneration in *M.aucheri* [11]. Results of the present study differ from this study, the contrasting results may be related with different endogenous plant growth regulators in explants obtained from different types of geophyte species used in the above mentioned reports. But are in agreement with Huetteman and Preece (1993) who reported that TDZ increased bulblet regeneration [12]. In the present study high bulb regeneration was obtained with media using 3 mg/l TDZ + 0.4 mg/l 2 NAA. Similarly Nasircilar et al., (2011) reported positive increase in mean number of bulb regeneration when TDZ was used with NAA [13]. Our results agreement with this finding and showed efficient bulblet regeneration from stems explants using TDZ + NAA. The concentration of TDZ or NAA in the culture medium was found to have a critical role for bulblet regeneration. Suzuki and Nakano (2001) showed that the importance of NAA on bulb regeneration in culture medium [14]. This finding agreement that NAA increase in culture medium, increase bulblet regeneration too. The findings of the study confirm that the stems can be used successfully as explants and can form an alternative source for regeneration without destruction of stock plant and positively evaluate potential of expediently isolated explants from inflorescence stems for an efficient micropropagation.

4. CONCLUSION

In conclusion, culturing stems explants on MS medium containing BAP and NAA is an effective method for in vitro propagation of *M. aucheri*. This method can be used for propagation and conservation of endemic *M. aucheri.*

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Determination of antioxidant properties of *Rumex crispus* and *Scrophularia canina* subsp. *bicolor*

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Abstract: Methanol and ethyl acetate extracts of *Rumex crispus* L. and *Scrophularia canina* L. subsp. *bicolor* (SM.) Greuter were tested for their antioxidant activity using the DPPH method. Extracts were prepared from the above-ground parts of these plants. Significant antioxidant activity was determined for methanol (IC_{50}: 4.16 µg/mL) and ethyl acetate (IC_{50}: 8.71 µg/mL) extracts of *Rumex crispus*. Moreover, methanol (IC_{50}: 60.78 µg/mL) and ethyl acetate (IC_{50}: 149.33 µg/mL) extracts of *Scrophularia canina* subsp. *bicolor* (SM.) Greuter were shown to have important free radical scavenging antioxidant activity.

Keywords: Antioxidant activity, *Rumex crispus*, *Scrophularia canina* subsp. *bicolor*

1. INTRODUCTION

The genus *Rumex* L. (Polygonaceae) consists of about 200 species growing worldwide and 23 species and 5 hybrids naturally growing in Turkey. *Rumex crispus* L. is a perennial plant, and its basal leaves are acute and narrowly lanceolate to oblanceolate [1, 2]. Various parts of *Rumex* species, including roots and fresh leaves, have been used in traditional medicine in Turkey. *Rumex* roots have important uses because of their laxative property. Decoctions prepared from the underground parts have been claimed to be therapeutically useful as cholagogue, tonic and laxative and for blood cleansing. Fresh leaves are used to treat eczema and also consumed as vegetable in Anatolia [3].

The name of *Scrophularia* comes from "scrofula", a kind of tuberculosis, since some species have been used for treatment of tuberculosis [4]. In the flora of Turkey, *Scrophularia* is represented by 59 species, 23 of which are endemic. Some *Scrophularia* L. species, especially *S. nodosa* L. are used in folk medicine as a diuretic and for the treatment of wounds and hemorrhoids [5, 6]. Different species of the genus *Scrophularia* (Scrophulariaceae) have been used in traditional medicine to treat some diseases, including dermatosis and inflammatory affections [7]. Besides that, some of *Scrophularia* species has shown anticancer and cell growth enhancing activities [8].

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Contrary to *Scrophularia canina* L. subsp. *bicolor* (SM.) Greuter there have been reports on the antioxidant activity of *Rumex crispus* species. The leaves, seeds and fruits of Rumex *crispus* have been shown to have antioxidant activity [9-10]. In the present study it is aimed to investigate the DPPH radical scavenging activity of methanol and ethyl acetate extracts prepared from *Rumex crispus* and *Scrophularia canina* subsp. *bicolor*.

2. MATERIAL and METHODS

2.1. Plant Material

Rumex crispus and *Scrophularia canina* subsp. *bicolor* were collected from Soma, Manisa. These plants were identified by Volkan Eroğlu and Hasan Yıldırım, respectively (Ege University, Faculty of Science). Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Above-ground parts of *Rumex crispus* and *Scrophularia canina* subsp. *bicolor* were powdered and 5 g powder of both plants were extracted with methanol and ethyl acetate (30 ml) three times. Extraction was followed by filtration and the filtrate was evaporated to dryness by a rotary evaporator.

2.2. Free Radical Scavenging Activity by DPPH Method

Radical scavenging activity of the extracts were determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) method [11] with slight modification. Methanol and ethyl acetate extracts of *Rumex crispus* were tested within the range of 1.25 - 20 µg/mL concentrations. DPPH radical scavenging activity of methanol extract of *Scrophularia canina* subsp. *bicolor* was performed within the range of 25-400 µg/mL concentrations. Antioxidant activity of ethyl acetate extract of *Scrophularia canina* subsp. *bicolor* was determined by using a concentration range of 50-800 µg/mL. DPPH solution was added to the extracts and the absorbance of the reaction mixture was measured at 517 nm after 30 minutes. Inhibition of free radical DPPH in percent (I %) was calculated and a sample concentration providing 50 % inhibition (IC_{50}) was calculated by plotting inhibition percentages against different concentrations of samples. The experiments were carried out in triplicate.

3. RESULTS

In DPPH assay, significant antioxidant activity was determined for methanol (IC_{50}: 4.16 µg/mL) and ethyl acetate (IC_{50}: 8.71 µg/mL) extracts of *Rumex crispus*. The IC_{50} values of *Scrophularia canina* subsp. *bicolor* (methanol IC_{50}: 60.78 µg/mL) and (ethyl acetate IC_{50}: 149.33 µg/mL) extracts were higher than the extracts of former species pointing to a weaker antioxidant activity.

4. DISCUSSION

Rumex crispus and some *Scrophularia* species contain phenolic compounds [4-12]. Phenolic compounds are among the major secondary metabolites in plants responsible for their antioxidant activity (12). Therefore, antioxidant activity of these plant species may be due to their phenolic composition.

In conclusion, all of the extracts prepared from *Rumex crispus* and *Scrophularia canina* subsp. *bicolor* have been found to possess antioxidant activity. Future studies, regarding the determination of the components responsible for the free radical scavenging activity may be carried out to clarify the antioxidant potentials of these plants.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Essential Oil and Fatty Acid Composition of *Centaurea solstitialis* ssp. *solstitialis*

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Abstract: *Centaurea* is a widespread genus from Asteraceae family in Turkey. There are plenty of researches about fatty acid and essential oil profiles of *Centaurea* species. Essential oils were obtained using a Clevenger apparatus by hydrodistillation from aerial part of the plant. The essential oil composition of the plant was identified by GC-MS using FID detector. 31 compounds representing 91.5% were identified. Hexadecanoic acid (50.2%) and tetradecanoic acid(10.1%) were found to be the major compounds. For fatty acids, fatty acid methyl esters (FAMEs) were prepared. The fatty acid compositions were analyzed by GC. Saturated fatty acids (SFAs) were totally 25.05%, monounsaturated fatty acids (MUFAs) were 19.60% and polyunsaturated fatty acids were 19.86%. The major compounds were found as oleic acid (18.54%), linoleic acid (10.07%), palmitic acid (8.28%), stearic acid (6.82%) and γ- linoleic acid (6.75%).

Keywords: *Centaurea*, essential oil, fatty acid

1. INTRODUCTION

Asteraceae family which is included in Cynarae ordo comprises 4 sub-ordo; Carduinea, Carlineae, Centaureinae and Echinipsideae. *Centaurea* is included in Centaureinae. Asteraceae family has 130 genus in Turkey. *Centaurea* is a widespread genus including 180 species [1, 2]. *Centaurea* genus is widely used in folk medicine as sedative, antipyretic and against allergy in Turkey. *Centaurea solstitialis* ssp. *solstitialis* is an also widespread species from this genus. According to the etnobotanic researches, *C. solstitalis* ssp. *solstitialis* is mostly used for urinary diseases [3]. When we examine the the general content of the species it is well known that sesquiterpene lactones, flavonoids and polyacetylenes are major groups of seconder metabolites [4-6].

Fatty acids have many important biological functions such as presenting in biological membran structure and being an energy source. PUFAs and MUFAs are useful for decreasing LDL [7]. Essential oils have also different biological activities such as antimicrobial and

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antioxidant. There are lots of essential oil and fatty acid researches about different *Centaurea* species [8].

In this study we present the volatile oil profile and fatty acid content of *C. solstitialis* ssp. *solstitalis*. To the best of our knowledge, this is the first report for determining essential oil and fatty acid profile of *Centaurea solstitialis* ssp. *solstitialis* from Elazığ, Turkey.

2. MATERIAL and METHODS

2.1. Plant Material

C. solstitalis ssp. *solstitialis* was collected from Elazığ, Turkey in 2011. The plant was identified by one of the authors (B. Kivcak) from Ege University. Voucher specimen was deposited by number 1468 in the Herbarium of Faculty of Pharmacy, Department of Pharmacognosy, Ege University, Izmir, Turkey. The dried and powdered aerial parts of the plant material (40 g) have been extracted by petroleum ether (400 ml) for 6 h at 60ºC by Soxhlet extractor. The solvent was evaporated by a rotary evaporator. The obtained oil was esterified to determine the fatty acid composition. Additionally the air-dried aerial parts of the plant was subjected to hydrodistillation for 3 h using a Clevenger-type apparatus to obtain essential oil.

2.2. Fatty acid methyl esters (FAMEs) preparation

The fatty acids were esterified into methyl esters by saponification with methanol (50%) containing 5% sodium hydroxide at 100 ºC for 10 min and transesterified with 14% (v/v) boron trifluoride (BF_3) in methanol 100 ºC for 5 min (A).

2.3. GC Conditions

Fatty acid methyl esters (FAMEs) were analyzed on a HP (Hewlett Packard) Agilent 6890 N model gas chromatograph (GC), equipped with a flame ionization detector (FID) and fitted to a Supelco SP-2380 Fased Silica capillary column (60 m, 0.25 mm i.d. and 0.2 µm). Injector and detector temperatures were set at 250ºC and 260ºC, respectively. The oven was programmed at an initial temperature of 140ºC and an initial time of 5 min. Thereafter the temperature was increased up to 240°C at a rate of 3°C min^{-1}. The total run time was 41.33 min. Helium was used as the carrier gas (1 ml min^{-1}). Identification of fatty acids was carried out by comparing sample FAME peaks from samples with standarts (26). The results were expressed as FID response area in the relative percentages. Each reported result is given as the average value of three GC analyses. The results are offered as means ±S.D.

2.4. Gas Chromatography-mass spectrometry (GC-MS) Conditions

Agilent gas chromatograph model 6890 equipped with an Innowax FSC column (60 m x 0,25 mm x 0,25µm). Instrument conditions were programmed from 60ºC (10 min) to 220ºC (5ºC/min), stayed 10 min at 220ºC and to 240ºC for 1 min. Split ratio was 40:1; injector and detector temperatures were 250 and 280ºC, respectively. The MS conditions were programmed as ionization potential at 70 eV, between 35-450 mass range. In order to identificate the components by comparison, their relative retention times and their relative retention indices (RRI) were used by analyzing Wiley GC/MS Library, Adams Library, MassFinder Library and in Baser Library. Retention indices were determined by using standard alkanes (C_9-C_{30}) and also by comparison of literature data.

3. RESULTS AND DISCUSSION

The results of fatty acid analyses are shown in Table 1. Saturated fatty acids (SFAs) were totally 25.05%, monounsaturated fatty acids (MUFAs) were 19.60% and polyunsaturated fatty acids were 19.86%. The major compounds were found as C 18:1 ω9 (oleic acid) (18.54%), C 18:2

ω6 (linoleic acid) (10.07%), C 16:0 (palmitic acid) (8.28%), C 18:0 (stearic acid) (6.82%) and C 18:3 ω6 (γ- linoleic acid) (6.75%).

Table 1. Fatty Acid Profile of *C. solstitialis* ssp. *Solstitialis*

Fatty Acids	*Centaurea solstitialis* ssp. *solstitialis*
C 4:0 (Butyric acid)	-
C 6:0 (Caproic acid)	-
C 8:0 (Caprilic acid)	1.76
C 12:0 (Lauric acid)	2.87
C 13:0 (Tridecyclic acid)	-
C 14:0 (Miristic acid)	2.45
C 15:0 (Pentadecanoic acid)	1.06
C 16:0 (Palmitic acid)	8.28
C 17:0(Heptadecanoic acid)	-
C 18:0 (Stearic acid)	6.82
C 21:0(Heneicanoic acid)	1.08
C 22:0 (Behenic acid)	-
C 23:0 (Tricosanoic acid)	0.56
C 24:0 (Lignoseric acid)	0.17
∑SFA[b]	25.05
C 18:1 ω9 (Oleic acid)	18.54
C 20:1 ω9 (Gondoic acid)	-
C 24:1 ω9 (Nervonic acid)	1.06
∑MUFA[b]	19.60
C 18:2 ω6 (Linoleic acid)	10.07
C 18:3 ω6 (γ-linolenic acid)	6.75
C20:3ω3(Eicosotrienoic acid)	3.04
∑PUFA[b]	19.86

[a]n: 3; SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

The hydrodistillation of the aerial part of *C. solstitialis* ssp *solstitialis* gives essential oil of yellow colour. The identified components of essential oils and percentages are shown in Table 2. 31 compounds representing 91.5% were identified. Hexadecanoic acid (50.2%) and tetradecanoic acid(10.1%) were found to be the major compounds.

Table 2. Identified Essential Oil Components from *C. solstitalis* ssp. *solstitialis*

RRI	COMPOUND	PERCENTAGE (%)
1400	Nonanal	0.3
1705	Zizanene	0.3
1740	α-Muurolene	0.4
1827	(*E*,*E*)-2,4-Decadienal	0.7
1830	Tridecanal	
1838	(*E*)-β-Damascenone	0.3
1868	(*E*)-Geranyl acetone	0.3
1870	Hexanoic acid	0.1
1945	1,5-Epoxy-salvial(4)14-ene	0.6
1958	(*E*)-β-Ionone	0.6
2008	Caryophyllene oxide	1.1
2037	Salvial-4(14)-en-1-one	0.6
2041	Pentadecanal	0.3
2080	Junenol (=Eudesm-4(15)-en-6-ol)	0.5
2084	Octanoic acid	0.6
2098	Globulol	0.5
2130	Salviadienol	0.3
2131	Hexahydrofarnesyl acetone	1.0
2144	Spathulenol	4.4
2209	T-Muurolol	0.8
2255	α-Cadinol	1.1
2278	Torilenol	0.9
2300	Tricosane	1.1
2369	Eudesma-4(15),7-dien-4β-ol	1.0
2500	Pentacosane	0.8
2503	Dodecanoic acid	4.4
2622	Phytol	2.7
2670	Tetradecanoic acid	10.1
2700	Heptacosane	2.5
2822	Pentadecanoic acid	1.3
2900	Nonacosane	0.7
2931	Hexadecanoic acid	51.2
		91.5

In previous studies for fatty acid profile; C 18:2 ω6 (linoleic acid) and α-linoleic acid seemed to be the major compoud and C 18:1 ω9 (oleic acid) and C 16:0 (palmitic acid) are also predominantly major compounds. Our results are compatible with literature about fatty acid profile of *Centaurea* species. Oleic acid; monounsaturated fatty acid (MUFA) is the major compound of our research and this result can show that *C. solstitialis* ssp. *solstitialis* can be beneficial for decreasing LDL [7, 9].

According to the literature an essential oil research about *C. solstitialis* from Iran showed different profile than our research about *C. solstitialis* ssp. *solstitialis.* In that research hexadecanoic acid was also found to be a major (30.8 %) compound with a different amount and caryophyllene oxide (25.2 %) was also a major compound [10]. This difference may be because of the region or the variation of subspecies. Additionally other researches of essential

oil composition of *Centaurea* species showed; caryophyllene oxide, spathulenol, tetracosane, arachidic acid, hexadecanoic acid, isononane and tetradecanoic acid were mostly found major compounds. Consequently our results are compatible with other *Centaurea* species [11, 12].

Conflict of Interests

Authors declare that there is no conflict of interests.

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Study of Orchids Growing in The Niğde City and Their Habitat Qualities and Threat Factors

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Abstract: Niğde city is located at the intersection of Iranian-Turanian and Mediterranean phytogeographical areas and is located within B5 and C5 grids according to Davis' (1965) Grid system. This study was carried out in Altınhisar (6 towns, 2 villages), Bor (5 towns, 19 villages), Çamardı (2 towns, 18 villages), Çiftlik (4 towns, 9 villages), And Ulukışla (4 towns, 34 villages) within the boundaries of Niğde which is in the specified grids. Niğde and its surroundings are composed of rural areas, having very few forest areas, and due to the destruction caused by various reasons, many plant species are destroyed and face the danger of extinction. One of these plant species is the orchid species. In the research, primarily the types of orchids that have been recorded in the research area within the literature studies have been determined. Orchid species were examined on site, during the field works conducted in March-July 2015-2016. Areas of orchid species were identified in the study and was assessed according to Braun Blanquet cover abundance scale method (1964). In the field studies, various data about the habitat of the species (height, view, sunlight condition, plant species which found together) were collected. *Epipactis helleborine, E. Persica, Orchis stevenii, Dactylorhiza saccifera* species stated as endemic in Niğde according to TÜBİVES data and *E. helleborine* have been found in the area of the study. As a result of the field studies in general, orchid species were found only in Çamardı (Pınarbaşı, Çukurbağ, Demirkazık villages) and Ulukışla (Maden Village) districts. Harvesting orchids in the villages of Pınarbaşı and Çukurbağ still continues. As a result of the research, 14 orchid species belonging to the 7 genus which are; *Anacamptis laxiflora, Cephalanthera damasonium, Dactylorhiza romana, Epipactis helleborine, E.purpurata, Ophrys İsaura, O. reinholdii, Orchis mascula, O.anatolica, O.purpurea, O. Boryi, O.palustris,* ve *Serapias vomeracea* were determined to live in the area. Morphological information about each species has been given; some suggestions have been made to provide effective protection according to the existing area usage in the habitats. It has been mentioned that harvesting of orchids around Çamardı still continues and the Directorate of Provincial Food Agriculture and Livestock's work on the orchid cultivation has been mentioned.

Keywords: Salep, Niğde, Tübives, Orkide, Toros

1. INTRODUCTION

Orchids are a plant family that spread from 0 to 5000 meters altitude in almost every part of the world, from the tropical climate to the temperate climate, which can adapt to all kinds of climate and soil conditions from bogs to rocky fields [4]. The reasons for rich plant diversity and a rich flora in Turkey are, being in an area at the three phytogeographies (Mediterranean, Iranian-Turanian and Siberian), being a bridge between Southern Europe and South-West Asia and Anatolia being the center of origination and differentiation of many species [5]. Turkey is

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one of the countries with rich floras with about 12,000 ferns and seedy plant taxon. If we consider that the continental flora of Europe has species close to 12,000 and the continent is about 15 times larger than our country, the floristic richness of our country becomes more clear. The interesting part of the Turkish flora arises from the richness of its species, as well as the abundance of endemic species. While the sum of endemic taxa in European countries is about 2750, 3925 of the taxa in our country are endemic and endemism rate is around 34% [9]. Endemic and non-endemic plants grown in Turkey are endangered due to industrialization and urbanization of agricultural areas and the collection of these plants for overgrazing, tourism, export or domestic use, rehabilitation of arid (salt water) areas, uncontrolled and excessive use of agricultural chemicals, pollution and fires. The conservation of plant species is of great importance in the protection of biodiversity, because the destruction of habitats cause the irreversible destruction of species. It is known that there are about 800 orchid genus in the world and around 20000 species under those genus [9]. According to different research results; Papua New Guinea is known to have 3,000 species of Orchidaceae [9]. 1600 in India and 369 in Bhutan which 16 are endemic. In our country there are 268 orchid taxa belonging to 26 genuses. They are named such as dilçıkık, dildamak, çam çiçeği, çobançoluk, tavşan topuğu, katırtırnağı, çayır çiçeği in different regions [4]. Tamer et all. (2006) reported that there are 90 orchid species of 24 genuses used in the production of salep in Turkey [9].

The importance of natural plant sources is increasing day by day. However, because of the overuse of plants that are traditionally used, some plant species are gradually disappearing and the sustainable use of natural resources is being discussed by biologists. For this reason, it is important to investigate many plants that potential have not yet been discovered, both traditionally used and potential, without being destroyed. One of the species that is being destroyed is the salep. The aim of the study is to determine the genus and threat factors of orchid species belonging to the Orchidaceae family present in our country flora [5]. Making a contribution to these kinds of work in our country, to shed light on the new works, and to make the plants consciously used among the people constitute other purposes in this work.

The purpose of this study is; Identifying species of orchids in Niğde and its surroundings together with their habitats, developing the characteristics of the distribution area together with the elements of repression and developing suggestions considering the protection-use balance in the light of all this information.

2. MATERIAL and METHODS

2.1. Material

The study was carried out in the boundaries of Altunhisar in the west, Çamardı in the east in Niğde region in 2015-2016 (Image 1). The elevation, view, cover status of the area has been determined. Braun Blanquet cover-abundance (1964) method has been used in order to determine the current status of the area. Woody plant taxa and orchid species naturally grown in the area has been exemplified for identifying by sampling and photographing. In plant samples; the orchid plants were taken together with the tubers during the flowering periods and the other plant species were taken to contain the parts that could help the identification of the plants.

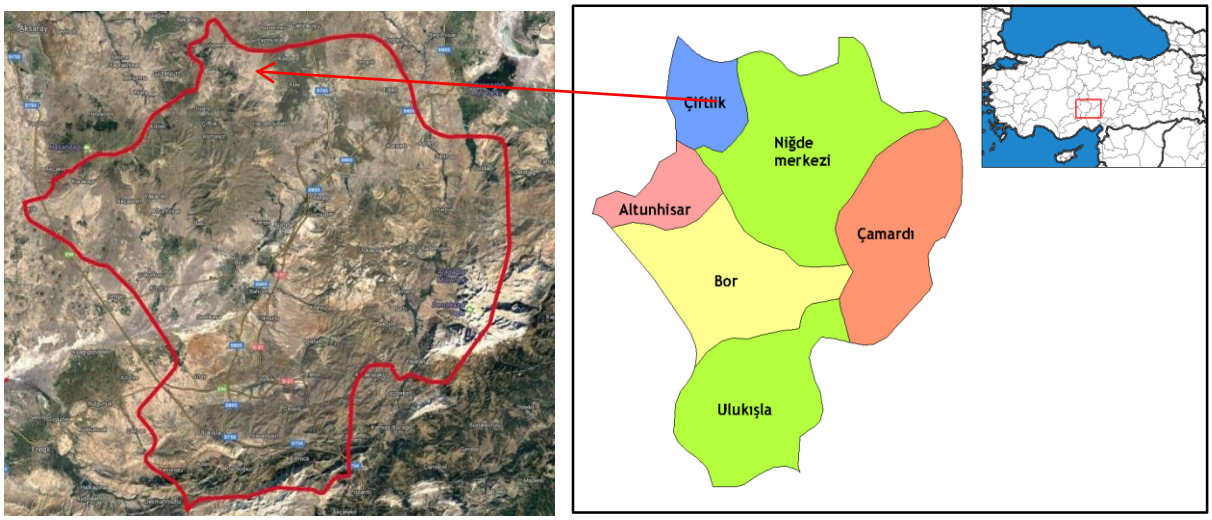

Image 1. Research Area

Butler (1986), Fanfani and Rossi (1988) and Kreutz (1998) were used to identify the orchid species found in the parcels. The distribution of orchid species in Turkey has been determined with the help of Davis' (1984) 8th volume and Ekim et all. (2000). "Türkiye Bitkileri Kırmızı Kitabı, Eğrelti ve Tohumlu Bitkiler" was used for to help with determining the conditions of the species.

Table 1. Locations of Research Parcels

City	Districts	Villages	Height from sea level
Niğde	Niğde	Merkez	1229
	Çamardı	Pınarbaşı	1513
		Çukurbağ	1550
		Demirkazık	1250
	Ulukışla	Maden	1427

In our country, plant belts that have been determined are generally designated as "Upper Mediterranean and Mediterranean Mountain vegetation layers" between 1000-2000 meters. Our research area is located in this belt. 5 areas that contain orchid species were found in the study area.

2.2. Method

In the first stage of the research, in order to reveal the characteristics of the growing environment of orchid species in the fields, properties such as altitudes and directions from the sea level were recorded. In addition, the characteristics of orchid species (group or single presence, presence of sunny or shaded area, etc.) were also determined. The Braun Blanquet (1964) method was used to determine the cover status of plant species in the parcels. Woody plant taxa and orchid species naturally grown in the area has been exemplified for identifying by sampling and photographing. In taking plant samples; the orchid plants were taken together with the tubers during the flowering periods and the other plant species were taken to contain the parts that could help the identification of the plants.

Plant samples and woody plant taxa that found in the area has been identified with the help of Altan (2000), Yılmaz (1996), Mataracı (2004) and Tekin (2007). Support from Ömer Halisdemir University, Department of Biology, Botanical Science lecturers have been taken.

The Braun Blanquet (1964) method was used to determine the cover status of the plant species in the parcels (Table 2).

Table 2. Cover-abundance scale

Scale Value	Cover (%)	Scale Value	Cover (%)
r	1 individual, %1>cover	2b	%12.5 to %25 cover
+	1-5 individual, %1>cover	3	%25 to %50 cover
1	6-50 individual, %5>cover	4	%50 to %75 cover
2m	More than 50 individual, %5>cover	5	%75 to %100 cover
2a	%5 to %12.5 cover		

3. RESULTS and DISCUSSIONS

14 orchid species belonging to 7 genuses were found between 1200-1600 m elevations around Niğde province in submediterranean belt in the research area. Findings obtained from the areas are given below, taking the city borders into consideration. Some of the qualities determined within the borders of Niğde city and the numbers of orchid individuals in the parcels are given in Table 3.

Table 3. Features of the Research Areas

Research Area		Direction	Altitude	Orchid species	Vegetation
Niğde	Merkez	North	1229	*O.palustris, Serapias vomeracea*	*Malva neglecta Linum mucronatum*
	Çamardı /Pınarbaşı	North	1513	*Anacamptis laxiflora, Orchis mascula*	*Abies cilicica Cedrus libani*
	Çamardı /Çukurbağ	South	1550	*Ophrys isaura, O. reinholdii*	*Ranunculus demissus Plantago crassifolia*
	Çamardı /Demirkazık	South	1250	*Dactylorhiza romana Orchis purpurea, O. boryi*	*Isatis glauca Convolvulus lineatus*
	Ulukışla/ Maden	North	1427	*Cephalanthera damasonium Epipactis helleborine, E.purpurata, O.anatolica*	*Alyssum linifolium Matthiola longipetala Capparis spinosa*

Following species have been determined in the area; *Anacamptis laxiflora, Cephalanthera damasonium, Dactylorhiza romana, Epipactis helleborine, E.purpurata, Ophrys isaura, O. Reinholdii, Orchis mascula, O.anatolica, O.purpurea, O. Boryi, O.palustris, ve Serapias vomeracea*.

These orchid species are not found in any category in the "Türkiye Bitkileri Kırmızı Kitabı". Following species have been found together with orchids in the area; *Malva neglecta, Linum mucronatum, Abies cilicica, Cedrus libani, Ranunculus demissus, Plantago crassifolia, Isatis glauca, Convolvulus lineatus, Alyssum linifolium, Matthiola longipetala* ve *Capparis spinosa*.

The orchid species in the center of Niğde are being destroyed because of the settlement areas, the salep collecting in Çamardı and its surroundings is still continuing and in the Maden village, the orchid species are being destroyed due to agricultural areas.

Epipactis persica and *Orchis stevenii* species were not found in the research area that have been determined in the TÜBİVES data. Orchid species found in the research areas are not included in any of the IUCN threat categories.

4. CONCLUSION

Due to the fact that Turkey is on the transition belt, it has been the settlement area of various plant species. As in other countries, various protection statutes are inadequate for conservation. Species that need to be protected can be preserved and multiplied when placed in the nature conservation status. On the other hand, species that grow and be in threat in close proximity to the areas can be protected within protected areas.

In recent years, efforts to protect natural life and biodiversity have gained considerable importance in Turkey and in the world. Protection of orchids and especially endangered species is very important. For this purpose, necessary legal arrangements have been made and exports of orchid species and salep in each form are prohibited [9]. Despite any prohibitions on the protection of species, destruction is still ongoing. The work to be done in areas exposed to unauthorized collecting, development in the natural environment, can contribute to the multiplication of orchid individuals. In 2013, similar work has started in the region. Orchid tubers obtained from Aegean Agricultural Research Institute Vegetable Production Development and Alternative Product Project was planted in Çardakık Village in Çamardı District by Niğde Provincial Directorate of Food, Agriculture and Livestock [2]. In Kırklareli forest villages (Yıldız Mountains), natural salep farming has started. Since Salep was also accepted as a product outside woody plant in the forest, the General Directorate of Forestry prepared a salep action plan for 2014 - 2018 for the protection of orchid species and for raising public awareness. Besides these, scientific studies have increased as well. Similar activities can be intensified in nature to protect our botanical riches and ecological environments.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Effect of Salt Stress on Seed Germination, Shoot and Root Length in Basil (*Ocimum basilicum*)

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Abstract: Salinity is one of the most environmental problems in arid and semi-arid region. It is an abiotic stress factor which restricts crop production and affects development of plants. One of the these plants is basil (*Ocimum basilicum*). It is an annual medicinal and aromatic plant from Lamiaceae family. The present study was carried out to determine the response of different salt concentrations from 0 to 240 mM doses which increasing 20 mM. The experiment was conducted with randomized complete block design with 3 replications and placed 20 number from seeds of each plant in petri dishes. 39 petris were used consisting from 1 plant x 13 salt levels x 3 replicats. Germination tests were made at constant temperature (29±1 °C), dark field and drying oven in laboratory conditions. Appropriate test solution was placed at each petri dish being 5 ml and was changed with an interval of two days. According to the study results, germination speed and power of basil seeds completed within 3-15 days. The results noted that root lenght changed between 0.08-5.07 cm, shoot lenght changed between 0.1-5.82 cm in the basil and they changed between 10-100% germination rate between the 0-240 mM salt concentrations. The lowest germination speed and power were seen in 240 mM and the highest germination speed and power were seen in 20 mM except control condition. Germination percentage of basil seeds were decreased from 0 (control) to 240 mM. In addition to this, the highest shoot and root lenght were obtained from 20 mM and the lowest shoot and root lenght were obtained from 240 mM except control condition. Root lenght/shoot lenght was also determined changing between 0.43-1.27. According to results, it was determined that germination number and rate, shoot and root lenght were statistically affected by different salt doses. Considering different salt doses, the highest number of germination was obtained from 20 mM salt dose, the lowest value was determined in 240 mM salt application compared to control application. It is suggested that salt tolerence studies should be preferred under 200 mM salt dose to obtain the germination number and rate over 80% in different salt doses and in order to grow the basil under salted areas.

Keywords: Basil, Salt, Germination, Root and shoot lenght

1. INTRODUCTION

Medicinal and aromatic plants are used for different purposes because of their active components. So, they are cultivated [1]. However, the quality and the quantity of secondary metabolites of medicinal plants strongly depend on environmental conditions. To obtained high yielding genotypes of these plants under different environmental conditions, in many of

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literatures has been given about response of medicinal and aromatic plants againts the salinity stress [2].

Salt stress effects different physiological life of medicinal plants on different stages. One of the most important part of plant life is germination period on salt conditions [3]. For the survival and perpetuation of seeds, they need to germination of seeds under salt concentration in the soil. Seed germination takes place after high downfall in saline habitats as decreased soil saline [4].

Salt stress is becoming a significant global factor as affects nearly 20% of global irrigated land because it limits production in terms of agricultural in the worldwide [5]. Besides many substantial crops are salt-sensitive such as pepper, eggplant, potato, lettuce, and cabbage [6]. Therefore, depending on growing the world population and increasing of soil salinization, crops which are adapt to salt stress, need to develop. Salt stress is also affected essential oil and its component of medicinal and aromatic plants. For this reason, determination of salt tolerance has a great importance in medicinal plants.

It is reported that some plant seeds showed major reduce under salt stress i.e. *Ocimum basilicum* [7], *Petroselinum hortense* [8], sweet marjoram [9] and *Thymus maroccanus* [10]. The other stage is seedling growth which influenced by salinity negatively. It has been reported that, seedling growth of *Thymus maroccanus* [10], basil, chamomile and marjoram [9] were severely decreased depend on salt stress. Some researchers said that morphological characteristics of number of medicinal plants were effected under salt stress conditions such as number of leaves, leaf area and leaf biomas in reduced form as *Majorana hortensis* [11], peppermint [12] , geranium [13], *Thymus vulgaris* [14], sage [15] and *Mentha pulegium* [16].

In this study, sweet basil was used as an experimental material. This plant is commonly used by local people in treatments of various diseases. For example, it is used for treatment of dry mouth and dental complaints, diarrhea and chronic dysentery, respiratory disorders, and effective in the treatment of fungal diseases and stomach discomfort in addition, the influential antitussive, diuretic, anthelminthic, tranquilizer and expectorant roles in medicinal approach [17, 18].

The aim of this study was to investigate the effects of thirteen salt doses (0-240 mM, increasing 20 mM) on basil seed germination, shoot and root length.

2. MATERIAL and METHODS

Seed of *Ocimum basilicum* was obtained from field experiment in 2016. The present study was carried out to determine the response of different salt concentrations from 0 to 240 mM which increasing 20 mM. The experiment was conducted with randomized complete block design with 3 replications and placed 20 number from seeds of each plant in petri dishes. 39 petris were used consisting from 1 plant x 13 salt levels x 3 replicats. Germination tests were made at constant temperature (29±1 °C), dark field and drying oven in laboratory conditions. Appropriate test solution was placed at each petri dish being 5 ml and was changed with an interval of two days. Germination trials were conducted in 9 cm sterile petri dishes lined with Whatman No.1 filter papers and moistened with distilled water to ensure adequate moisture for the seeds. The seed was examined daily and considered germinated when the radicle was visible. The germination percentage, like shoot length and root length were measured at the end of the germination periods.

Statistical analysis of the experimental data was conducted with the SPSS statistical program. Differences between the average values were compared by Duncan at a 5% probability level (Table 1).

Table 1. Analysis of variance table

Treatment		SS	df	MS	F	Sig.
Number of germinating seeds	Between groups	878.308	12	5.524	22.657	0.000
	Within Groups	40.667	26	0.224		
	Total	918.974	38			
Germination ratio	Between Groups	21957.7	12	73.192	46.795	0.000
	Within Groups	1016.67	26	1.564		
	Total	22974.4	38			
Shoot lenght	Between Groups	103.513	12	1829.808	46.795	0.000
	Within Groups	7.922	26	39.103		
	Total	111.435	38			
Root Lenght	Between Groups	66.288	12	8.626	28.312	0.000
	Within Groups	6,339	26	0.305		
	Total	72.627	38			
Root/Shoot Lenght	Between Groups	0,859	12	0.072	1.948	0.075
	Within Groups	0.955	26	0.037		
	Total	1.813	38			

SS: sums of squares; df: degrees of freedom, MS: mean squares, F: test statistic, Sig.: significance

3. RESULTS and DISCUSSIONS

3.1. Germination Dates and Number of Germinating Seeds

The results indicated that salinity doses had a significant effect on the measured traits (Table 1). According to the data obtained from the petri trials, generally, salt stress caused significant decrease in shoot lenght, root length, germination of basil. Increasing salinity doses led to decrease in germination of this plant. This decrease started after 40 mM salinity dose. The germination stopped at 240 mM salinity dose. The germination varied from 3 to 14 days and the highest germination obtained from the control applications followed by the others. In sweet basil, average 19.66 seeds germinated in control application and 3.33 seed germinated in 240 mM. The highest germination was seen in 20-120 mM salt doses.

Table 2. Number of germinating seeds in basil

Doses (mM)	Number of Germinating seed	Doses (mM)	Number of Germinating seed
0	19.67^{a}	140	17.00^{bc}
20	19.33^{ab}	160	16.67^{c}
40	19.00^{abc}	180	16.67^{c}
60	18.33^{abc}	200	13.00^{d}
80	18.33^{abc}	220	7.33^{e}
100	17.33^{abc}	240	3.33^{f}
120	17.33^{abc}		

Different letters at each column indicate significant differences at 5% probability level.

3.2. Germination Percentage

According to results of variance analysis, effect of salinity stress dose on germination percentage was statistically significant (Table 3). The results showed that the germinations decreased sharply with increasing salinity doses. While the lowest germination was obtained from 240 mM NaCl dose application in basil, the highest germination was obtained from control

concentration application. Comparing control, germination reductions were 1.68, 6.77, 15.25 and 83.05% in 20, 80,160 and 240 NaCl doses, respectively.

Table 3. Effect of different doses (mM) of NaCl on the germination percentage for evaluated plant (%)

Doses (mM)	Germination Percentage (%)	Doses (mM)	Germination Percentage (%)
0	98.33^{a}	140	85.00^{bc}
20	96.68^{ab}	160	83.33^{c}
40	95.00^{abc}	180	83.33^{c}
60	91.67^{abc}	200	65.00^{d}
80	91.67^{abc}	220	36.67^{e}
100	86.67^{abc}	240	16.67^{f}
120	86.67^{abc}		

Different letters at each column indicate significant differences at 5% probability level.

3.3. Shoot Lenght

The effect of salinity stress on shoot lenght has been showed in Table 4. Comparison of shoot length mean in salinity different dose showed that when salinity dose increase, shoot length decrease. The most reduction in shoot length obtained from 240 mM salt dose with 0.12 cm and the highest rate was seen 20 mM with 4.72 cm. On control conditions, shoot lenght found 4.67 cm after 20 mM salt application. Comparing control, while shoot lenght increased with 1.08% in 20 mM salt dose, reductions were found 25.27, 79.01 and 97.43% in 80, 160 and 240 NaCl doses, respectively.

Table 4. Effect of different doses (mM) of NaCl on shoot lenght for evaluated plant (cm)

Doses (mM)	Shoot Lenght (cm)	Doses (mM)	Shoot Lenght (cm)
0	4.67^{a}	140	1.30^{de}
20	4.72^{a}	160	0.98^{efg}
40	3.62^{b}	180	0.44^{fg}
60	3.10^{bc}	200	0.31^{fg}
80	3.49^{b}	220	0.17^{g}
100	1.67^{de}	240	0.12^{g}
120	2.33^{cd}		

Different letters at each column indicate significant differences at 5% probability level.

3.4. Root Lenght

Root lenght is one the most important features for salt stress because of contacting with soil directly. Impact of salinity on root lenght has been showed Table 5. As you see by increasing salinity doses, rate of root lenght decreased extremely. In this case, on control conditions we had the tallest root lenght with 3.82 cm and the smallest shoot length was seen 0.10 cm in 240 mM. Comparing control, root lenght reductions were determined as 12.40, 22.33, 69.50 and 96.86% in 20, 80, 160 and 240 NaCl doses, respectively.

Table 5. Effect of different doses (mM) of NaCl on the root lenght for basil (%)

Doses (mM)	Root Lenght (cm)	Doses (mM)	Root Lenght (cm)
0	3.18[a]	140	0.89[d]
20	3.63[ab]	160	0.97[d]
40	2.93[bc]	180	0.35[d]
60	2.58[c]	200	0.19[d]
80	2.47[c]	220	0.13[d]
100	0.91[d]	240	0.10[d]
120	1.04[d]		

Different letters at each column indicate significant differences at 5% probability level.

3.5. Root/Shoot Lenght

Basil showed different responses to the salinity doses applied in terms of root/shoot lenght ratio in Table 6. Generally, the root/shoot lenght ratio decreased with increasing salinity doses, which showed that a more reduction in root lenght than shoot lenght. In other words, the root lenght was more negatively affected than shoot lenght by increasing salinity doses. Among the salinity doses, 160 mM salt dose gave a higher root/shoot lenght ratio at all salinity doses with 1.069 cm. It was also observed that 160 mM had higher values than control condition. The lowest root/shoot lenght was seen in 120 mM salt concentration with 0.445 cm. Comparing control, root lenght reductions were determined as 2.57, 12.24 in 20 and 80 mM NaCI concentrations and they were found increasing 160 and 240 mM NaCl concentrations with 30.84 and 5.26%, respectively.

Table 6. Effect of different doses (mM) of NaCl on the root/shoot lenght for basil (%)

Doses (mM)	Root/Shoot Lenght (cm)	Doses (mM)	Root/Shoot Lenght (cm)
0	0.817[abc]	140	0.687[bc]
20	0.796[abc]	160	1.069[a]
40	0.798[abc]	180	0.785[abc]
60	0.824[ab]	200	0.672[bc]
80	0.717[abc]	220	0.889[ab]
100	0.561[bc]	240	0.860[ab]
120	0.445[c]		

Different letters at each column indicate significant differences at 5% probability level.

In the earlier studies in the literature, harmful effects of high salinity effects on crops are multidirectional and effect plants in several ways as drought stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization and reduction of cell division and expansion [19-24]. Therefore, it is reported that plant growth, development and survival are reduced [25, 26]. Salinity effects seedling growth slowly or less mobilization of food which is reserved, deferring the cell division, growing and injuring hypocotyls [27]. Reduced seedling growth has been reported on basil, chamomile and marjoram by increasing salt doses [8, 9, 28]. Salinity stress is an important distinctive when a variety is selected for tolerance of salinity [29]. It is reported that number of germination, germination percentage, shoot and root lenght of sage (*Salvia officinalis* L.), black cumin (*Nigella sativa* L.) and flaxseed (*Linum usitatissimum* L.) were effected by different salt concentrations [30]. The results of present investigation are in agreement with the findings of many workers in different plant species [8, 9, 28,30].

4. CONCLUSION

The results in this study showed that the application of NaCl from 0-240 mM had significantly effect on basil seed germination, shoot and root length. Highest germination percentage was seen under control condition, and followed by 20 mM salt dose. While the highest root lenghts were found under control condition and followed by 20-80 mM salt doses. The highest shoot lenght were seen under 20 mM salinity level and it was higher than control condition. Depending on the increased salt levels, germination, shoot and root lenght were decreased. In conclusion, 200 mM salt dose should be preferred to grow basil in salt area.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Biosynthesis and Characterization of Copper Oxide Nanoparticles using Cimin Grape (*Vitis vinifera cv.*) Extract

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Abstract: Nowadays, nanoparticle synthesis has been a very important research area because of the wide use of nanoparticles in many fields. Green synthesis is one step ahead of other synthesis methods due to both cost reduction in production and environmentally friendly approach. For these reasons, we chose green synthesis method which is nature friendly in our research. In this study, it was aimed synthesis of copper nanoparticles by the green synthesis method using the water extract of Erzincan Cimin grape (*Vitis vinifera cv.* Black plum) from 0.1 M $CuCl_2.2H_2O$ in the reaction medium. Then, optimum conditions for the green synthesis reaction had been determined and product optimization of copper nanoparticles was made. Optimum conditions for synthesis were measured at a wavelength of 355 nm (for CuO nanoparticles). It was observed that the best synthesis reaction was occurred at pH 5, temperature 60 °C, 0.05 M metal ion concentration and within the 60 minutes.The characterization of the CuO nanoparticles was obtained then was characterized using spectroscopic techniques such as UV-VIS, FTIR, XRD and SEM. When the chromatograms obtained using these techniques were examined, it was understood that 25-50 nm morphologically homogeneous nanoparticles were synthesized. Due to the new physical and chemical properties of obtained copper nanoparticles using a cost-effective and environmentally friendly green synthesis method using water extract of Erzincan Cimin Grape; it was thought that in many areas (optical, biosensors, etc.) could be used.

Keywords: Copper(II)oxide NPs, Green synthesis, Nanotechnology

1. INTRODUCTION

Green synthesis is an important application that it is preferred by many environmentally friendly approaches like chemistry. Nowadays, the synthesis of nanoparticles with green synthesis has resulted in green nanosynthesis. Nature and human-friendly nanomaterial synthesis is performed by this method. The most important advantage of this method is that the harmful toxic chemicals are either not used at all or the less harmful ones are used. The reaction

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media in green nanosynthesis can be live in tissue, cells, organisms or extracts from them like enzymes, carbohydrates, lipids and proteins. There are three types commonly used [1-3].

First, enzymes are used in green nanosynthesis since they now have a wide range of chemical reaction forces. Secondly, microorganisms (bacteria, yeast or mold) are also important reaction factors for green synthesis reactions. Finally, other used components are plant or animal extracts. Plants and extracts have been preferred due to their advantages of growing in short time, abundant quantity and storage. [4-6]. By some research groups, the synthesis of silver nanoparticles and gold nanoparticles have been achieved using plant extracts *Nephelium lappaceum L.* and *Plectranthus amboinicus* [7-8].

It comes from the historical sources that grape fruit was known and consumed 6.000 years ago by the Egyptians. In the whole grape and seeds, there are commonly phenolic compounds, antioxidants, organic acids, fatty acids, carbohydrates and water. Nowadays grape plant; proteins and biomolecules have begun to be used in metal nanoparticle synthesis due to their reducing properties [1-3].

Figure 1. Cimin grape (*Vitis vinifera*, variety Cimin)

Nanoparticles have entered into many fields, mainly energy storage, textile, paint industry and health field. Moreover, it is noteworthy that the share of nanotechnology in these areas has increased day by day. Nano materials show different properties according to the non-nano size. This situation varies depending on the particle size. All metal nanoparticles in which copper oxide are synthesized have different optical, conductivity, antimicrobial and thermal properties than non-nanoscale metal oxides. For this reason, metal oxide nanoparticles are found in different application areas. They have extensive range of uses, such as fuel cells, catalysts, magnetic recording, optoelectronic, magnetic fluid, materials, photovoltaic supplies, pigments, and sensors [7-9].

Because of the unique properties (physicochemical, optical, catalysis, magnetic, electronic and antimicrobial) of metal oxide nanoparticles in their dimensions below 100 nm, the number of studies directed to them has also increased significantly. There are now numerous methods for synthesizing nanoparticles with the desired properties. However, most of these methods are expensive and the synthesis conditions are heavy. It also contains many elements that can create a biological threat to the environment. Copper oxide nanoparticles, which have become of great interest since the late 20th century due to their catalytic, optical and electrical properties, in terms of their lubricants and catalysts in the chemical industry, and conductivity properties, they have a very wide application network in the electricity and the chemical sector [10-11]. Because copper oxide nanoparticles are semiconductor, they are used in catalysts, gas sensors and photovoltaic cells. Because of its electronic and magnetic properties, it is one of the basic components of superconducting materials [12].

In this study, synthesis of copper oxide nanoparticles was aimed. An easy, non-toxic and rapid method for green synthesis has been investigated. For this reason, easily obtained, non-toxic, renewable grape fruit can be chosen to be synthesis material.

2. EXPERIMENTAL

2.1. Chemical and reagents

Copper(II)chloride ($CuCl_2.2H_2O$), Sodium acetate ($NaCH_3COO$), Tris ($(HOCH_2)_3CNH_2$), Sodium carbonate (Na_2CO_3), Sodium hydroxide (NaOH), Hydrochloric acid (HCl) were purchased from Sigma-Aldrich GmbH, (Sternhe I Germany). The other chemicals were obtained from Merck. Deionized water was used to prepare all solutions.

2.2. Preparation of Cimin Grapes Extract

Grape extracts containing a big amount of polyphenols. The grape extracts may a significant source of natural and economical antioxidants and it can be used as a reducing agent during metal nanoparticle preparation (13). For this reasons, The Cimin grape (*Vitis vinifera cv.*), used in this study was collected from Üzümlü providence in Erzincan City, Turkey at the season August-September, was identified with the help of taxonomists. They were stored in a cold air store until working. The Cimin grape was separated from branches and washed with deionized water three times for removing their dust. The grape (250 g) was thoroughly shattered to form a homogeneous mixture in a blender, along with a 250 mL, deionized water. The mixture was centrifuged at 5.000 *xg* for 10 min and the supernatant was used for in the green synthesis reaction.

2.3. Green Synthesis Reaction Medium

It was prepared 0.1 M $CuCl_2.2H_2O$ with deionized water for nanoparticle synthesis. 10 mg/mL grape extract was added reaction mixture. Green synthesis medium was mixed with a magnetic stirrer at 200 *xg* for 3 h.

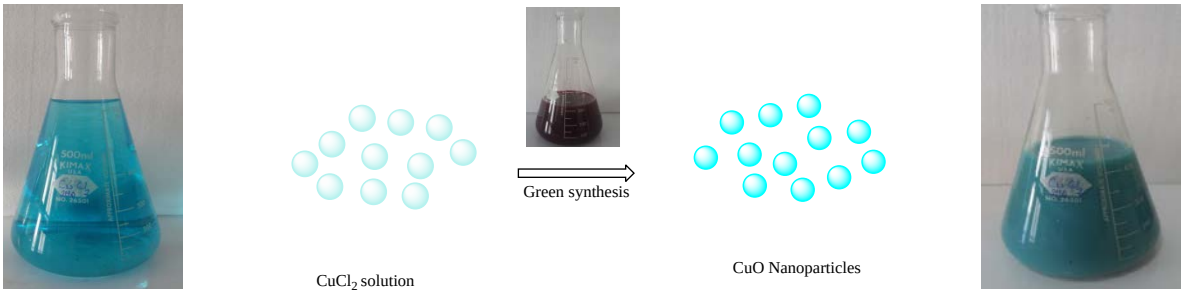

Figure 2. Grape extract and 0.1 M $CuCl_2.2H_2O$ was used for nanoparticle synthesis

Cu^{2+} with reacted with the grape extract was transformed into Cu^{+} biochemical reduction and it became nanoparticle as copper(I)oxide together with the oxygen molecules in the environment. Cu^{2+} which reacts with the resulting grape extract were converted to Cu^{+} by a biochemical reduction. When the reaction started the entire medium was clear blue. The formation of red–brown precipitate of the obtained Cu_2O nanoparticles was observed on the lower surface of the reaction chamber. Cu^{+} together the oxygen molecules in the air have become copper(II)oxide nanoparticles. Blurred blue Copper(II)oxide nanoparticles were synthesized by a bio oxide-reduction method [14].

For wavelength scanning, the maximum wavelength was determined by scanning the wavelength between 190-800 nm after treatment with 1 M $CuCl_2.2\ H_2O$ solution with Cimin grape extract for 3 hours. Nano copper(II)oxide formation was observed during this time. UV-Vis spectrometer was used for this purpose [15-16].

2.4. Optimization of Green Synthesis Reaction

2.4.1. Optimization of Green Synthesis Reaction

For determination of the optimum contact time, samples were spectrophotometrically measured between 0 and 180 min with 10-minutes intervals.

2.4.2. Determination of optimum pH

Synthesis of the CuO nanoparticles was performed at pH 3-5, an acetate buffer at pH 5-8, a phosphate buffer at pH 8-9, Tris buffer and at pH 10, a carbonate buffer. The pH was adjusted using 0,1 N HCI and 0,1 N NaOH. The values of absorbance were determined with an UV–Vis spectrophotometer.

2.4.3. Determination of optimum temperature

Green synthesis of the CuO nanoparticles was carried out separately from 5 °C to 80 °C respectively, and the changes in absorbance in the samples were measured by a UV–Vis spectrophotometer (Epoch Biotech nanoDrop Spectrophotometer).

2.4.4. Determination of optimum concentration of metal ion

Green synthesis of the CuO nanoparticles was performed using a copper (II) chloride solution between 4.10^{-1} to $1.25.10^{-2}$ M and the absorbance changing of the examples was measured by a UV–Vis spectrophotometer at 355 nm.

2.5. Characterization of CuO Nanoparticles

The synthesized CuO nanoparticles were characterized by scanning at a range of 190–800 nm by using an UV-VIS spectrophotometer (Epoch Biotech nanoDrop Spectrophotometer). For investigating features parameters of examples, XRD (X-Ray Diffractometer), SEM (Scanning Electron Microscope) (Sigma 300, SEM Zeiss, Germany) and EDX (energy dispersive X-ray) were used as exploration tools. Structural and morphological studies were carried out by XRD (PANalytical, Empyrean, Netherlands) using Cu Kα (λ=1.5405Å) radiation. And they were performed at DAYTAM (Eastern Anatolian High Technology Applications and Research Center).

3. RESULTS and DISCUSSIONS

Cimin grape (10 mg/mL) was added in sample of copper (II) chloride solution $CuCl_2.2H_2O$, (0.1 M) and incubated and mixed in a closed space for 1 hours. The reaction mixture was changed from blue to intense blue and at the bottom red-brown, which indicates the presence of copper(II)oxide nanoparticles. The maximum absorbance of synthesized copper(II)oxide nanoparticles was found to be 355 nm in fig. 3.

Then, water was removed with the help of an evaporator and the copper(II)oxide nanoparticles were examined after they were dried at 70 °C for 72 hours. The changes in absorbance were measured to determine the optimum time in the sample taken with 10 minutes intervals from the reaction mixture prepared. The optimum time required to complete the reaction was determined to be 1 hour. The result of the analysis was given in figure 4.

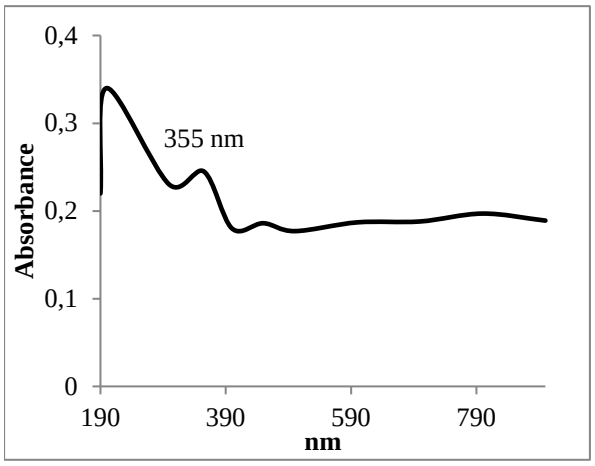

Figure 3. Wavelength-absorbance graph for copper(II)oxide nanoparticles

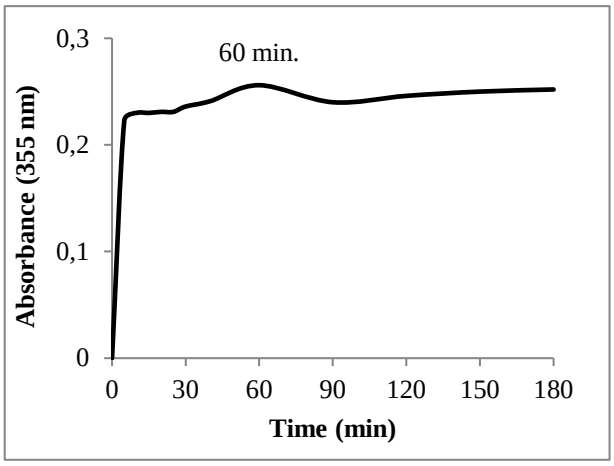

Figure 4. Absorbance diagram of copper(II)oxide nanoparticles versus time. (pH: 5. temperature: 60 ^{o}C and concentration: 0.05 M)

In order to determine the optimum pH, the pH was varied from 3 to 10 at 8 different pH conditions. The obtained results were shown in figure 5. As a result of experiments, we had determined that the optimum pH was 5.

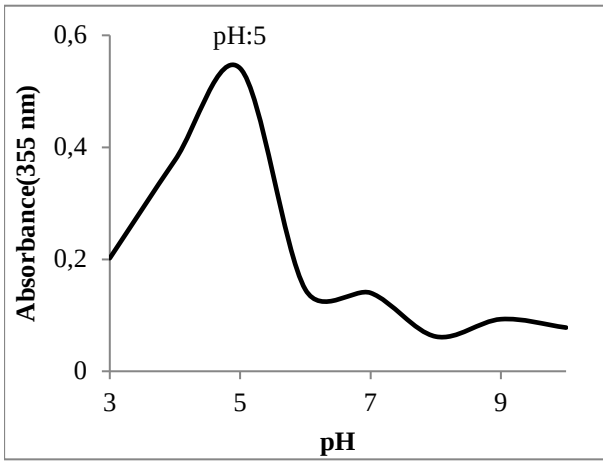

Figure 5. Absorbance graph of copper(II)oxide nanoparticles versus pH (time:60 min. temperature: 60 ^{o}C and concentration: 0.05 M).

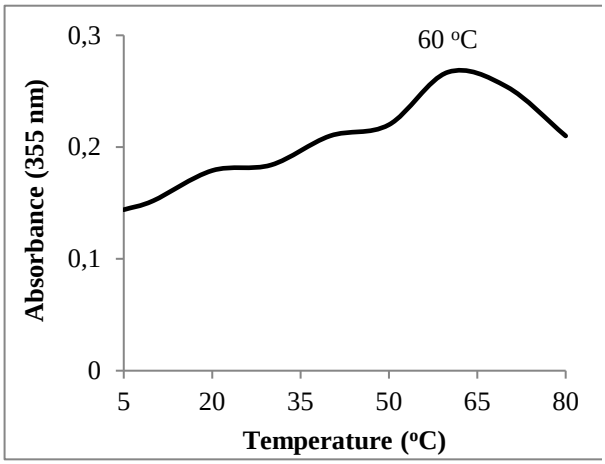

Figure 6. Absorbance graph of copper(II)oxide nanoparticles versus temperature pH (time:60 min. pH:5 and concentration: 0.05 M).

The absorbance-temperature graph was drawn with the results obtained from the experiments. The results of the processes were given in Fig 6. The graph showed that the optimal temperature for green synthesis was 60 ^{o}C. The optimum metal ion concentration of the reaction was measured with a solution prepared at a concentration range of 4.10^{-1}-$1.25.10^{-2}$ M of $CuCl_2.2H_2O$. As a result of absorbance measurements at 355 nm, the optimum concentration was found to be 0.05 M in Figure 7.

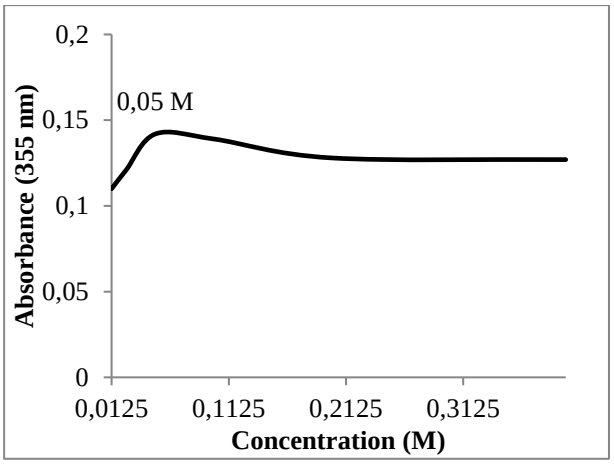

Figure7. Absorbance graph of copper(II)oxide nanoparticles versus concentration pH (time:60 min. temperature: 60 ºC and pH: 5).

Characterization of CuO nanoparticles synthesized by the green synthesis method was carried out the SEM chromatography (Figure 8). It was observed that the synthesized CuO nanoparticles according to SEM results are uniform spherical and are between 25-50 nm in size. This result showed that Cu_2O nanoparticles synthesized from Erzincan Cimin grape extract are compatible with the values in the literature and can be classified as nanoparticles. EDX (energy dispersive X-ray) chromatogram was shown the nanoparticles occurred from Cu and O molecules in Figure 9.

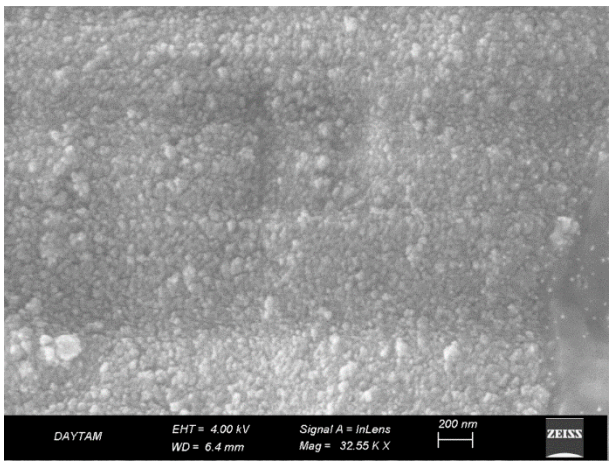

Figure 8. SEM chromatogram of copper (II) oxide nanoparticles

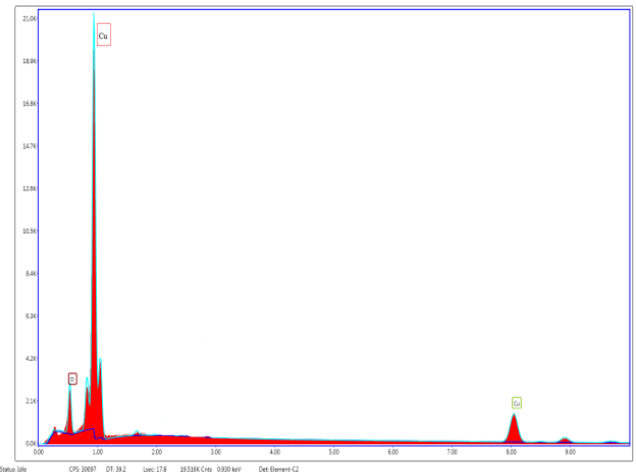

Figure 9. EDX chromatogram of copper (II) oxide nanoparticles.

In the work of Azam and his group, the characteristic peaks were found to be (110), (111), (220) and (211) taken in the XRD chromatogram for CuO [17]. These results supported our experiments.

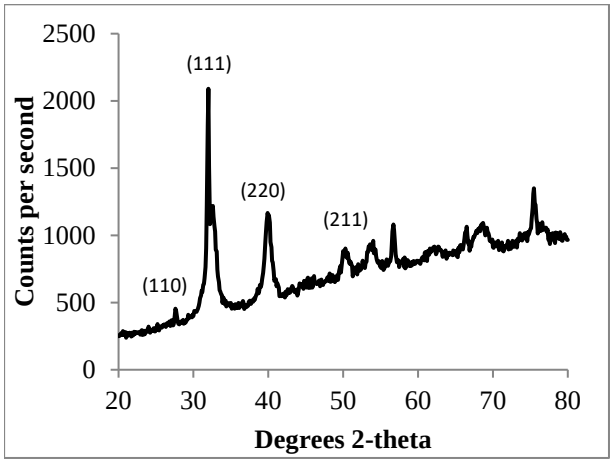

Figure 10. XRD chromatogram of copper(II)oxide nanoparticles

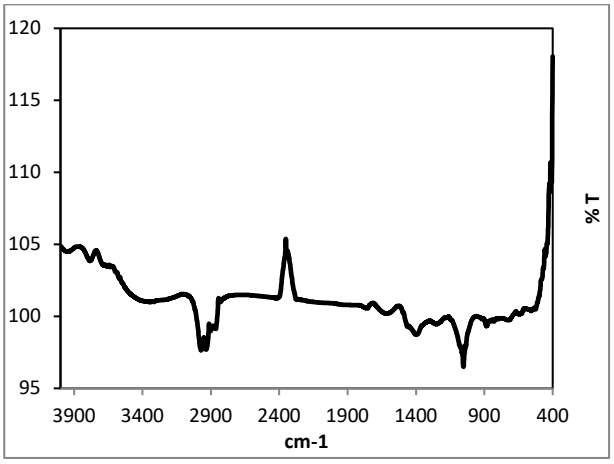

Figure 11. FTIR chromatogram of copper (II) oxide nanoparticles

FTIR spectrum was containing similar results before examination (18). The FTIR spectrum of grape extract mediated CuO nanoparticle shows that the broad absorption band at around 1650 cm^{-1} can be assigned to aromatic bending of alkene group (C=C). Absorption peak at 1060.0 cm^{-1} is stretching vibration of C–O group of primary and secondary alcohols (C–O).

4. CONCLUSIONS

As a result of all these studies, copper(II)oxide nanoparticles have been synthesized with a new and unique method using plant extracts. For this purpose, Cimin grape extract which was an endemic species and grown in Erzincan region was used. As a result of the work, the nanoparticle synthesis has been proven by chromatographic measurements. The reaction conditions were optimized by the performed experiments. Since the CuO nanoparticles were obtained in a cheap, environmentally friendly and mild condition using the green synthesis method, the developed method could be easily applied. It was also thought that CuO nanoparticles might find widespread use in industry for dye removal, catalyst, biosensor development and antimicrobial agent synthesis.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Neuroprotective Effects of Rutin and Quercetin Flavonoids in *Glaucium corniculatum* Methanol and Water Extracts

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Abstract: Neurodegenerative diseases (NDs) are characterized by loss of neurons. NDs are associated with development of inflammation. Existing drugs against NDs only delay the progression; however, they do not provide a cure. The studies for the treatment of NDs focused on to discover natural products that have the potential of anti-inhibition and anti-radical properties. The *Papaveraceae* family members are important for the synthesis of pharmaceutically compounds such as flavonoids which act like anti-inflammatory drugs. In this study, methanol and water extracts of *Glaucium corniculatum*, a member of the *Papaveraceae* family, were analysed for flavonoid compounds. The effects of extracts on neuronal PC12 cells viability was determined. The anti-inflammatory effects of extracts were assessed by measuring the levels of IL-6 and IL-10 cytokines on hydrogen peroxide (H_2O_2)-stimulated PC12 cells. As a result of our studies, Rutin and Quercetin flavonoids have been found to be as major. The amount of Rutin was higher in methanol (45 µg/ml) than water (41 µg/ml). Quercetin was better extracted with methanol (12 µg/ml) than water (10 µg/ml). None of the tested extracts were cytotoxic even to PC12 cells. Both extracts showed an anti-inflammatory effect in a dose dependent manner. The water extract showed the maximum anti-inflammatory effect, with IL-6 secretion decreased 79 fold according to the H_2O_2 treated group and IL-10 secretion increased to 87 fold according to the control group. This study is an evidence that the Rutin and Quercetin flavonoids detected in *G. corniculatum* methanol and water extracts have a neuroprotective effect through anti-inflammation.

Keywords: *Glaucium corniculatım,* flavonoid, neurodegenerative diseases, anti-inflammation

1. INTRODUCTION

Neurodegenerative diseases (NDs) such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) are defined as disorders with loss and damages of neurons. Approximately 30 million people worldwide are affected by NDs and it is estimated that until 2040, casualties caused by NDs will be the second after cancer. A common link between these diseases is chronic activation of innate immune responses, also known as inflammation [2]. Inflammation is a response that operates against the endogenous or exogenous agents such as infection, injury, and exposure to contaminants to continue the organism's life. The inflammation response that occurs in chronic conditions is caused the extracellular matrix damages and organ dysfunctions [3]. It has been proven that chronic inflammation is a part of the cause of NDs [4, 5]. Mechanisms responsible for the perception,

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conduction and replication of inflammatory processes that cause the production of neurotoxic mediators, such as cytokines and interleukins, are associated with NDs [6, 7]. The evidences of chronic inflammation in NDs are based on the findings on increased levels of proinflammatory cytokines such as interleukin 6 (IL-6) [8] and deceased levels of anti-inflammatory cytokines such as interleukin 10 (IL-10). Understanding the interaction between the inflammation and nervous systems is crucial as a strategy to intervene with chronic NDs.

There are some drugs approved by U.S. Food and Drug Administration (FDA) such as dopamine agonists, cholinesterase inhibitors, anti-inflammatory medications and calcium channel blockers that are used for the treatment and prevention of NDs [9]. The existing drugs against NDs, only delay the progression of the disease by alleviating the symptoms; however, they do not provide a complete cure.

In recent years, the studies for the discovery of novel drugs and biomarkers for the treatment of NDs focused on strategies to discover natural products that have the potential of anti-inflammatory properties. These studies usually focus on drugs used in traditional medicine, most of which are plant extracts. Plant derived bioactive compounds such as steroidal-piperidine-alkaloids, fatty acids, phenols, alkaloids, saponins, terpenes and flavonoids play a major role in the slowing of many NDs. Some plants have traditionally been used for centuries to treat memory impairment. One of these species is *Glaucium corniculatum*, belonging to the family of *Papaveraceae* [10]. The species of the *Papaveraceae* biosynthesize pharmacologically active flavonoids, known to exhibit different pharmacological effects such as anti-inflammatory and neuroprotective [11]. Rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside) and Quercetin **(**3, 30, 40, 5, 7-pentahydroxyflavone) are bioflavonoids of the flavonol type abundantly present in *Papaveraceae.* Many studies have been carried out to understand the medicinal importance of Rutin and Quercetin. They have been reported to exert numerous biochemical and pharmacological activities, such as the suppression of cellular immune and inflammatory [12] and neuroprotective [13] responses.

The goal of this study is to thoroughly examine the flavonoid content of methanol and water extracts of *Glaucium corniculatum* by high-performance liquid chromatography (HPLC) method. However, this study involved testing the ability of these extracts to protect differentiated PC12 (dPC12) cells by nerve growth factor (NGF) from neuroinflammation and neurodegeneration when exposed to neurotoxic insult induced by H_2O_2 as an *in vitro* model, by analyzing the effect of on IL-6 and IL-10 production.

2. MATERIAL and METHODS

2.1. Plant Material

Crube plants were collected and verified by Prof. Dr. Zeki Aytaç (Gazi University, Faculty of Science, Department of Biology). The voucher specimen was kept in the Herbarium of Gazi University, Faculty of Science, Department of Biology. *G. corniculatum (L.) RUD.* subsp. *refractum* (NAB.) CULLEN was collected from Beypazari district in the northwest of Ankara on 9.07.2012.

2.2. Preparation of Plant Extracts

Above-ground tissues of the plant samples were dried, powdered with an electric grinder and stored in laboratories of the Faculty of Science Department of Biology, Gazi University, Turkey. The plant powder (30 gr) were macerated with 300 mL of methanol and water, respectively at the room temperature for 6 hours using soxhlet device (LabHeat). The extracts were filtered by whatman filter paper and evaporated to dryness (45°C) under reduced pressure by rotary evaporator (Heidolph Laborota 4000) and stored in a refrigerator at 4°C until the time of use [14].

2.3. Quantitative HPLC Analysis of Flavonoids in Plant Extracts

Flavonoids in the samples were identified on an Agilent Technologies HPLC 1200 series (Santa Clara, CA, USA) equipped with a quaternary pump, a manual sampler and an ultraviolet/ visible (UV-Vis) detector with a loop size of 20 µl. The reversephase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (150 mm × 4.6 mm i.d., particle size 5 µm, Agilent Zorbax Eclipse XDB-C18) at 25 °C. Simultaneous monitoring was performed at 254 nm and 330 nm and a flow rate of 1 mL/min. The amount of each phenolic compound was expressed as µg per mg of the extracts [15].

2.4. PC12 Cell Culture

Pheochromocytoma PC12 cells were obtained from Gazi University, Biotechnology Laboratory Collection and they were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), 1% antibiotic mixture comprising penicilin streptomycin (100 units/mL penicilin-100 µg/mL streptomycin) and 1% (2 mM) L-glutamine in a humidified CO_2 (5%) incubator at 37°C. The medium was changed every other day. The cells were differentiated for 4 days using 100 ng/mL NGF [16]. The differentiation medium was refreshed every two days.

2.5. Cell Viability Assay

In order to determine the toxicity of H_2O_2, the cells were plated at an appropriate density ($1x10^4$ cells/200 µL) in a collagen-coated 96-well plate. After that, the cells were treated with 200 µM H_2O_2 for 12 and 24 h.

To determine the toxicity of *G. corniculatum* extracts, the cells were plated at an appropriate density ($1x10^4$ cells/200 µL) in a collagen-coated 96-well plate at 37°C in 95% humidified air with 5% CO_2. dPC12 cells were treated with 100, 250, 500 and 1000 µg/ml plant extracts which were diluted with differentiation medium for 12 and 24 h. The cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay. After incubation, to each well 20µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added and incubated at 37° C for 4h. MTT medium was carefully aspirated from the wells and the formed formazan crystals were solubilized in 200µl of dimethyl sulfoxide (DMSO). Using a micro plate reader the absorbance was measured at 570 nm (Epoch, BioTek) [17]. The % cell viability was determined using the following formula:

% Cell Viability = [Abs (sample) / Abs (control)] x 100 [18].

2.6. Anti-inflammatory Effect of Extracts

Interleukin-10 (RT IL-10-Life Tech. (KRC0101)) and Interleukin-6 (RT IL-6-Life Tech. (KRC0061)) ELISA kits were used to test the anti-inflammatory effect of the extracts. The cells were seeded in 96-well plates ($1x10^4$ cells per well) and pretreated with 100, 250, 500 µg/ml for 24 h prior to exposure to 200 µM H_2O_2. After 24 h, the cell culture media was collected. The IL-10 and IL-6 levels in cell culture mediums of the samples and control groups were measured using IL-10 and IL-6 commercial kits according to the manufacturer's instruction. In each assay, the duplicates of each sample, standard, and blank control (zero standard) were run on the same microplate. Furthermore, IL-10 and IL-6 levels were expressed in pg/mL of the cell culture medium [19].

2.7. Statiscal Analysis

The analysis of the data was performed by analysis of variance (ANOVA) followed by Tukey's post-test to test for significance, which was set at 5%. The results were presented as mean value ± standard deviation (n=5 for the MTT assays, n=3 for all other analysis).

3. RESULTS and DISCUSSIONS

3.1. Qualitative Flavonoid Analysis

The identification of the individual flavonoids was performed through HPLC, by comparing their masses and λmax with the literature data (Table 1). Rutin and Quercetin were indentified, but Luteolin, Biochanin, Kaemferol, Genistein, Catechin and Apigenin could not be identified (Ni) in methanol extract. Luteolin, Rutin and Quercetin were indentified, but Biochanin, Kaemferol, Genistein, Catechin and Apigenin could not be identified in the water extract. As a result of our studies, Rutin and Quercetin flavonoids were found as major and the other flavonoid contents were insignificant. The amount of the Rutin was higher in methanol (45 μg/ml) than in water (41 μg/ml). Quercetin was also better extracted with methanol (12 μg/ml) than with water (10 μg/ml).

Table 1. Quantitative analysis of flavonoids in *G. corniculatum* methanol and water extracts

Extracts	**Flavonoids (μg/ml)**							
	Luteolin	Biochanin	Rutin	Quercetin	Kaemferol	Genistein	Catechin	Apigenin
Methanol	Ni	Ni	45	12	Ni	Ni	Ni	Ni
Water	0.23	Ni	41	10	Ni	Ni	Ni	Ni

3.2. Effects of H2O2 on viability of dPC12

In our assays, H_2O_2 did not show any significant cytotoxic effects at 100 μM concentrations for 12 and 24h (data not shown). Therefore, the 200 μM concentrations of H_2O_2 were used for 24h in the subsequent experiments.

3.3. Effects of G. corniculatum extracts on viability of dPC12

In our assays, generally, all the extracts were found to have high IC50 values than 1000μg/ml IC50 concentrations (1287±7μg/mL for methanol and 1150±8 μg/mL for water) for 24h. The results of cell viability were presented in Table 2. According to the U.S. NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with an IC50 value ≤20μg/mL [20]. Consequently, all the extracts demonstrated less toxicity on PC12 cells. These results have proven that the extracts do not cause anti-inflammatory effect resulting from cell death in PC12 cells.

Table 2. The effect of *G. corniculatum* methanol and water extracts on cell viability of PC12

	Cell Survival (IC_{50}) (μg/ml)	
Extracts	**12 hour**	**24 hour**
Methanol	1650±5	1287±7
Water	1527±6	1150±8

Values expressed as mean ± SD.

3.4. Anti-inflammatory effects of G. corniculatum extracts

For the determination of anti-inflammatory effects of the plant extracts, IL-6 and IL-10 were measured in dPC12 cells pretreated with 100, 250, 500 μg/ml for 24 h plant extracts prior to exposure to 200 μM H_2O_2 (As shown in Figure 1 and 2).

The results showed that the extracts (100, 250, 500 μg/mL) significantly ($P<0,05$) inhibited IL-6 secretion by H_2O_2-stimulated dPC12 cells and increased anti-inflammatory cytokine IL-10 secretion in a dose-dependent manner.

However, dPC12 cells which were treated with 200 μM H_2O_2 for 24 hours showed a significant increase in the secretion of IL-6 (1106±2 pg/ml) when compared to the control (27±3 pg/ml). Although there was no significant difference between water and methanol extract, the water extract showed the maximum anti-inflammatory effect. The water extract decreased IL-6 secretion 79 fold according to the H_2O_2 treated group for 500 μg/ml for the highest applied plant extract dose.

In our study, IL-10 levels were found to be 21±3 pg/ml in the control group (untreated dPC12 cells). The secretion of IL-10 in *G. corniculatum* methanol extract treated dPC12 cells was found as 1173±3, 1401±5 pg/ml 1810±4 pg/ml and in *G. corniculatum* water extract treated dPC12 cells as 1224±5 1472±3 1845±4 pg/ml, respectively 100, 250 and 500 μg/ml. Treatment with *G. corniculatum* water extract showed the maximum anti-inflammatory effect with increased IL-10 by 87 fold according to the control group for 500 μg/ml, for the highest applied plant extract dose.

Our results suggested that *G. corniculatum* methanol and water extracts exhibited an anti-inflammatory effect against H_2O_2-induced inflammation of dPC12 cells via increasing anti-inflammatory cytokine IL-10 and decreasing pro-inflammatory cytokine IL-6 secretions.

Linear regression analysis proved that the amount of flavonoid and anti-inflammatory activity showed a negative correlation with extract type. Therefore, the inhibition of the inflammation by the extracts may be partially due to the amount of their flavonoid content but might be attributed to the diversification of flavonoid types.

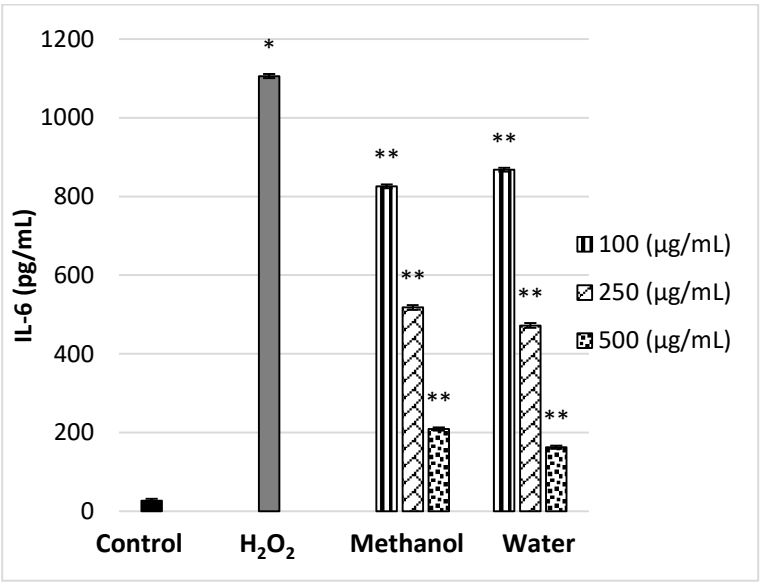

Figure 1. Dose-dependent inhibition of H_2O_2 mediated production of IL-6 by *G. corniculatum* methanol and water extracts in dPC12 cells. $^{*}p<0,05$; statistically different compared to negative control (untreated cells). $^{**}p<0,05$; statistically different compared to H_2O_2 treated group.

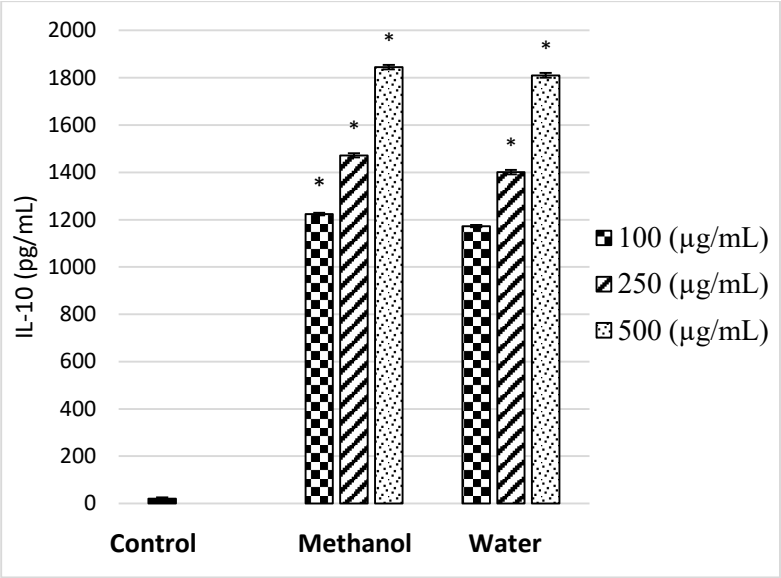

Figure 1. Dose-dependent inhibition of IL-10 TNF-α by *G. corniculatum* methanol and water extracts in PC12 cells. *p<0,05; statistically different compared to the control (untreated cells).

4. DISCUSSION

Glaucium-derived products have been used in traditional medicine in the treatment various inflammatory diseases. However, only limited numbers of studies have been conducted to elucidate the action mechanisms of these products on inflammatory diseases [21-23].

Oxidative stress due to the imbalance between ROS and antioxidant defenses will lead to the activation of survival pathway that involves in inflammation. H_2O_2 was thought to be the major precursor of reactive free radicals [24]. H_2O_2 activated macrophages and stimulated the production of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, leukotrienes, and nitric oxide (NO) [25]. It is known that NDs are associated with neuronal loss, triggered by neurodegenerative agents leading to oxidative stress and the development of inflammation. The levels of IL-6 are normally low and tightly regulated in a cell, but elevated levels of IL-6 have also been associated with various neurological disorders, including AD, PD, ALS and HD [26-28].

The anti-inflammatory effects of *G. corniculatum* methanol and water extracts were assessed here by measuring the expression and the release of pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10. However, in this article, the effects of *G. corniculatum* flavonoids in the suppression of inflammation and the preservation of neurons were investigated. In recent years, various herbal extracts and phytochemicals have been found to have anti-inflammatory properties. For instance, several flavonoids, such as Rutin and Quercetin were found to block IL-6 production [29, 30]. It has been found that flavonoids activate the endogenous antioxidant status in neuronal cells hence protecting them from undergoing neurodegeneration [31]. Polyphenols such as Quercetin and Rutin have distinct features in upregulating the production of intracellular antioxidant enzymes induced in PC-12 cells [32-34]. However, it has been reported that Quercetin and Rutin flavonoids produced anti-inflammatory effects by inhibiting proinflammatory cytokines [36, 36]. Hu et al. demonstrated that Rutin ameliorates inflammasome activation, leading to suppressed inflammation in the kidney of rats [37]. Similarly, Javed et al. reported the activity of Rutin in preventing cognitive impairments in rats [38].

The results show that *G. corniculatum* methanol and water extracts inhibit the H_2O_2 induced IL-6 production and increased the production of IL-10 in dPC12 cells. Previous studies

have shown an antagonist effect of IL-10 on secretion of pro-inflammatory cytokines, suggesting that *G. corniculatum* extract mediated inhibition of the H_2O_2 induced IL-6 secretion may pass through the induction of IL-10 production. These outcomes were consistent with the results of anti-inflammatory properties; however, the amount of flavonoids showed a negative correlation with extract type. Therefore, the results suggest that anti-inflammatory effect may be related to flavovoid diversity rather than flavonoid amounts. The ability of *G. corniculatum* extract to modulate the pro-inflammatory and anti-inflammatory cytokines suggests that it may be an alternative agent for the treatment and / or prevention of inflammatory diseases and NDs.

5. CONCLUSSION

The amount of flavonoid content of methanol and water extracts of *G. corniculatum* was successfully screened. Rutin and Quercetin flavonoids are major flavonoids and *G. corniculatum* methanol extract has the highest flavonoid amount.

All of the *Glaucium* extracts used in this study provide anti-inflammatory effect without damaging dPC12 cells. These results bring attention to the inhibitory effects of *G. corniculatum* on inflammation and can explain why this plant is considered as a traditional medicinal plant. According to these results, *G. corniculatum* extracts may have a favourable pharmacological profile in the treatment of NDs and inflammation. Moreover, this study proves that the Rutin and Quercetin flavonoids detected in *G. corniculatum* methanol and water extracts have a neuroprotective effect through anti-inflammation.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Effect of Lavender Powder on Microbial, Physicochemical, Sensory and Functional Properties of Yoghurt

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Abstract: The objective of this research was to determine changes in microbial flora and functional properties of yoghurts containing lavender powder at rates of 0.010, 0.025, 0.050 and 0.075%. The effect of lavender powder on the potential flora during fermentation was determined, furthermore, alterations in microbiological properties of yoghurts were examined during the storage period (1 d, 7 d and 14 d). Yoghurt samples containing 0.010, 0.025, 0.050 and 0.075% lavender powder, were coded A1, A2, A3, and A4, respectively. Streptococcal counts were 8.89±0.21, 8.71±0.09, 8.77±0.13, 8.66±0.04 and 8.91±0.13 log CFU/g, respectively, in the control, A1, A2, A3, and A4 samples. No decrease in streptococcal counts was observed during storage in all samples. Lactobacilli counts were 8.58±0.06, 8.43±0.26, 8.34±0.26, 8.02±0.02 and 8.53±0.34 log CFU/g, respectively, in the control, A1, A2, A3 and A4 samples. While the lactobacilli count of the control sample decreased during the storage period, it remained constant in A1, A2, A3 and A4 samples at the end of the storage time. It was determined that the lavender powder supplementation contributed the viability of lactobacilli during storage. No statistically significant difference was found in the total bacterial counts between the samples on the first day, however the highest total bacteria content was determined in A4 sample (8.31±0.01 log CFU/g) at the end of the storage period (14 d).

Keywords: antioxidant effect, lavender, microbial potential, yoghurt

1. INTRODUCTION

Yoghurt is a fermented milk product that is produced by *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*) and consumed all around the world [1]. Yoghurt fortification has an important role to develop nutritional, sensorial, functional and health promoting effects of the yoghurt [2]. In this context, vegetables [3, 4], fruits [5, 6], fibers [7, 8], seed extracts [9] and plants [10, 11] have been used in yoghurt production.

Lavandula officinalis L. is a fragrant herb belonging to Lamiaceae family and called lavender [12]. It is known as having therapeutic and antimicrobial effects [13]. It was reported that lavender showed high antioxidant properties [14]. Lavender is rich with phenolic contents and ferulic acid is the major phenolic compound of it [15].

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In this study, the effect of lavender powder used at different ratios on potential yoghurt bacteria was investigated. For this purpose, 0.010, 0.025, 0.050 and 0.075% lavender powders were added to yoghurt samples. The effect of lavender powder on the kinetic parameters of acidification during fermentation was examined, furthermore, changes in microbial growth were monitored during the storage period (1 d, 7 d and 14 d). The antioxidant activities and total phenolic contents of yoghurt samples were investigated. Yoghurt samples were subjected to sensory testing to determine consumer acceptance.

2. MATERIAL and METHODS

2.1. Materials

Medium-heat skim milk powder (34.5 % protein, 3.5 % moisture, 7.2 % ash, 55 % lactose, pH:6.55, % titratable acidity 0.112) were obtained from ENKA Dairy Co. Ltd. (Konya, Turkey). Milk was ensured farm of Selcuk University dairy farm. YF-L901 consisting of *S. thermophilu*s and *L. bulgaricus* were used as starter cultures in yoghurt production and they were supplied from Chr's Hansen-Peyma (Istanbul). Harvested lavender plants (June-July) were dried for 1 month at room temperature in light-free environment and then powdered with blender.

2.2. Production of Set-Type Yoghurt and Sample Design

Five yoghurt formulations were performed by described by Akın [1]. A1, A2, A3 and A4 samples contained lavender powder at 0.010, 0.025, 0.050, and 0.075%, respectively, whereas no lavender powder was added to control sample. All yoghurt formulations were standardized to 16% dry matter content by milk powder. In A1, A2, A3 and A4 samples, milk powder and lavender powder were mixed with fresh cow milk, while only milk powder was added to fresh cow milk in control sample. Afterwards the mixes were pasteurized at 85 °C for 10 min and cooled 42 °C. The yoghurt mixes were inoculated with 2% (w/v) starter culture and then distributed in 150 mL sterile plastic containers and incubated at 42 °C until a pH was reached to 4.6. Yoghurt samples were stored in a refrigerator (4 °C). during storage time (1 d, 7 d, and 14 d). Yoghurt samples were manufactured each in 2 replications.

2.3. Monitoring of Fermentation Kinetics

The changes in pH and titratable acidity during the fermentation were monitored until the pH 4.6. Maximum acidification rate (V_{max}), the time to reach the maximum acidification rate (T_{max}) and the time at which was the end of the fermentation (T_e) were determined.

2.4. Physicochemical Analysis

Fat [16], water holding capacity [17], syneresis [18], and color [19] were done on day 7, pH and titratable acidity [20] were monitored during the storage period.

2.5. Assessment of Antioxidant Activity

Antioxidant activity was determined by two different radical scavenging methods on day 7. ABTS [21] and DPPH [22] radical scavenging methods were used due to differences in susceptibility of the methods. ABTS radical scavenging activity results were explicated in terms of Trolox equivalent antioxidant capacity (TEAC) value, while DPPH radical scavenging results were reported as % inhibition.

2.6. Total Phenolic Content

The total phenolic compounds in yoghurts were determined by using the method of McCue and Shetty [23] on day 7. The results that were calculated based on the gallic acid curve were given as milligram gallic acid equivalents (GAE) per gram of yoghurt samples.

2.7. Determination of Changes in Microbial Flora During Storage Time

S. thermophilus and *L. bulgaricus* counts of yoghurt samples were determined during the storage time. *S. thermophilus* colonies were enumerated in M17 agar under aerobic incubation at 37 °C for 24-48 h [24], while *L. bulgaricus* colonies were counted in MRS agar under anaerobic incubation at 45 °C for 24-48 h [25]. Potato Dextrose Agar was used to determine yeast and fungi under aerobic incubation at 25 °C for 5 d [20]. Total bacteria were enumerated in Plate Count Agar (PCA) at 30 °C for 48 h [26].

2.8. Sensory Analysis

Sensorial tests of yoghurt samples were evaluated by a panel of seven members using seven-point hedonic scale [27] (1: strongly disliked; 7: strongly liked). Panelists appraised the appearance, taste and flavor, smell, texture, acidity and general acceptability. Sensory evaluation was done on the 7th day of storage time.

2.9. Statistical Analysis

The parameters of experimental yoghurts were assessed by General Linear Model ANOVA by MiniTab 7.1 [28]. Different groups were compared by the Tukey test at $P<0.05$, and statistically significant differences among them were indicated by different letters.

3. RESULTS and DISCUSSIONS

3.1. Fermentation Kinetics

The fermentation kinetic of each sample group was obtained by calculating the maximum acidification rate (V_{max}), the time to reach Vmax (T_{max}) and the time required to reach the end of fermentation ($T_{pH4.6}$). V_{max}, T_{max} and $T_{pH4.6}$ values are given in Table 1 and the changes in the pH and titratable acidity values occurring throughout fermentation are shown in Figure 1.

Table 1. Acidification kinetics of yoghurt samples during fermentation.

Samples	V_{max} (10^{-3} pH units min^{-1})	T_{max} (h)	$T_{pH4.6}$ (h)
Control	11.00 ± 0.00^{a}	2.75 ± 0.01^{ns}	6.16 ± 0.02^{a}
A1	10.12 ± 0.01^{b}	2.25 ± 0.00^{ns}	5.41 ± 0.28^{b}
A2	10.16 ± 0.01^{b}	2.25 ± 0.00^{ns}	5.33 ± 0.22^{b}
A3	9.16 ± 0.02^{c}	2.75 ± 0.01^{ns}	5.12 ± 0.01^{b}
A4	10.00 ± 0.00^{b}	2.25 ± 0.00^{ns}	5.37 ± 0.17^{b}

A1: with 0.010% lavender powder; A2: with 0.025% lavender powder; A3: with 0.050%; A4: with 0.075% lavender powder. V_{max}: maximum acidification rate; T_{max}: time corresponding to V_{max}; $T_{pH4,6}$: time to obtain pH 4.6. Different letters in the same column are significantly different ($P<0.05$); ns: not significant.

Statistically significant differences were observed in V_{max} and $T_{pH4.6}$ values of the yoghurt samples ($P<0.05$). There was significant effect of lavender powder addition on V_{max} (Table 1). This effect may have been caused by components with buffering capacity such as phenolics present in the lavender [15]. Similar results were seen in the passion fruit peel added yoghurt [29]. There was no difference between the times to reach maximum acidification rates (T_{max}) of the samples. However, $T_{pH4.6}$ was significantly reduced by adding lavender powder ($P<0.05$) and the highest decrease in $T_{pH4.6}$ was observed in A3 sample.

Titratable acidity increased with time in all samples, but the highest increase occurred in the A3 and A4 samples. The formation of lactic acid advanced due to the increase in the amount of lavender powder. This may be due to the positive effect of lavender powder on lactic acid bacteria. De Martino and colleagues [30] reported that lavender oil exhibited no inhibitory activity against lactic acid bacteria.

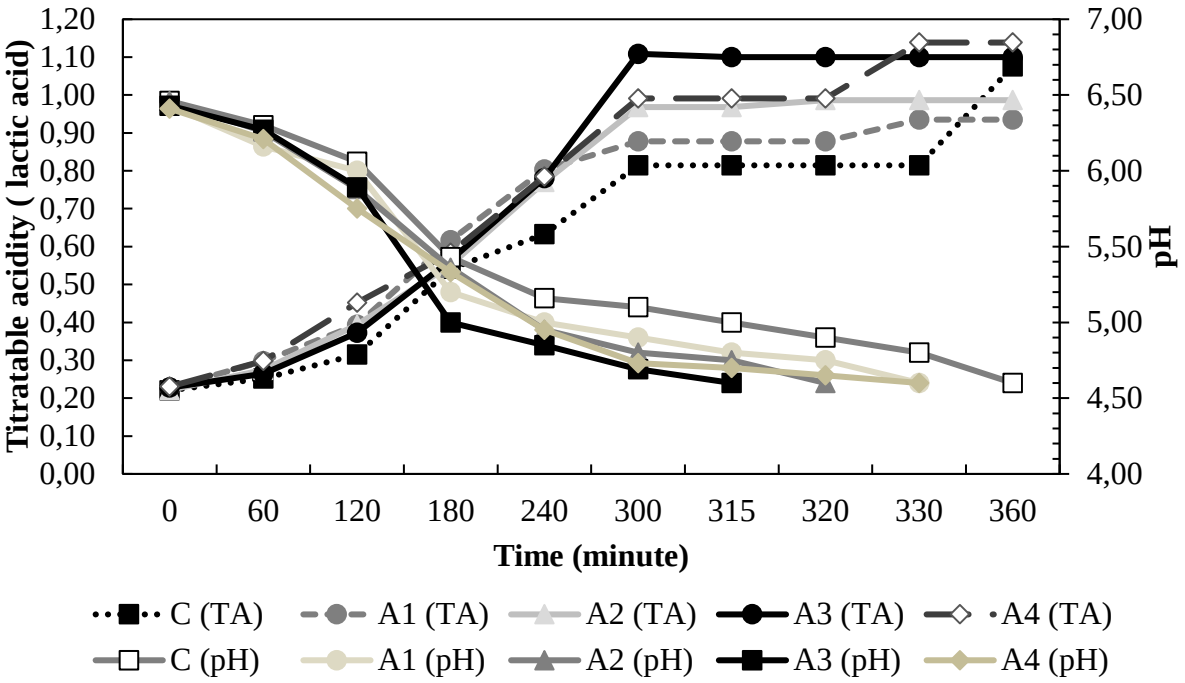

Figure 1. Changes in the pH and titratable acidity during fermentation. C: control; A1: with 0.010% lavender powder; A2: with 0.025% lavender powder; A3: with 0.050%; A4: with 0.075% lavender powder.

3.2. Physicochemical Characteristics of Yoghurt Samples

Physicochemical properties of yoghurt samples are shown in Table 2. Water holding capacity and syneresis values of yoghurt samples were found between 56.55-61.20%and 8.86-10.94%, respectively and there were no statistical differences between the yoghurt samples (P>0.05). Fat values of samples were determined between 2.70-2.90%, the differences in fat values were not statistically significant. While there were no statistical differences in the brightness and yellowness values of the yoghurt samples (P>0.05), the highest redness value was determined in the A4 sample (P<0.05).

Table 2. Some physicochemical properties, total phenolic contents and antioxidant activities of yoghurt samples (on day 7).

		Sample[1]				
		Control	A1	A2	A3	A4
WHC[2](%)		58.95±1.06^{ns}	61.20±0.71^{ns}	57.20±4.67^{ns}	56.55±0.78^{ns}	58.10±0.42^{ns}
Syneresis (%)		10.06±2.01^{ns}	8.86±0.54^{ns}	9.16±0.11^{ns}	9.78±0.42^{ns}	10.94±1.95^{ns}
TPC[3] (mg GAE/g)		0.05±0.00^{c}	0.07±0.01^{bc}	0.07±.0.01^{b}	0.08±0.01^{ab}	0.10±0.01^{a}
DPPH (inhibition %)		2.82±0.09^{d}	3.32±0.27^{c}	3.76±0.18^{b}	4.01±0.36^{b}	4.70±0.09^{a}
ABTS (μM Trolox)		170.89±0.06^{e}	190.87±0.13^{d}	240.47±0.12^{c}	284.75±6.50^{b}	311.01±0.91^{a}
Fat (%)		2.75±0.07^{ns}	2.80±0.00^{ns}	2.70±0.14^{ns}	2.80±0.00^{ns}	2.90±0.14^{ns}
Color	*L**	88.39±0.10^{ns}	89.66±0.01^{ns}	89.29±0.15^{ns}	88.98±0.12^{ns}	88.18±0.38^{ns}
	*a**	-4.13±0.05^{b}	-4.15±0.03^{b}	-3.95±0.15^{b}	-4.05±0.03^{b}	-3.13±0.07^{a}
	*b**	9.11±0.02^{ns}	9.20±0.14^{ns}	9.12±0.02^{ns}	9.10±0.16^{ns}	9.15±0.27^{ns}

[1]A1: with 0.010% lavender powder; A2: with 0.025% lavender powder; A3: with 0.050%; A4: with 0.075% lavender powder. [2]WHC: Water holding capacity; [3]TPC: Total phenolic content. Values in same row having different superscripts differ significantly (P<0.05); ns: not significant.

The pH and titratable acidity values monitored during storage time are given in Table 3. After one day of cold storage the pH values of control, A1, A2, A3 and A4 samples were determined to be 4.48, 4.52, 4.58, 4.68, and 4.56, respectively. After 14 days of storage time, the pH values of all yoghurt samples decreased significantly ($P<0.05$) and the decrease in pH values were about 6.14-6.70%. Titratable acidity values varied from 1.14 to 1.29 mg lactic acid/g at the beginning of the storage. Titratable acidity of all samples increased by 11.40-20.33% at the end of the cold storage. These results could be attributed to metabolic activities of microbial flora existed in yoghurt samples. Bonczar and colleagues [31] and Do Espírito and colleagues [29] reported that the increase in the titratable acidity values of yoghurts at the end of the 14 and 28 days was determined.

Table 3. Changes in pH and titratable acidity of yoghurt samples during the cold storage.

Samples[1]	pH			Titratable acidity (lactic acid%)		
	d 1	d 7	d 14	d 1	d 7	d 14
Control	4.48 ± 0.02^{cA}	4.45 ± 0.00^{cA}	4.18 ± 0.00^{dB}	1.23 ± 0.28^{abB}	1.17 ± 0.08^{nsB}	1.48 ± 0.04^{aA}
A1	4.52 ± 0.00^{bcA}	4.52 ± 0.00^{bA}	4.23 ± 0.00^{cB}	1.29 ± 0.00^{aB}	1.31 ± 0.04^{nsB}	1.46 ± 0.00^{abA}
A2	4.58 ± 0.02^{bB}	4.63 ± 0.04^{aA}	4.28 ± 0.00^{bC}	1.20 ± 0.02^{bcC}	1.31 ± 0.03^{nsB}	1.44 ± 0.00^{abA}
A3	4.68 ± 0.07^{aA}	4.51 ± 0.00^{bB}	4.37 ± 0.00^{aB}	1.14 ± 0.04^{cNS}	1.18 ± 0.12^{nsNS}	1.27 ± 0.21^{cNS}
A4	4.56 ± 0.01^{bcA}	4.55 ± 0.00^{bA}	4.28 ± 0.00^{bB}	1.19 ± 0.00^{bcB}	1.35 ± 0.00^{nsA}	1.40 ± 0.03^{bA}

[1]A1: with 0.010% lavender powder; A2: with 0.025% lavender powder, A3: with 0.050%; A4: with 0.075% lavender powder. d 1, d 7 and d 14: days 1, 7 and 14 after fermentation. Values in same row and column having different superscripts differ significantly ($P<0.05$); ns, NS: not significant. Lower case letters indicate differences between samples; capital letters show differences between storage times.

3.3. Antioxidant Activities and Total Phenolic Contents of Yoghurt Samples

Antioxidant activities and total phenolic contents of yoghurt samples are shown in Table 2. The highest ABTS and DPPH radicals scavenging activities were determined in the A4 sample ($P<0.05$). The DPPH inhibition % values for the control, A1, A2, A3 and A4 samples were detected as 2.82, 3.32, 3.76, 4.01 and 4.70, respectively. The TEAC values of control, A1, A2, A3 and A4 samples were determined to be 170.89, 190.87, 240.47, 284.75 and 311.01 µM Trolox, respectively. The results of the ABTS and DPPH tests were consistent in terms of radical scavenging activity of each yoghurt samples. Gülçin and colleagues [14] reported that free radical scavenging activity of lavender increased depending on the ratio of lavender powder used in the formulation.

The highest total phenolic content (0.10 mg GAE/g) was determined in A4 sample ($P<0.05$), while there was no statistically difference in total phenolic contents of other sample groups ($P>0.05$). The total phenolic content of lavender was 27.42 mg GAE/g [32], thus the use of lavender powder at very low levels (0.01, 0.025, 0.050 and 0.075%) did not generally affect the total phenolic contents of yoghurts.

3.4. Microbial Dynamic During Cold Storage

The changes in microbial flora of yoghurt samples during the storage period are shown in Table 4. *S. thermophilus* counts were statistically stable in the control, A1, A2 and A4 samples during the storage time and the mean counts of it were defined between 8.75-8.88 log CFU/g. Do Espírito and colleagues [29] asserted that *S. thermophilus* counts remained constant for 28 days at cold storage of yoghurts containing passion fruit peel powder. On the other hand, the *S. thermophilus* counts in A3 sample containing 0.050% lavender powder increased 0.61 log unit after 14 days. It may be due to the fact that *S. thermophilus* dominated in the yoghurt flora because of the antimicrobial effect of lavender on other microorganisms [13]. 0.050%

lavender powder supported the growth of *S. thermophilus* in the yoghurt media. Higher and lower lavender powder ratios than 0.050% were not observed to have a negative effect on *S. thermophilus* counts.

The counts of *L. bulgaricus* in control sample decreased at the end of the storage time. Do Espírito and colleagues [29] and Shori [33] determined a decrease in *L. bulgaricus* counts of control yoghurt samples at the end of the storage time. In A1, A2 and A4 samples, the mean *L. bulgaricus* counts were found to be 8.38, 8.35 and 8.40 log CFU/g, respectively and no statistically change was observed in its counts during the storage period. Compared to the control, lavender powder was found to support growth of lactobacilli. Marhamatizadeh and colleagues [34] reported that dried lavender increased the number of lactobacilli such as *L. acidophilus*, *L. casei* and *L. paracasei*. The decrease in the number of *L. bulgaricus* on the 14th day of storage in A3 sample may be due to increase in *S. thermophilus* counts.

Table 4. Changes in microbial flora of yoghurt samples during the storage period (log CFU/g).

Yoghurt bacteria	Samples[1]	d 1	d 7	d 14
S. thermophilus	Control	8.89 ± 0.21^{nsNS}	8.61 ± 0.08^{nsNS}	9.08 ± 0.30^{nsNS}
	A1	8.71 ± 0.09^{nsNS}	8.67 ± 0.08^{nsNS}	8.86 ± 0.21^{nsNS}
	A2	8.77 ± 0.13^{nsNS}	8.82 ± 0.12^{nsNS}	9.05 ± 0.30^{nsNS}
	A3	8.66 ± 0.04^{nsB}	8.63 ± 0.01^{nsB}	9.24 ± 0.19^{nsA}
	A4	8.91 ± 0.13^{nsNS}	8.72 ± 0.11^{nsNS}	8.86 ± 0.02^{nsNS}
L. bulgaricus	Control	8.58 ± 0.06^{nsAB}	8.78 ± 0.09^{aA}	8.36 ± 0.13^{aB}
	A1	8.43 ± 0.26^{nsNS}	8.46 ± 0.17^{abNS}	8.26 ± 0.01^{abNS}
	A2	8.34 ± 0.26^{nsNS}	8.50 ± 0.19^{abNS}	8.22 ± 0.01^{abNS}
	A3	8.02 ± 0.02^{nsB}	8.19 ± 0.04^{bA}	8.01 ± 0.04^{bB}
	A4	8.53 ± 0.34^{nsNS}	8.45 ± 0.04^{abNS}	8.24 ± 0.00^{abNS}
Total bacteria	Control	8.60 ± 0.00^{nsA}	8.54 ± 0.23^{nsA}	8.03 ± 0.01^{cB}
	A1	8.29 ± 0.04^{nsNS}	8.21 ± 0.01^{nsNS}	8.20 ± 0.02^{bNS}
	A2	8.30 ± 0.04^{nsNS}	8.30 ± 0.04^{nsNS}	8.24 ± 0.05^{bNS}
	A3	7.97 ± 0.04^{nsNS}	8.13 ± 0.16^{nsNS}	8.25 ± 0.02^{bNS}
	A4	7.72 ± 0.59^{nsNS}	8.17 ± 0.09^{nsNS}	8.31 ± 0.01^{aNS}

[1]A1: with 0.010% lavender powder; A2: with 0.025% lavender powder; A3: with 0.050%; A4: with 0.075% lavender powder.d 1, d 7 and d 14: days 1, 7 and 14 after fermentation. Different letters in the same row and column indicate significantly different ($P<0.05$). ns, NS: not significant. Lower case letters indicate differences between samples; capital letters show differences between storage times.

No yeast and fungi were observed in all yoghurt samples during the storage time. The total bacteria count was statistically reduced in the control sample at the end of the storage ($P<0.05$), while it remained constant in other yoghurt samples containing lavender powder during the storage time. On the 14th day of the storage time, statistical differences were detected between the total bacterial counts of the yoghurt samples and the lowest total bacterial count was determined in control sample ($P<0.05$). This difference may be due to the positive effect of lavender powder on the growth of *S. thermophilus* and *L. bulgaricus*.

3.5. Sensorial Characteristics of Yoghurt Samples

Sensorial characteristics of yoghurt samples are shown in Figure 2. The best appearance score (5.88) was determined in A1 sample according to the sensory acceptability test ($P<0.05$). The appearance score of A2, A3 and A4 samples decreased with the use of lavender powder at higher ratios. The highest texture score (5.44) was detected in A1 sample ($P<0.05$), generally, the texture scores of yoghurts added with lavender powder were found higher than the control. Acidity scores were higher in A3 and A4 than control ($P<0.05$), it may have been caused by supporting effect of the higher amounts of lavender powder on acidic taste.

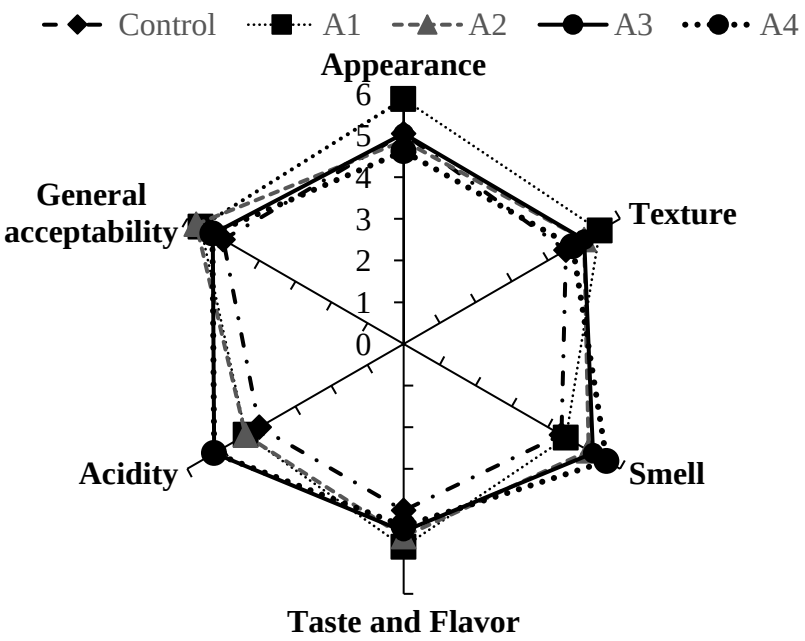

Figure 2. Sensorial characteristics of yoghurt samples (on day 7)

The highest smell score was determined in A4 sample, the smell score increased depending on the amount of lavender powder. However, the difference between smell scores of A1 and the control samples was statistically insignificant. The highest taste and flavor score was determined in A1 sample, taste and flavor scores decreased with the use of lavender powder at higher ratios. The highest general acceptability score was determined in the A1 and A2 samples. According to general acceptability score, A1 and A2 were the most preferred yoghurt samples for consumers.

4. CONCLUSION

In this study, the effect of lavender powder on lactic acid bacteria was determined. The results showed that the lactic acid production during the fermentation and the viability of *S. thermophilus* and *L. bulgaricus* during the cold storage can be enhanced by supplementation of lavender powder. It was determined that the antioxidant activity of yoghurt increased with the addition of lavender powder. The antioxidant activity of yoghurt increased about 2-fold by addition of 0.075% lavender powder. Considering the scores of sensory properties and functional properties of lavender powder, it can be thought to be a good supplement for yoghurt.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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***Special Issue 1**: Research Article*

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The Medicinal Plant of Genus *Paronychia* and the Karyotype Analysis of *Paronychia adalia*

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Abstract: The genus *Paronychia* Miller is placed in the family Caryophyllaceae. It contains approximately 110 species of annual or perennial found all over the world except Southern Africa, Southeast Asia. Genus *Paronychia* is known as Algerian tea in the world. In our country, the genus commonly known as et yaran, kepek otu and dolama otu is used as medicinal tea because of relieving inflammation between the hands and toes, aphrodisiac, diuretic and blood purifier. Antimicrobial and antioxidant properties of the genus are known. The chromosome number is $2n = 36$ in many species of *Paronychia*. But there are various chromosome numbers as $2n = 10, 14, 16, 18$ and 28. In this study, the chromosome number of *P. adalia* Chaudhri was reported for the first time. The chromosome number and karyotype formula are $2n = 2x = 36 = 34m + 2sm$. Total haploid length, centromeric index and karyotype asymmetry were calculated with detailed chromosomal measurements.

Keywords: *Paronychia*, medicinal plant, chromosome.

1. INTRODUCTION

The genus *Paronychia* Miller is placed in the family Caryophyllaceae. It contains approximately 110 species of annual or perennial widespread around the world except Southeast Asia and Southern Africa. It contains total of 41 taxa as 29 species, five subspecies and seven varieties in Turkey, too. In other words, the species in our country cover approximately 35% of the world *Paronychia*. There are 28 endemic taxa for our country and the rate of endemism of the genus is 68.3%. The genus, which was previously in the family Illecebraceae, has now been taken into the subfamily Paronychioideae in the family Caryophyllaceae with recent studies [1].

Genus *Paronychia* is known as Algerian tea in the world. In our country, the genus commonly known as et yaran, kepek otu and dolama otu is used as medicinal tea because of relieving inflammation between the hands and toes, aphrodisiac, diuretic and blood purifier. Antimicrobial and antioxidant properties of the genus are known [2].

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Paronychia adalia Chaudhri is a plant with white coloured and small flowers. It is an endemic species in Turkey and grows at rocky-stone areas, limestone rocks and forest openings. No data available about the biological activity and chromosomal data of *P. adalia* in literature.

In this study, we examined the karyotype analyses of *P. adalia* collected from Mediterranean region, Turkey (Figure 1). The diploid chromosome number is 2*n* = 36 in many taxa of *Paronychia* [3-5]. But there are different chromosome numbers as 2*n* = 10 (*P. echinulata* Chater), 2*n* = 14 (*P. polygonifolia* (Vill.) DC.), 2*n* = 16 (*P. suffruticosa* (L.) DC.), 2*n* = 18 (*P. aretioides* Pourr. ex DC., *P. kurdica* Boiss., *P. macrosepala* Boiss., *P. caespitosa* Stapf), 2*n* = 28 (*P. argentea* Lam.), *2n* = 32 (*P. pulvinata* A. Gray) and *2n* = 64 (*P. sessiliflora* Nutt.) [3-5]. These species are only given the diploid chromosome number in the studies. There is not the data regarding chromosomal measurements. The cytogenetic characters, especially chromosome size, karyotype formula and chromosome asymmetry are important characters as chromosome number. These characters are also important to elucidate the origin, speciation and phylogenetic relationships of species and genus [6-7].

2. MATERIAL and METHODS

Collection information regarding the species is listed below.

Paroncyhia adalia – TURKEY. Antalya: Elmalı, Susuz mountain, close to the top, rocky slopes, 2080 m, 01-VIII-2013, Budak 2756 & Hamzaoğlu (Bozok Hb.).

The cytogenetic procedure is listed below, respectively. (i) germination at room temperature, (ii) pretreatment with α-monobromonaphthalene, (iii) fixation with Carnoy's fixative, (iv) hydrolysis with 1N HCl, (v) staining with aceto orcein, (vi) preparation with acetic acid, (vi) permanent preparation with DPX [6-7].

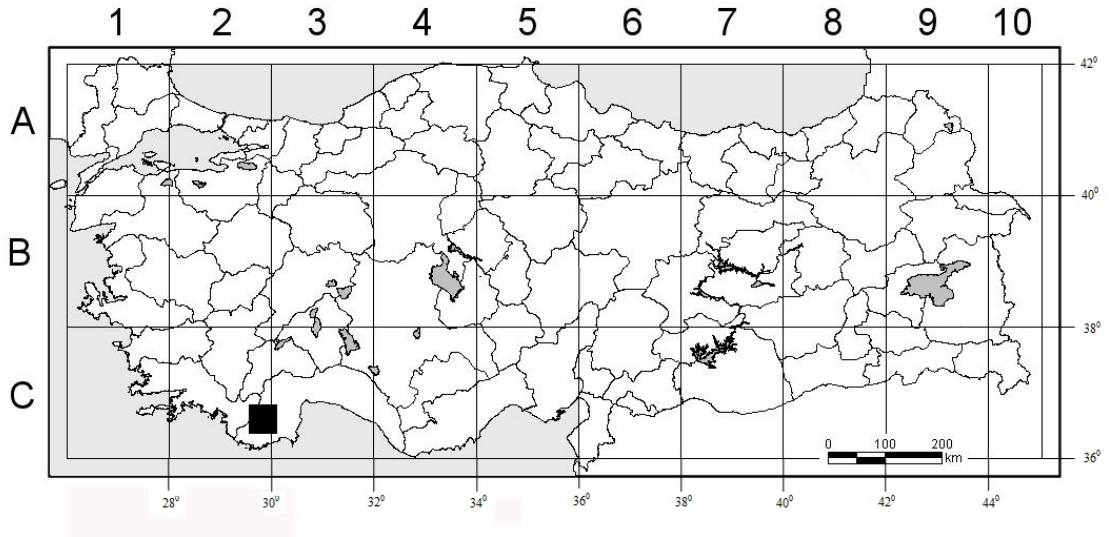

Figure 1. Distribution in Turkey of *Paronychia adalia*

The chromosomes were photographed with Olympus DP72 camera and measured with KaryoType software [8]. Chromosome classifications were made according to the Levan et al. [9]. The parameters were calculated to characterize of karyotypes numerically: long arm length of chromosome (LAL), short arm length of chromosome (SAL), total chromosome length (TCL) = [L + S], arm ratio of chromosome (AR) = [L / S] and centromeric index (CI) = [S / (L + S) × 100]. The ideogram was drawn based on length of chromosome size (arranged large to small). Karyotype asymmetries were estimated by M_{CA} [10] and CV_{CL} [11].

3. RESULTS

The somatic metaphase chromosomes and monoploid ideogram of *Paronychia adalia* are given in Figure 2 and 3. The measurement data of chromosomes are given in Table 1. Somatic metaphases analysis showed that the diploid chromosome number of *P. adalia* is *2n* = 2x = 36. The karyotype formula is *2n* = 2x = 36 = 34m + 2sm. All chromosomes are median type outside sub-median chromosome 8. No satellite was observed in the chromosomes.

The chromosome lengths range between 0.93 and 2.55 μm. The chromosome 1 has the longest both long arm length (1.35 μm) and short arm length 1.20 μm). The chromosome 18 has the shortest both long arm length (0.57 μm) and short arm length 0.36 μm). The arm ratio of chromosome 8 is quite high, unlike the arm ratio of chromosome 2, 5 and 6 are quite low.

Total haploid length and mean haploid length are 27.34 and 1.52 μm, respectively. The centromeric indexes range between 31.29 and 49.09. The low centromeric index is characterized with median zone, unlike very high centromeric index is characterized with telocentric zone.

The M_{CA} and CV_{CL} values are 12.20 and 27.24, respectively. The M_{CA} and CV_{CL} decrease with decreasing asymmetry. The centromeric position changes in intrachromosomal karyotype asymmetry (M_{CA}). The M_{CA} varies between 0 (most symmetrical) and 100 (most asymmetric). Besides, the chromosome sizes are quite different in interchromosomal karyotype asymmetry (CV_{CL}). The CV_{CL} varies between 0 and 100.

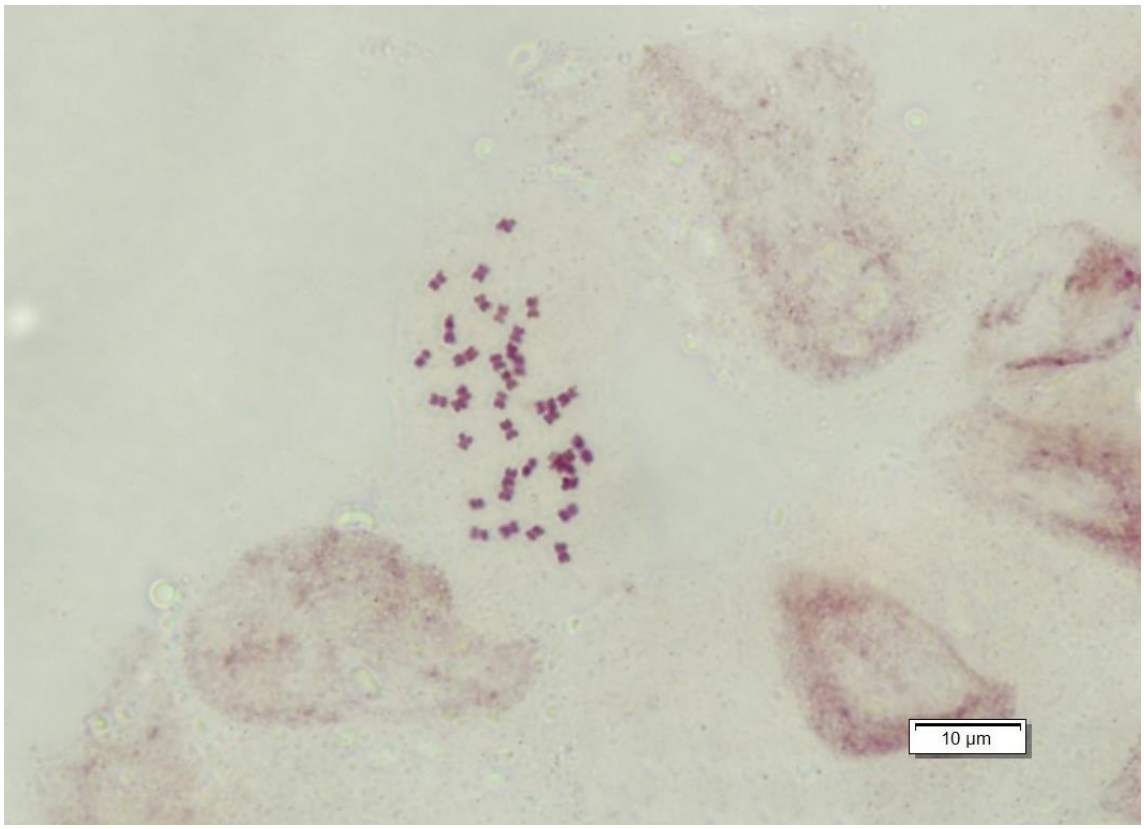

Figure 2. Somatic metaphase chromosomes of *Paroncyhia adalia*.

Table 1. The chromosomal data of *Paronychia adalia*.

Chromosome Pair	TCL (µm)	LAL (µm)	SAL (µm)	AR	Type	CI
1	2.55	1.35	1.20	1.12	m	47.06
2	2.20	1.12	1.08	1.04	m	49.09
3	1.87	1.05	0.82	1.28	m	43.85
4	1.75	1.05	0.70	1.50	m	40.00
5	1.73	0.90	0.83	1.08	m	47.98
6	1.66	0.86	0.80	1.07	m	48.19
7	1.64	0.87	0.77	1.13	m	46.95
8	1.63	1.12	0.51	2.20	sm	31.29
9	1.51	0.82	0.69	1.19	m	45.70
10	1.48	0.77	0.71	1.08	m	47.97
11	1.32	0.70	0.62	1.13	m	46.97
12	1.30	0.71	0.59	1.20	m	45.38
13	1.22	0.70	0.52	1.35	m	42.62
14	1.21	0.65	0.56	1.16	m	46.28
15	1.14	0.69	0.45	1.53	m	39.47
16	1.11	0.65	0.46	1.41	m	41.44
17	1.09	0.64	0.45	1.42	m	41.28
18	0.93	0.57	0.36	1.58	m	38.71

Abbreviations: TCL, total chromosome length; LAL, long arm length; SAL, short arm length; AR, arm ratio; CI, centromeric index; m, median; sm, sub–median.

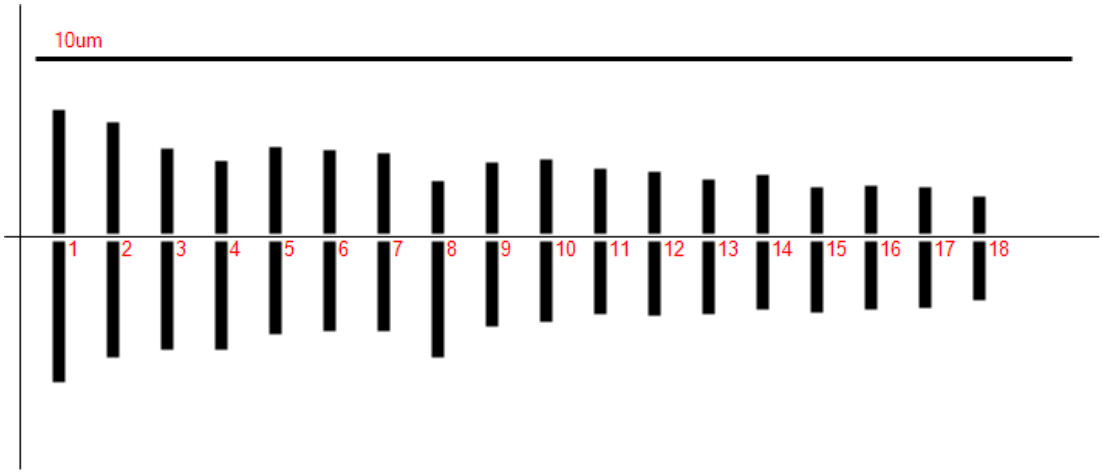

Figure 3. Ideogram of *Paroncyhia adalia*.

4. DISCUSSION

The chromosome number of *Paroncyhia adalia* is 2n = 36. The species has small chromosomes between 0.93-2.55 µm. There is very little variation between chromosomes (Table 1). It reported that the chromosome number was 2n = 36 in many species of *Paronychia*. But the genus was showed different chromosome numbers as 2n = 10, 14, 16, 18 and 28 [3-5].

Karyotype asymmetry is an important parameter for karyotype studies [12-13]. The M_{CA} and CV_{CL} are the most reliable values among karyotype asymmetry indexes [10]. The M_{CA} and CV_{CL} values of *Paroncyhia adalia* are 12.20 and 27.24, respectively. According to these values, the karyotype is quite symmetrical.

In the study, the karyological data of *Paroncyhia adalia* was showed for the first time. The karyological data are not distinctive or connective characters in plant taxonomy; however they can support these characters. The chromosomal variations can support to diversification of the species [6-7]. There are still many taxa unknown chromosomal data in genus *Paroncyhia*. More chromosomal data are needed to contribute to the cytotaxonomy of *Paroncyhia*. With future studies, the chromosomal data of other taxa will be determined.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Optimization of Ultrasonic Extraction of Total Flavonoids from *Cinnamonum zeylaniccum*

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Abstract: Cancer is the most widespread disease in the world currently. The availability of using controlled-drug-releasing capsules for those cancer diseases had become more and more important. The initial operation of the production process of the capsules requires the extraction of the active ingredients from the plant. Phytochemicals exert positive effects on human health and thus they are important compounds found in medicinal plants like *Cinnamonum zeylaniccum*. Flavonoids are the most effective phytochemicals having more than four thousand different types and they are well known antioxidants especially used in treatments of cancer diseases. In the light of these information, the aim of this study was to optimize the ultrasonic extraction of flavonoids (as a raw-material fort he capsules) from *Cinnamonum zeylaniccum* with methanol by using Response Surface Methodology. Batch extraction experiments had been carried out with the parameters of solid-to-liquid ratio, extraction temperature and time, by applying ultrasounds on sweep mode. Multi-parameter optimization was carried out with three-parameter, three-level, three-centered Box-Behnken design considering the optimum values obtained in single-parameter optimization. Design-Expert software was used to obtain the function representing the extraction yield surface depending on the selected parameters. As a result of the statistical analysis, the function expressing the effect of the parameters on the resulting amount of quercetin production in industry had been derived as reduced cubic model. Solid-to-liquid ratio was found as the most effective parameter on the extraction yield. At the optimum conditions (30°C, 1/10 g/ml, and 15 minutes), 12.34 mg quercetin equivalent total flavonoids were extracted.

Keywords: *Cinnamonum zeylaniccum*, Extraction, Flavonoid, Response surface methodology

1. INTRODUCTION

Currently cancer is the second leading cause of the death globally. Analysis showed that about a third of the most common cancer types can be prevented through diet, weight and physical activity. Herbal plants containing phytochemicals are gaining more and more consideration especially for cancer treatments. Phytochemicals exert positive effects on human health and thus they are important compounds found in medicinal plants. In order to take acceptable amount of those phytochemicals, they should be taken as capsules. As a result, several studies focusing on producion of cancer-focusing drug delivery systems by loading phytochemicals of medicinal plants are an important subject for this area.

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Several plants have been present in different families. Cinnamon, a member of the Laurel family, native to the South and Southeast Asia, is a genus of evergreen aromatic fragrant tree. Substantially dried bark of the cinnamon tree is used in general consumption and as a spice in daily life. There are about 100 kinds of cinnamons present. Countries performing the most of cinnamon production in the World are China, Indonesia and Sri Lanka. This plant was known with its special property of releasing the ureteral and kidney stones [1].

One of the phytochemicals, flavonoids are the most effective antioxidants having more than 4000 different types [2, 3]. They are capable of binding free radicals and they show high specificity on interacting with the cancer cells without damaging the normal ones. Those properties make them attractive for curing cancer diseases [4]. Catechin and catechin derivatives, gallic acid, and quercetin are the most common polyphenols in plants. They are found in fruits, vegetables, and also in spices [5, 6]. One of the most important properties of the quercetin is formation of the chelates with heavy metals [3, 7-10]. It reacts with titanium-based anions in 1:2 ratios [7]. This chelate formation characteristic is especially preferred in curing of food poisoning and an adsorption of radiation [7, 9]. In some of the researches, quercetin was found to be effective on cancer treatments and curing.

The type and the amount of the flavonoids of a specific plant are strongly affected not only from the harvest date and growing conditions but also by the process conditions, especially the extraction conditions. From its first usage in nineteenth century to today, several different types of extraction techniques have been developed, namely ultrasonic, microwave, and supercritical. These new methods some advantages over classical extractions: short extraction times, less solvent requirement, less amount of waste production etc. Almost all of the researchers focus on the type of flavonoid and the amount found in the plant material, but they do not deal with the the economics, and thus the optimization of the process. Response Surface Methodology is the most approved model for the optimization of any process. It is a combination of statistical and mathematical techniques used for analyzing several independent variables and also interactive effects among the variables on the response. This method has been used in several optimizations including adsorption [11], extraction [12], fermentation [13], and production processes [14].

In the light of the lierature cited above, the aim of the study was determined as optimization of ultrasonic extraction of total flavonoids from cinnamon for its possible use in controlled drug releasing applications in cancer treatment. In order to investigate the combinational effects of the parameters, a software program called Design-Expert used with Response Surface Methodology due to the fact that this method has an advantage of prediction of the effects of several parameters simultaneously without doing any more experiment [11-14]. Three-dimensional extraction surfaces are constructed by the software. The derivation of an industrially applicable equation representing the extraction surface of the quercetin equivalents of total flavonoids of the cinnamon via ultrasonic extraction was also realized.

2. MATERIAL and METHODS

Cinnamonum zeylaniccum was purchased from a herbalist and the analytical grade chemicals (aluminum chloride, sodium acetate, and acetic acid) were purchased from Sigma Co. The experimental design optimization was applied in two steps; firstly single-parameter optimization was used by changing the range of the selected parameter while keeping all the others at their respective value, and then multiple-parameter was achieved with three-parameter-and-three-level Box-Behnken Design by using the results of the single-parameter optimization.

In order to study the effects of temperature, solid-to-liquid ratio, and time on the extracted amount of total flavonoids, independent variables were coded according to (1) in multiple-parameter optimization studies:

$$x_i = \frac{x_i - x_0}{\Delta x} \quad (1)$$

where x_i is the dimensionless coded value of ith independent variable, x_0 is the value of x_i at the center point, and Δx is the step change value. The parameters of the study were summarized in Table 1. The center points (coded as "0") of the parameters were obtained from the single-parameter optimization results.

Table 1 Box-Behnken Design parameters used in the study

Parameters	**-1**	**0**	**+1**
x_1: Extraction temperature (^{0}C)	30	40	50
x_2: Extraction time (min)	5	15	25
x_3: Solid/liquid (g/ml)	1/10	1/30	1/50

The surface of the system is explained by the polynomial model given in (2) and higher orders:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where Y is the predicted response, x_i, x_j,...,x_k are input variables, which affect the response Y, x_i^2, x_j^2,..., x_k^2 are the square effects, β_0 is the intercept term, $x_i x_j$, $x_j x_k$ and $x_i x_k$ are the interaction effects, β_i (i = 1, 2, . . . , k) is the linear effect, β_{ii} (i = 1, 2, . . . , k) is the squared effect, β_{ij} (i = 1, 2, . . . , k) is the interaction effect, and ε is the random error [15], [16].

The Design-Expert 9.0 (Stat-Ease Inc., Minneapolis, MN, USA) software was used for regression and graphical analysis of the experimental data to fit the equations developed and evaluation of their statistical significance. BBD is frequently used under response surface method due to its suitability to fit quadratic surface that usually works well for process optimization. The optimum values of the selected variables were obtained by solving the regression equation at desired values of the process responses was fixed at the optimization criteria.

Classical extraction was realized batch-wise in a 250 mL Erlenmeyer flask with methanol as a solvent according to the Box-Behnken design conditions. Ultrasound application was set at sweep mode at all extractions. At the end of the extraction, the content of the flask was filtered through 110 mm filters (FilterLab) and filtered samples were used for quercetin equivalent of total flavonoid analysis. Quercetin was measured by colorimetric method of aluminum chloride [17]. 1ml of sample was mixed with acetic acid-sodium acetate buffer solution of pH-4 and 2 ml of $AlCl_3$ solution. The absorbance of the resultant solution was recorded at 415 nm by UV- Spectrophotometer (LANGF, DR 5000). In the analysis, methanol-buffer containing blank was used. The amount of quercetin equivalent of total flavonoids extracted were calculated with the (3) (R^2= 0.9997) derived from the absorbance values of solutions prepared by solving different amounts of pure quercetin in a 200 ml of methanol.

$$Absorbance@415nm = (0.0025).Concentration\ (mg/g) + 0.0192 \quad (3)$$

3. RESULTS and DISCUSSIONS

In the study, the results of batch experiments at the conditions of Box-Behnken design were summarized in Table 2. They were entered into the software, and statistical tests were applied in each of the suggested functions of Design-Expert program. The model having the highest regression coefficient and the lowest coefficient of variance value was chosen as the predicted best function for representation of the extraction surface. Those values for the suggested reduced cubic model of the software were 0.9998 and 1.05, respectively. The predicted R-squared was found reasonably in agreement with the R-squared value of the model. The symmetrical relationship between the experimental data (actual) and their respective calculated values from the function (predicted) approved the applicability of the function chosen (Fig. 1). As a result, the model function was chosen as reduced cubic model.

Table 2. Box-Behnken Design and experimental results.

No	x_1	x_2	x_3	Absorbance@415 nm	Quercetin eq.of total flavonoids (mg)
1	-1	-1	0	0.0420	0.7776
2	+1	-1	0	0.0752	1.5744
3	-1	+1	0	0.0955	2.0616
4	+1	+1	0	0.1061	2.3160
5	-1	0	-1	1.5518	12.3376
6	+1	0	-1	0.9745	7.7192
7	-1	0	+1	0.1006	1.6280
8	+1	0	+1	0.1228	2.0720
9	0	-1	-1	0.9251	7.3240
10	0	+1	-1	0.9402	7.4448
11	0	-1	+1	0.0613	0.8420
12	0	+1	+1	0.1248	2.1120
13	0	0	0	0.7207	5.6888
14	0	0	0	0.7096	5.6000
15	0	0	0	0.7172	5.6608

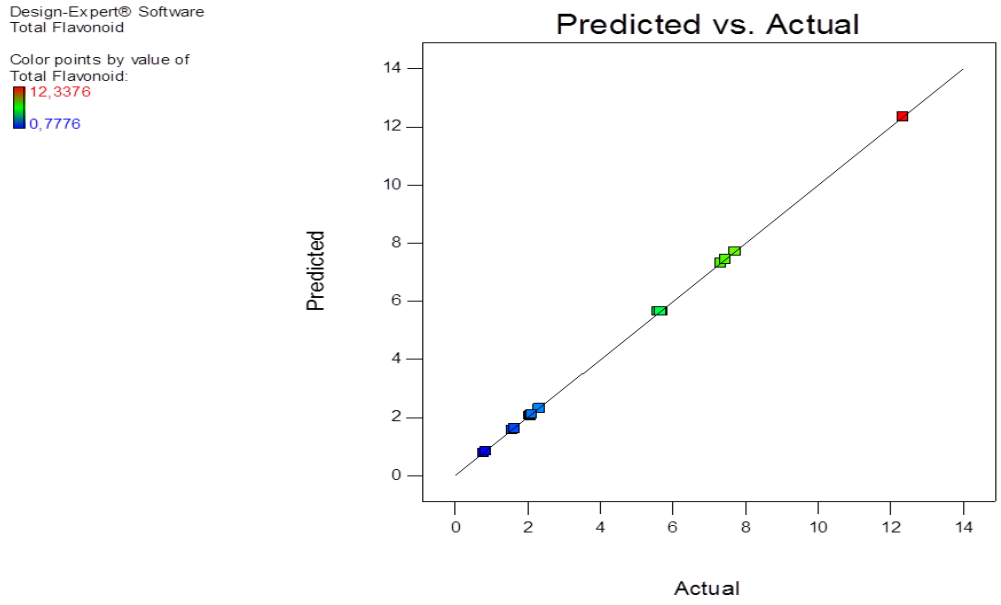

Figure 1. Statistical approval of the reduced cubic model

The Analysis of Variance (ANOVA) for the reduced cubic model was constructed by the software (Table 3). In that, A demonstrates the temperature; B and C were representing time and solid-to-liquid ratio, respectively. In the statistical analysis, the reduced cubic model was "significant", lack of fit was "insignificant", and model had nearly 0.004% pure error. The larger the magnitude of the F-value and the smaller the p-value, the more significant the effect of corresponding parameter on the yield of extraction. Thus, according to the ANOVA table, the most effective single parameter on the extraction of quercetin equivalent of total flavonoid from *Cinnamonnum zeylaniccum* were found as solid-to-liquid ratio, followed by the extraction temperature. This result was acceptable since at higher temperatures, the solubility of flavonoids increases and also as the ultrasound application time increases the solvent diffusion into the solid plant particles increases. Additionally, the results showed that temperature and the solid-to-liquid parameters were mostly interrelated parameters on this operation. The same relation was also observed in our previous research considering the microwave-assisted extraction of flavonoids from the same plant [18].

Generally, second order polynomials found as the most appropriate functions when response surface methodology was applied to the systems [18]. This observation results from the interrelation of parameters on the process response. If all of the parameters are highly interrelated with the other, then quadratic model was acceptable. However, as in this study, the interactive effects between some of the parameters (Figure 2) were well representable with the reduced cubic model then the quadratic one.

Table 3. Analysis of variance (ANOVA) for reduced cubic function model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	158,40	12	13,20	6404,51	0,0002
A-Temperature	4,36	1	4,36	2113,72	0,0005
B-Time	0,48	1	0,48	234,63	0,0042
C-Solid/liquid	34,90	1	34,90	16932,14	< 0.0001
AB	0,074	1	0,074	35,69	0,0269
AC	6,41	1	6,41	3108,65	0,0003
BC	0,33	1	0,33	160,20	0,0062
A^2	5,58	1	5,58	2708,09	0,0004
B^2	27,68	1	27,68	13430,08	< 0.0001
C^2	8,52	1	8,52	4132,64	0,0002
A^2B	0,050	1	0,050	24,44	0,0386
A^2C	2,58	1	2,58	1251,19	0,0008
AB^2	3,41	1	3,41	1656,16	0,0006
Pure Error	4,122E-003	2	2,061E-003		
Core Total	158,40	14			

Model: Significant; Lack of fit: Not significant; R-Squared: 1.0000; Predicted R-Squared: 0.9998

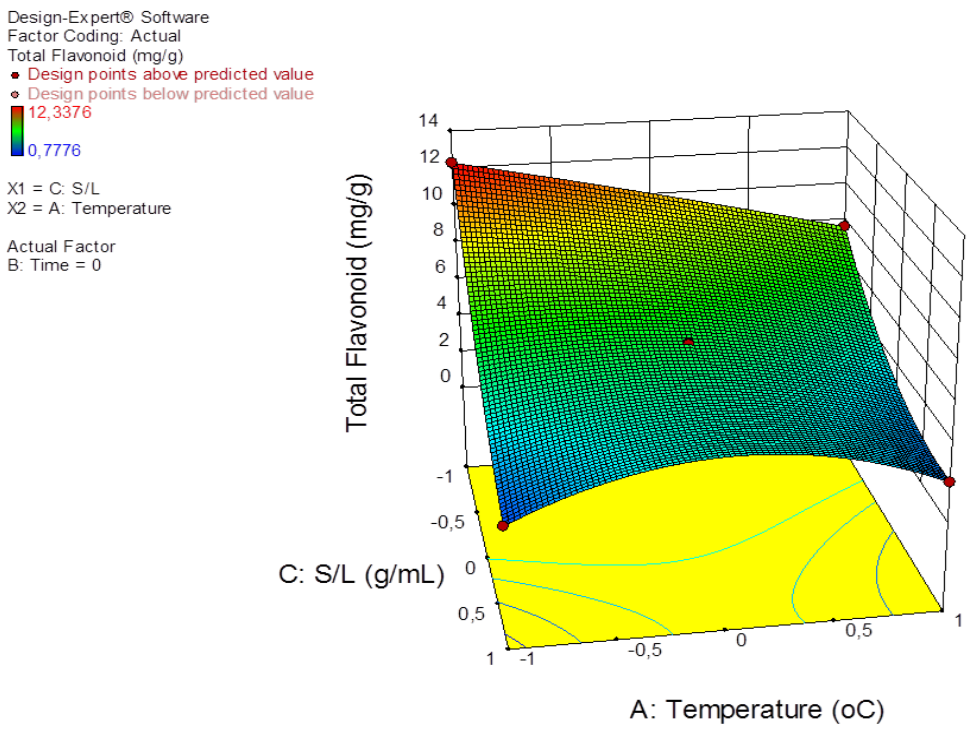

Figure 2. Three-dimensional response surface of extraction yield depending on solid-to-liquid ratio and temperature

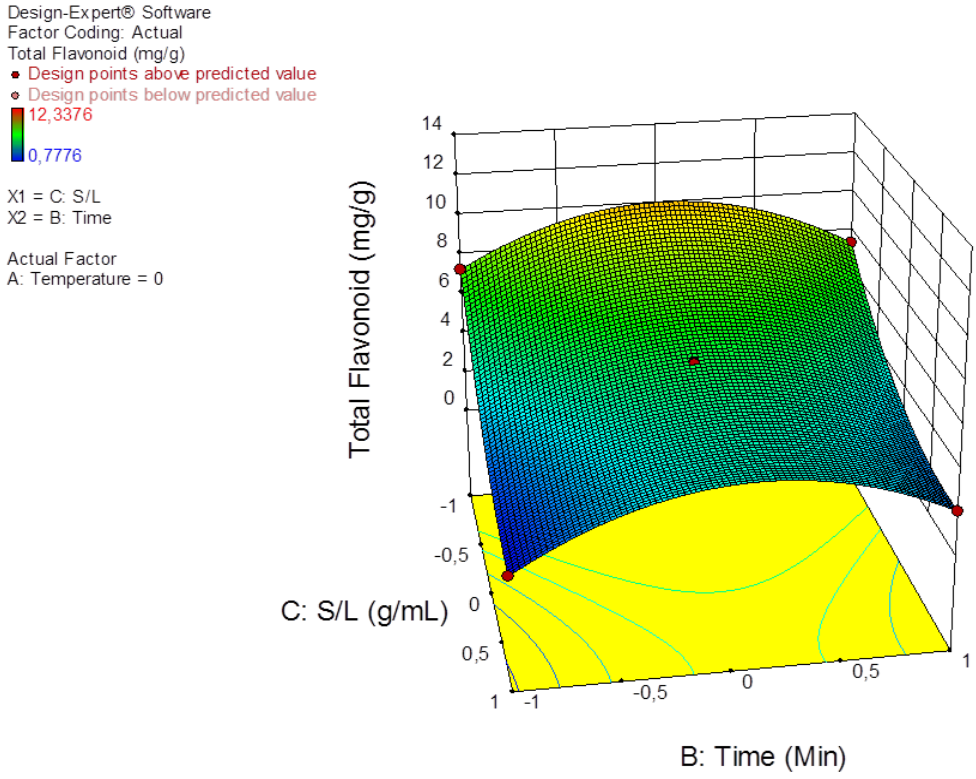

Figure 3. Three-dimensional response surface of extraction yield depending on solid-to-liquid ratio and time

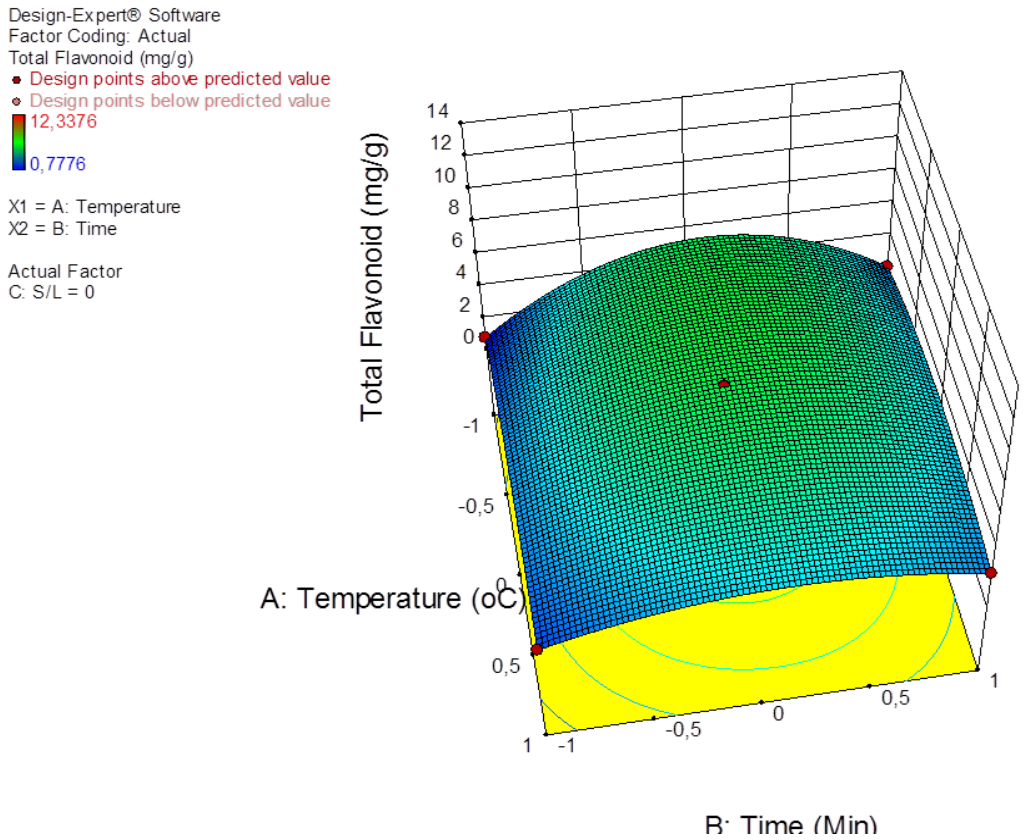

Figure 4. Three-dimensional response surface of extraction yield depending on temperature and time

At this point, three-dimensional response surfaces were constructed in this study by using software. The interactive effects of the parameters were shown in Figures 2-4. In those, red regions shows the highest amount of total flavonoids extracted, yellow and blue parts represent the lower and much lower extraction yields than those. As it can be seen from figures, solid-to-liquid ratio and the extraction temperature combinations can produce the highest yield, and neither temperature-and-time nor solid-to-liquid ratio-time combinations can reach it. As it can be concluded from ANOVA table and Figure 4, extraction temperature and time relations were so low. In order to reach the highest flavonoid amount, both the solid-to-liquid ratio and the temperature must be in the coded range of [-0.5;-1] (Figure 2).

4. CONCLUSION

In this study our aim was to optimize the ultrasonic extraction conditions of quercetin equivalent of total flavonoids from *Cinnamonnum zeylaniccum* by investigating the effect of parameters for each and also in combination. In addition, in order to use the results of the study in an industrial production processes, modeling equation was derived. According to the results of statistical analysis, reduced cubic model function was chosen as the resultant equation (Eqn. 4) for the industrial production of flavonoids from this plant:

$$Quercetin\ eq.of\ total\ flavonoids = 5.65 - (1.04)A + (0.35)B - (2.95)C - (0.14)AB + (1.27)AC + (0.29)BC - (1.23)A^2 - (2.74)B^2 + (1.52)C^2 + (0.16)A^2B - (1.14)A^2C + (1.31)AB^2 \quad (4)$$

Finally, the required extraction conditions were analyzed by using this equation and numerical analysis section of the software. In the analysis, restrictions of the parameters were selected as “in range” (has a meaning that they are in the experimental range), and the response

criterion was determined as “max”. As a result of the multi-parameter optimization, the optimum conditions producing the highest yield (12.34 mg QAE/g) were determined as 30ºC, 15 minutes, 1/10 g/mL. Also, it was determined that, the yield was not directly proportional with the solid plant material used. The highest flavonoid amount increased up to 19 mg (data is not shown). As a conclusion ultrasonic extraction was found much more effective method than the microwave-assisted extraction [18] of flavonoids from this plant.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Microwave Extraction of Gallic Acid Equivalent of Total Flavonoids from *Nigella sativa*

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Abstract: Unfortunately, cancer is the most widespread disease in worldwide. Colorectal cancer is declared as the third most common cancer type. The usage of controlled-drug-releasing capsules for treating the cancer diseases has becoming more and more popular in the whole world. In the production of drug raw material, the first operation is an extraction of the phytochemicals from the herbal plant. The yield of the extraction operation is an important step for the economical and efficient production of such capsules. Thus the aim of this study was to optimize the microwave extraction of total flavonoids from *Nigella sativa* with methanol by using Response Surface Methodology based on Box-Behnken design. Microwave extraction experiments had been carried out with the parameters of microwave power, solid/liquid ratio and the and the application time. The optimum values were determined as 0.62 kW, 1/(47) g/mL, and 10.84 second, respectively. According to the quadratic surface of the extraction yield depending on those parameters, 309.60 mg/100g of gallic acid equivalent of total flavonoids can be obtained at the optimum conditions. As a result of the statistical analysis, this function was found as capable of expressing the effect of the parameters on the amount of total flavonoid production in industry. Nowadays, drying of the extracts and loading of the aqueous solutions of them into the prebiotic-coated probiotic capsules have been under consideration.

Keywords: *Nigella sativa*, Extraction, Flavonoid, Response surface methodology

1. INTRODUCTION

In the past several years, polyphenols received considerable attention because of their contribution to the human health. Flavonoids are type of antioxidants and are member of polyphenols. They are also called as phenolic hydrogens because their hydrogen supplies electron to the free radicals resulting them to neutralize [1]. These bioactive compounds are capable of scavenging free radicals, avoiding lipid oxidation and reducing the formation and progression of certain types of cancers including breast, colon and prostate [2, 3]. Vegetables, fruits, herbs and cereals are main sources of polyphenols [4-6]. The essential oil of *Nigella sativa* was investigated and especially thymoquinone, carvacrol, t-anethole and 4-terpineol components were determined as capable of radical scavenging activity [7]. Due to this antioxidant property, the herb is supposed to be used in cancer treatments since the antioxidant molecules inside have ability to bind the free radicals damaging the cell. Antioxidants also prevent DNA damaging due to lipid peroxidation resulting from the oxygen production of free

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radicals [8]. The crude extracts of the seeds of *Nigella sativa* have been reported as pharmacologically active; they protect against nephrotoxicity and hepatoxicity induced by either disease or chemicals, they also have antiinflammatory, analgesic, antipyretic, antimicrobial activity [9].

Solid-liquid extraction is a very important stage for later use of those bioactive compounds. The type and the amount of the flavonoids obtained from the herbs are strongly affected by the extraction. From the development of soxhlet extraction by F. Soxhlet in the nineteenth century, several different types of extraction techniques have been developed including ultrasonic, microwave, and supercritical [10-13]. Microwave extraction requires small amount of solvent and less extraction time, but the local temperature increases quickly due to the cavity effect. Thus, the optimization of the extraction conditions is inevitable.

Mathematical modeling of the extraction is a useful engineering tool which facilitates the understanding, optimization, design and control of the processes with minimal time and energy consumption. Todays the most approved model for the optimization, namely response surface method is a combination of statistical and mathematical techniques used for analyzing several independent variables and also interactive effects among the variables on the response. This method has been used in several optimizations including adsorption [14], extraction [15], fermentation [16], and production processes [17]. In addition, the final equation found by this method can be easily adaptable to any situation faced in the industrial scale production.

In the light of the literature cited above, the aim of the study was determined as optimization of microwave extraction of total flavonoids from *Nigella sativa* for its possible use in controlled drug releasing applications in cancer treatment. In order to investigate the combinational effects of the parameters, a software program called Design-Expert used with Response Surface Methodology due to the fact that this method has an advantage of prediction of the effects of several parameters simultaneously without doing any more experiment [11-14]. Three-dimensional extraction surfaces are constructed by the software. The derivation of an industrially applicable equation representing the extraction surface of the quercetin equivalents of total flavonoids of the herb via microwave extraction was also realized.

2. MATERIAL and METHODS

Nigella sativa was purchased from a herbalist and the analytical grade chemicals (aluminum chloride, sodium acetate, and acetic acid) were purchased from Sigma Co. The experimental design optimization was applied in two steps; firstly single-parameter optimization was used by changing the range of the selected parameter while keeping all the others at their respective value, and then multiple-parameter was achieved with three-parameter-and-three-level Box-Behnken Design by using the results of the single-parameter optimization.

In order to study the effects of temperature, solid-to-liquid ratio, and time on the extracted amount of total flavonoids, independent variables were coded according to (1) in multiple-parameter optimization studies:

$$x_i = \frac{x_i - x_0}{\Delta x} \tag{1}$$

where x_i is the dimensionless coded value of *i*th independent variable, x_0 is the value of x_i at the center point, and Δx is the step change value. The parameters of the study were summarized in Table 1. The center points (coded as "0") of the parameters were obtained from the single-parameter optimization results. The surface of the system is explained by the polynomial model given in (2) and higher orders:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \varepsilon \tag{2}$$

where Y is the predicted response, x_i, x_j,...,x_k are input variables, which affect the response Y, x_i^2, x_j^2,..., x_k^2 are the square effects, β_0 is the intercept term, x_ix_j, x_jx_k and x_ix_k are the interaction effects, β_i (i = 1, 2, . . . , k) is the linear effect, β_{ii} (i = 1, 2, . . . , k) is the squared effect, β_{ij} (i = 1, 2, . . . , k) is the interaction effect, and ε is the random error [18, 19].

Table 1. Box-Behnken Design parameters used in the study

Parameters	-1	0	+1
x_1: Extraction time (min)	10	25	40
x_2: Solid/liquid (g/ml)	1/30	1/45	1/60
x_3: Microwave power (kW)	0.3	0.6	0.9

The Design-Expert 9.0 (Stat-Ease Inc., Minneapolis, MN, USA) software was used for regression and graphical analysis of the experimental data to fit the equations developed and evaluation of their statistical significance. Box Behnken Design (BBD) is frequently used with response surface method due to its suitability to fit quadratic surface that usually works well for process optimization. The optimum values of the selected variables were obtained by solving the regression equation at desired values of the process responses was fixed at the optimization criteria.

Methanolic extraction was realized batch-wise in a 250 mL Erlenmeyer flask. Extraction time, solid-to-liquid ratio, and microwave power were chosen as the parameters of single- and multiple-parameter experimental designs. At the end of the specified extraction conditions the content of the flask was filtered through 110 mm filters (FilterLab) and filtered samples were used for total flavonoid analysis.

The concentrations of the total polyphenols in the extracts filtered were determined using the Folin-Ciocalteu method. In this paper, the main phenolics in *Nigella sative* were assumed as flavonoids, since they have the same effect on cancer disease treatments. In the analysis 0.4 ml of the extract was mixed with 5.1 ml of distilled water and 0.5 ml of Folin ciocalteu reagent. 1.5 ml of sodium carbonate solution (20% by weight) was added into the medium immediately and after mixing they kept in dark during two hours at room temperature. The color resulted from the colorimetric reaction between gallic acid in the sample and the Folin reagent was analyzed by UV-vis spectrophotometer (Perkin-Elmer) at 765 nm. The gallic acid equivalents (GAE) of total phenolics were calculated from the calibration curve (Absorbance = 0.01532 x Concentration (μg/ml); R^2=0.9989) and the results were expressed as mg GAE/g dry herb.

3. RESULTS

In the study, the parameters summarized in Table 1 was was used to construct the three-level, three-parameter Box-Behnken design and fifteen experimental conditions were obtained. Microwave extractions were realized at each of the coded conditions and the coded parameters and their respective yields (as it was summarized in Table 2) were entered into the software. The Design-Expert software statistically analyzes all the experimental conditions and yields together and provides a comparison of the possible functions for the user. The user selects the most appropriate function expressing the response surface by comparing the statistical data and graphs of the functions contained in the program. The model having the highest regression coefficient and the lowest coefficient of variance value was chosen as the predicted best function for representation of the extraction surface. Those values for the suggested quadratic model of software were 0.9949 and 7.27, respectively. The predicted R-squared was found reasonably in agreement with the adjusted R-squared value of 0.9858. Thus, the fitness in between the experimental data (actual) and their respective calculated values of the function

(predicted) (Fig. 1) was approved the statistical conclusions. As a result, the model function was chosen as quadratic.

Table 2. Box-Behnken Design and experimental results

No	x_1	x_2	x_3	Abs	GAE of total flavonoids (mg)
1	-1	-1	0	0.3164	61.9376
2	+1	-1	0	0.3845	75.2862
3	-1	+1	0	0.4823	188.8524
4	+1	+1	0	0.2160	84.5783
5	-1	0	-1	0.1713	50.3167
6	+1	0	-1	0.3168	93.0455
7	-1	0	+1	0.7342	215.6593
8	+1	0	+1	0.3041	89.3155
9	0	-1	-1	0.3100	60.6990
10	0	+1	-1	0.1521	59.5513
11	0	-1	+1	0.4195	82.1229
12	0	+1	+1	0.6135	240.2501
13	0	0	0	0.2642	77.6044
14	0	0	0	0.2288	67.1861
15	0	0	0	0.1644	45.3199

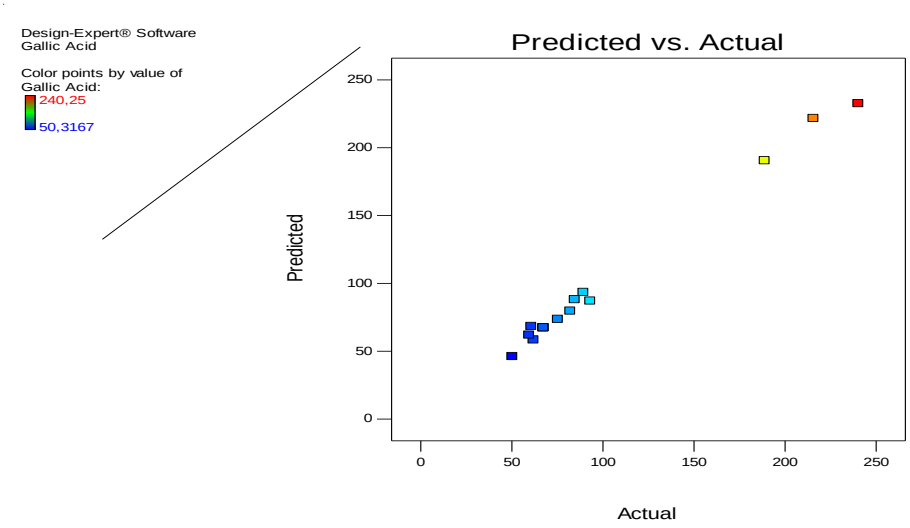

Figure 1. Statistical approval of the reduced cubic model.

The Analysis of Variance (ANOVA) for the quadratic model was constructed by the software (Table 3). In that, A demonstrates the time; B and C were representing solid-to-liquid ratio and microwave power, respectively. Since the larger the magnitude of the F-value and the smaller the p-value, the more significant the corresponding coefficient. The fact that the selected model is "significant" and "lack of fit" is "not significant" (Table 3) is the main reason for choosing the best expressing function as "quadratic". The most effective single parameters on the extraction of gallic acid equivalent of total flavonoids from *Nigella sativa* were found as microwave power followed by solid-to-liquid ratio. Although the extraction time seemed to be the least effective single parameter, the interrelation between time and other parameters selected were found considerable.

Table 3. Analysis of variance (ANOVA) for quadratic function

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	52297,92	9	5810,88	109,29	< 0.0001
A-Time	3808,05	1	3808,05	71,62	0,0004
B-S/L	10744,78	1	10744,78	202,09	< 0.0001
C-M.Power	16537,92	1	16537,92	311,05	< 0.0001
AB	3458,77	1	3458,77	65,05	0,0005
AC	7146,39	1	7146,39	134,41	< 0.0001
BC	6342,12	1	6342,12	119,28	0,0001
A2	1244,77	1	1244,77	23,41	0,0047
B2	1058,62	1	1058,62	19,91	0,0066
C2	2564,27	1	2564,27	48,23	0,0010
Residual	265,84	5	53,17		
Lack of Fit	265,75	3	88,58	1941,08	0,0005
Pure Error	0,091	2	0,046		
Cor Total	52563,76	14			

Model: Significant; Lack of fit: Not significant; R-Squared: 0.9949; Adjusted R-Squared: 0.9858; Predicted R-Squared: 0.9191

At this point, three-dimensional response surfaces were constructed in this study by using software. The interactive effects of the parameters were shown in Figures 2-4. In those, red regions shows the highest amount of total flavonoids extracted, yellow and blue parts represent the lower and much lower extraction yields than those. As it can be seen from figures, solid-to-liquid ratio and microwave combinations can produce the highest yield, and neither microwave power-and-time nor solid-to-liquid ratio-time combinations can reach it. In order to reach the highest flavonoid amount, both the solid-to-liquid ratio and the temperature must be in the coded range of [0.5;1] (Figure 3).

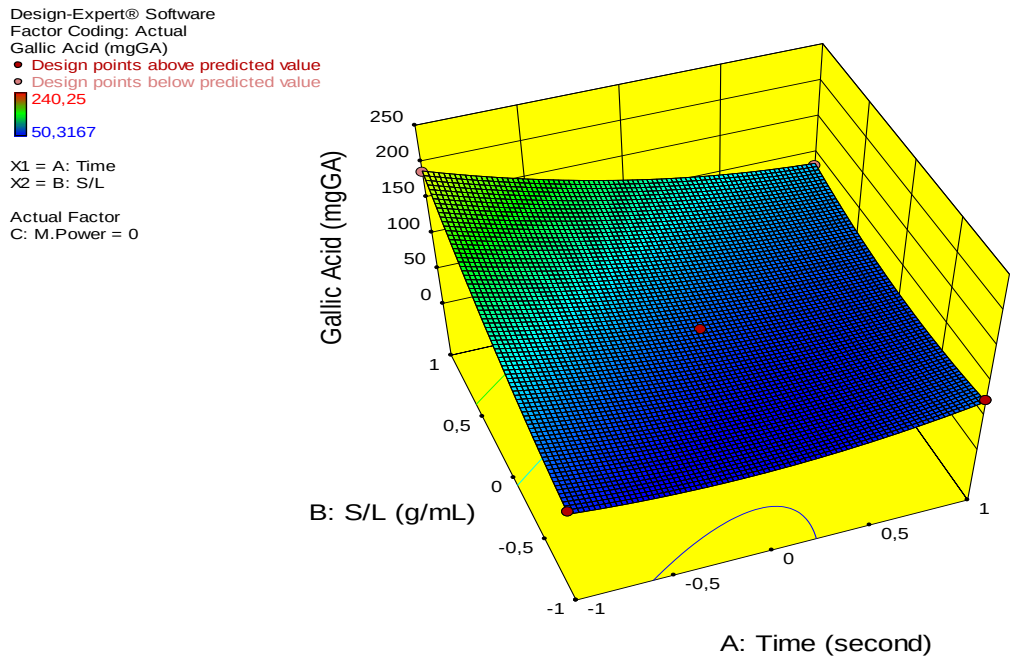

Figure 2. Three-dimensional response surface of extraction yield depending on solid-to-liquid ratio and extraction time.

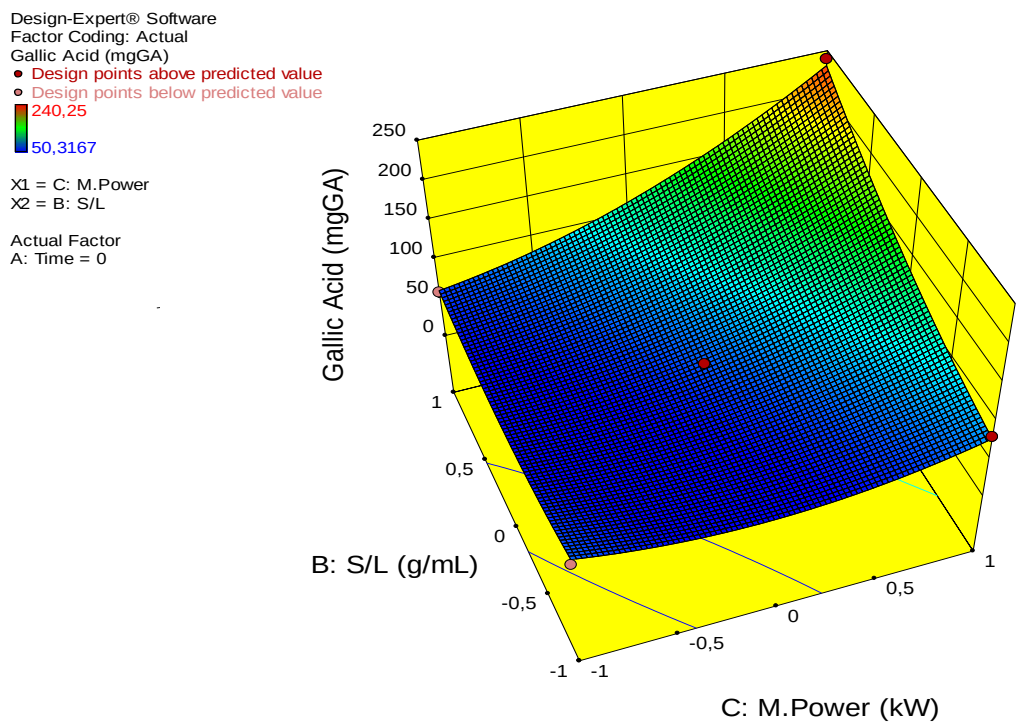

Figure 3. Three-dimensional response surface of extraction yield depending on solid-to-liquid ratio and microwave power.

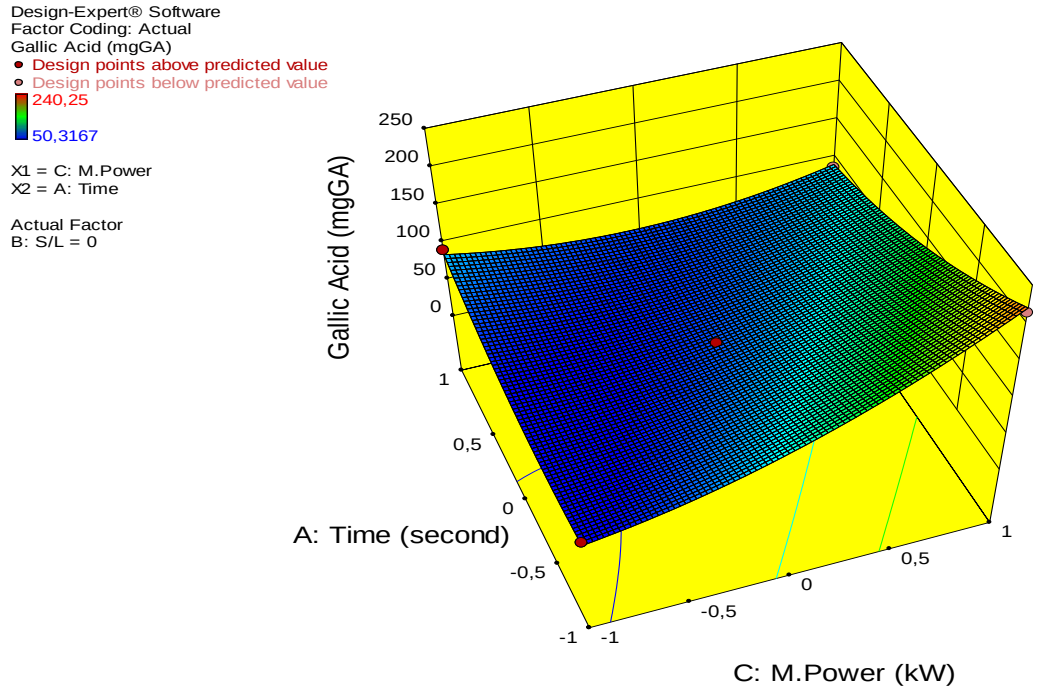

Figure 4. Three-dimensional response surface of extraction yield depending on extraction time and microwave power.

Here we demonstrated that *O. basilicum* effect differently on BM- and DP- mesenchymal stem cells. It is important to determine the differentiation, and proliferation of cells with promising inductive agents. Studies should avoid to suggest any plant extract as an agent without evidence of safe.

4. DISCUSSION and CONCLUSION

In this study our aim was to optimize the microwave extraction conditions of gallic acid equivalent of total flavonoids from *Nigella sativa* by investigating the effect of parameters for each (microwave power, extraction time and solid-to-liquid ratio) and also in combination. In addition, in order to use the results of the study in an industrial production processes, modeling equation was derived. Quadratic function (Eqn. 4) was found as the best function representing the extraction surface and all the statistical analysis approved this conclusion:

$$Gallic\ acid\ equivalent\ of\ total\ flavonoids = 67.37 - (21.82)A + (36.65)B + (45.47)C - (29.41)AB - (42.27)AC + (39.82)BC + (18.36)A^2 + (16.93)B^2 + (26.35)C^2 \quad (4)$$

The most and the least effective parameters of the extraction were determined as microwave power, and the application time, respectively. Finally, the required extraction conditions were analyzed by using this equation and numerical analysis section of the software. In the analysis, restrictions of the parameters were selected as "in range" (has a meaning that they are in the experimental range), and the response criterion was determined as "max". As a result of the multi-parameter optimization, the optimum conditions producing the highest yield (309.60 mg/100g of gallic acid equaivalent of total flavonoids) were determined as 0.62 kW, 10.84 seconds, 1/47 g/mL. Nowadays, drying of the extracts and loading of the aqueous solutions of them into the prebiotic-coated probiotic capsules have been under consideration.

Conflict of Interests

Authors declare that there is no conflict of interests.

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The Effects of Various Types of Press Wheels Mounted on Pneumatic Precise Drilling Machine on the Quality Criteria of Black Carrot

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Abstract: Black carrots are consumed as fresh vegetables in Turkey and are preferred in the form of fermented beverages in large quantities. Black carrots are cultivated in different regions of Turkey. The black carrot is cultivated heavily in the Eregli region. In this region, the highest yields for black carrots are obtained by planting on the ridge at narrow interval range with triplet drilling. In this study, the effects of front and rear stainless steel press wheel (BT_1), front and rear rubber press wheel (BT_2) and front and rear rubber press wheel and triple narrow intermediate rubber wheels (BT_3) on black carrot quality criteria in field conditions were investigated. According to results, yield, single carrot mass, diameter and length varied between, 15.11 and 41.61 t ha^{-1}, 76.96 and 226.43 g, 33.61 and 53.14 mm, and 193.65 and 237.33 mm respectively. It was found that total phenolic varied between 349.80 and 745.37 mg gallic acid equivalent $(100\ ml)^{-1}$ and the antioxidant activity value, determined by DPPH, varied between 26.71% and 54.80%.

Keywords: Press wheel, plant distribution on the row, yield, black carrot, quality criteria

1. INTRODUCTION

The carrot (*Daucus carota*) is a perennial plant from the *Umbelliferae* family. In Turkey, carrots are produced to a large extent in the Central Anatolia and Konya regions. Black carrots are an important agricultural product and source of income for Turkey, specifically in the Eregli District. It is estimated 40 000 to 50 000 decares of production are made in the area and according to the production area by years, approximately 100 000 to 150 000 tons of products have been manufactured. Although the fresh consumption of black carrots in our country is low, it is nevertheless preferred as a fermented beverage in high quantities.

Considering the production history of black carrots in the region until 2008, exports of black carrots were primarily as a raw material, and since then exports as a concentrate (i.e. as a fermented drink) have gained greater momentum. In production, approximately 80% is concentrated, while 20% is used in the turnip industry. The turnip juice obtained from the black carrot is a fermented product with many appealing properties [1]. One of the most important

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properties of turnip juice is the lactic acid content. Aside from the sour taste encountered in turnip juice, lactic acid is known also to be a digestive facilitator, refresher, pH regulator of the digestive system, and provides other more beneficial properties to the body in order to utilize more minerals [2].

Black carrots are a potential source of anthocyanin pigment with high antioxidant activity. Its composition is characterized by its high anthocyanin content (1750 mg kg^{-1}) and special quality parameters [3]. Anthocyanins are best known as natural food dyes that provide the bright red color in some foods such as blueberries and pomegranates, and moreover are considered an important alternative to synthetic dyes in the dyeing of many foods [4 and 5]. Fruit colored with black carrot juice provides significant benefits against chronic diseases due to the high anthocyanin content. In vascular diseases for instance, particularly arterial thickening, cancer, diabetes and disorders of nerve degeneration and in certain eye diseases, it has been shown to be especially therapeutic [6 and 7]. The pigment of the anthocyanin in black carrots is acylated with p-coumaric, ferulic, p-hydroxybenzoic acid and sinapic acids and thus more resistance to hydration, light and food pH. The black carrot extract gives an excellent strawberry red brilliance at acidic pH values. Fruit juice is used for coloring, softness, protection, glazing, and in pastries; as such, an e-number is not required in food labels because it is a natural additive [8]. Black carrot concentrate is also used as natural fabric dye. Synthetic fabric dyes is expected to be prohibited in the coming years and thus the use of natural dyes such as that from black carrots will be more important.

In recent years, narrow row spacing vacuum pneumatic precision sowing machines have begun to be used in the region. The pressure wheels used in these machines are flat sheet or flat tire. These machines also have pressure wheels with triple narrow tires in the middle. By planting two or three rows of carrots instead of a single row of seeds, agricultural products with high quality market value can be obtained. It has been reported that the seed quality can increase due to the uniformity of the habitat area within the narrow row spacing [9]. For this reason, it was aimed to determine the effects of press wheels on plant distribution uniformity, yield, and carrot quality in black carrot cultivation with a pneumatic sensitive vegetable sowing machine.

2. MATERIAL and METHODS

The research was carried out in the village of Kuzukuyusu, located in the Eregli District of Turkey, and according to the trial design of the divided parcels, three repetitions were conducted. In the experiments, a local ancient carrot genotype known with its common name "Eregli variety" (*Daucus carota* L.) was used. The seeds were uncoated, with a thousand seed weight of about 1.65 g and 88% germination rate as determined in the laboratory.

The sowing was done in three rows in the back and 7.5 cm between rows (Figure 1). In the experiments, the seeds were made at the same planting depth in all applications. The schematic view of the pneumatic precision vegetable seeding machine is given in Figure 2. The feed rate of the sowing machine was chosen to be 0.75 m s^{-1}.

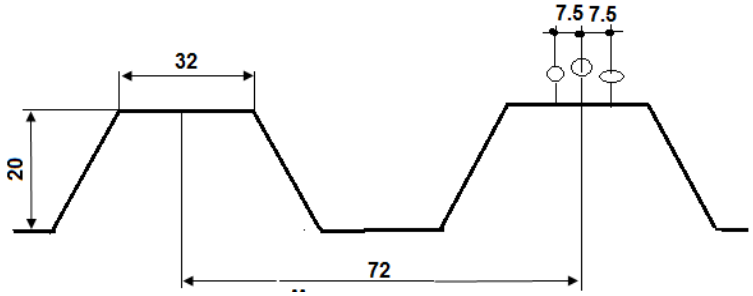

Figure 1. Measurements of the back ridge (Measurements cm)

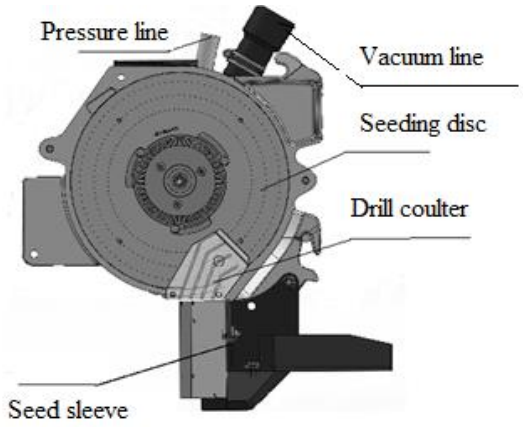

Figure 2. Schematic view of the sowing unit

The research was conducted on three different rows with varying sowing distances (Z_1=2.38, Z_2=4.65 and Z_3=6.78 cm) and on three different rollers. These pressure wheels consisted of a front and rear pressure tire (BT_1), a front and rear pressure sheet (BT_2), and a front and rear pressure tire with a triple narrow tire (BT_3) in the middle (i.e. Figures 3, 4 and 5, respectively). Each parcel is arranged to have a length of 50 m and a width of 2.8 m.

Material	: Tire
Diameter	: 250 mm
Width	: 200 mm
Mass	: 72.9 N

Figure 3. Front and rear pressure tire (BT_1)

Material	: Sheet
Diameter	: 217 mm
Width	: 200 mm
Mass	: 59.7 N

Figure 4. Front and rear pressure sheet (BT_2)

Narrow wheel	
Material	: Tire
Diameter	: 180 mm
Width	: 30 mm
Mass	:10.6 N
Number	: 3 pieces

Figure 5. Front and rear pressurized tire and pressure spring adjustable medium triple narrow tire (BT_3)

The clay content in the experimental field was determined to be 22.90%, with a silt content of 7.50%, and a sand content 69.90%. The soil texture class was categorized as Sandy-clay-loamy. Soils had a low organic matter content (1.45%) and aggregate stability value (5.90%). The soil was considered to be a very high calcareous soil, with lime content as 37.49% $CaCO_3$ and a pH value of 8.27, denoting a middle grade alkaline class.

Sowing was carried out from the end of April to mid-November 2015. Meteorological data from April to November covering these vegetation periods are given in Table 1. The seed bed was prepared in mid-February and plowed at the end of March 2015. Using a centrifugal distributor, a DAP fertilizer rate of 40 kg da^{-1} was distributed in late April 2015. The seed bed

was prepared by using horizontal rototill in late April and sowing ridges were formed with a ridge making machine (Table 2). The harvesting was carried out in mid-November 2015 and the experimental parcels were irrigation was supplied 23 times to, with 1 904 mm water given in total.

Table 1. Monthly meteorological data from the black carrot growing season [10].

Months (2015)	Average Monthly Maximum Temperature (^{0}C)	Average Monthly Minimum Temperature (^{0}C)	Monthly Average Temperature (^{0}C)	Monthly Average Rainfall (mm)
April	16.0	3.1	11.3	32.4
May	24.0	9.3	17.8	45.0
June	26.3	12.6	19.9	73.8
July	31.8	15.0	26.6	0.2
August	32.6	17.0	27.3	9.6
September	31.4	13.6	24.3	0.0
October	21.9	9.4	16.7	49.2
November	15.6	1.0	8.6	0.0

Table 2. Agricultural operations applied to the experimental plots in 2015 production

Dates	**Agricultural practices**
16.02. 2015	Seed bed preparation
30.03.2015	Plowing
23.04.2015	Centrifugal fertilizer distribution (DAP with 40 kg da^{-1} fertilizer norm)
25.04.2015	Preparation of seed bed with horizontal rototil
26.04.2015	Sowing preparation of ridges
26.04.2015	Sowing operation
21.05.2015	Inter-row hoeing
28.05.2015	Weed treatment (Linourin 150 g da^{-1})
04.06.2015	Sprinkler irrigation with 15 kg da^{-1} fertilizer UREA
17.06.2015	Fertile intermediate diameter (15 kg da^{-1} fertilizer UREA)
24.06.2015	Addition of humic acid with sprinkler irrigation 2 kg da^{-1}
29.06.2015	Removal of weeds
05.07.2015	Ammonium nitrate fertilizer application at 10 kg da^{-1} with sprinkler irrigation
14.07.2015	Ammonium nitrate fertilizer application at 10 kg da^{-1} with sprinkler irrigation
22.07.2015	Ammonium nitrate fertilizer application at 10 kg da^{-1} with sprinkler irrigation
25.07.2015	Inter-row hoeing
08.08.2015	Remove bolting plants
16.09.2015	Remove bolting plants
18.11.2015	Harvesting

Spacing measurements on row were performed on a 10 m length of row on randomly selected three rows of each plot 30 days after the seeding date and plant spacing was measured with a steel rule. Variation coefficient for on-row plant distribution was calculated by using the following equation.

$$VC=\sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}.\frac{100}{\bar{x}}$$

x : Average planting spacing on-row (cm)
$\bar{x}$: Measured planting spacing on-row (cm)
n : Number of measured planting spacing on-row
VC : Variation coefficient (%)

In yield measurements, five carrot samples of approximately 1.4 m in length were removed, cleaned and weighed from each plot. Black carrots were evaluated according to the extra class, based on the carrot boiling standard specified in TS 1193. Larger diameters of extruded carrots should be between 25 and 45 mm in diameter and masses between 50 and 200 g [11]. The method recommended by [12] for the determination of total phenolic materials of the samples taken from the field was used. This method is based on the fact that the phenolic compounds form a blue complex by reducing the phosphololybdic-phosphotungstic solution of the Folin-Ciocalteu solution and that this blue color is measured colorimetrically [13]. The results are given as Gallic acid equivalent (GAE). Antioxidant activity was determined by the DPPH (1,1-diphenyl-2-picrylhydrazyl) method [14].

It was analyzed by the Shapiro-Wilk test to determine whether the data showed a normal distribution. Variance analysis was performed using the MINITAB 16 program. LSD analysis was performed on the properties with variance at 1% and at least 5% significance level among the applications. These analyzes and calculations were made in the MSTAT-C package software.

3. RESULTS

Plant distribution uniformity and yield values for the 2015 black carrot growing season are given in Table 3. As it is shown in the table, under field conditions, the average rate of increase of plant per distance over the designated row was obtained. The lowest average was determined for the BT_2 press at 2.86 while the highest at 3.42 was obtained in the BT_1 press wheel. When the coefficient of variation was evaluated, the average coefficient of variation obtained at three distances was BT_1 with 97.85%, BT_2 with 87.07% and BT_2 with 86.88%. Yield values changed between 15.11 t ha^{-1} and 46.82 t ha^{-1}. The maximum yield value was shown to be with the BT_2 pressure wheel with an average of 30.70 t ha^{-1}, followed by the BT_3 pressure wheel (i.e. 30.62 t ha^{-1}) and the BT_1 pressure wheel with 30.07 t ha^{-1}. A variance analysis on yield values, indicated there was no statistically significant relationship between pressure wheels (F = 0.30). When the distance (F = 11.82) was examined, the yield value of 36.33_a t ha^{-1} was obtained at the Z_1 planting distance, 30.14_{ab} t ha^{-1} at the planting distance Z_2 and 22.91_c t ha^{-1} at the planting distance Z_3. Differences between the positions above and below were statistically significant at 1% level (LSD = 7.985), but no difference can be drawn between the Z_1 and Z_2 planting distances.

However, a significant interaction was found between pressure wheel and row distance (F=3.78) and the highest yield values were obtained at the Z_1 sowing of the pressure wheels. The average yield of agricultural enterprises in the region varies between 2 and 5 t da^{-1}. When an evaluation is made in this respect, it can be said that the regional average yields are achieved outside the Z_3 planting distance.

The mass, diameter and height values obtained after measurement of the black carrots taken from the parcels are given in Table 4. As a result of the analysis of variance applied to the mass, diameter and height values of black carrots, the pressure wheel was not found statistically significant, but the distance between row spacing and interaction pressure wheel by the distance between the row spacing was statistically significant. When the mass values of black carrots were examined, results showed there was a change between 76.96 g and 226.43 g. When the above distances were examined, it was found that the highest mass value was obtained at a distance of Z_3 with 178.97 g (Table 5). However, when the interaction was examined, the highest mass value (226.43 g) was obtained in the parameter of BT_3Z_3.

Table 3. Plant distribution smoothness and yield values on row in black carrot cultivation

Pressure Wheel	Row over theoretical distance (cm)	Average Row Spacing (cm)	The coefficient of variation (%)	Yield (t ha^{-1})
	2.38	11.44	113.25	41.61_{a}
BT_1	4.65	14.35	102.03	33.48_{abc}
	6.78	16.05	78.28	15.11_{d}
	2.38	8.67	84.36	31.95_{abc}
BT_2	4.65	12.49	91.25	30.61_{bc}
	6.78	15.24	85.59	29.56_{bc}
	2.38	7.58	86.40	35.44_{ab}
BT_3	4.65	13.56	91.08	26.34_{bc}
	6.78	21.05	83.16	24.07_{cd}
				LSD ($p<0.05$)=10.050

Table 4. Mass, diameter and height values of black carrots and applied LSD test

Pressure Wheel	Distance over the row	Mass (g)	Diameter (mm)	Height (mm)
	Z_1	76.96_{d}	33.61_{c}	208.63_{bcd}
BT_1	Z_2	142.59_{b}	41.05_{b}	209.25_{bcd}
	Z_3	152.60_{b}	43.40_{b}	213.82_{bc}
	Z_1	83.39_{d}	35.30_{c}	193.65_{d}
BT_2	Z_2	134.98_{bc}	42.79_{b}	216.63_{bc}
	Z_3	157.88_{b}	44.63_{b}	224.45_{ab}
	Z_1	81.11_{d}	33.74_{c}	201.85_{cd}
BT_3	Z_2	99.47_{cd}	41.33_{b}	216.03_{bc}
	Z_3	226.43_{a}	53.14_{a}	237.33_{a}
		LSD ($p<0.01$)=1.851	LSD ($p<0.05$)=5.598	LSD ($p<0.05$)=10.030

Table 5. LSD test applied to the mass, diameter and height values obtained from averages over distance

Distance over Row	Average Mass (g)	Average Diameter (mm)	Average Height (mm)
Z_1	80.49_{c}	34.21_{c}	201.38_{c}
Z_2	125.68_{b}	41.72_{b}	213.97_{b}
Z_3	178.97_{a}	47.06_{b}	225.20_{a}
	LSD ($p<0.01$)=1.068	LSD ($p<0.01$)=4.428	LSD ($p<0.01$)=10.03

When the diameter measurement values of black carrots were examined, it showed there was a change between 33.61 mm and 53.14 mm. When the sowing distances were examined, results showed that the highest mass value was obtained at Z_3 (47.06 mm) and at Z_2 planting distance (41.72 mm) and there was no statistical difference between them. When the spacing interaction with the pressure wheel was examined, the highest diameter value was found for BT_3Z_3 (53.14 mm).

The height values of black carrots varied between 193.65 mm and 237.33 mm. Results showed that at the planting distance Z_3 the highest height average of 225.20 mm was obtained, however when the distance between the pressure wheel and the row is measured, results showed that BT_2Z_3 (224.45 mm) and BT_3Z_3 (237.33 mm) had the highest values. In a study about yellow carrots it was reported that the maximum length value for Nantura F1 was 18.00 cm and 17.74

cm for Bertan F1 in mid-October of 1999. Furthermore, for the Asubeni F1 variety the highest mass average was 117.7 g and the lowest length value was 13.72 [15]. In this respect, our values correspond with those of the literature.

Carrots with large diameters entering the extra classifications, and classified according to the carrot boiling standard determined in TS 1193 (i.e. one of the quality criteria of black carrot), must have a diameter ranging between 25-45 mm and a mass value between 50-200 g. Hence, large diameter and mass interval values for our black carrot samples were set according to these standards, and the percentage ratios of the small and large ones from the limit value are given in Table 6.

Table 6. Distribution of diameter and mass values

Pressure Wheel	Row Spacing	Carrot diameter distribution (%)			Carrot weight distribution (%)		
		<24.99	25- 45	>45.01	<49.99	50- 200	>200.01
BT_1	Z_1	24.42	61.30	14.29	37.28	59.25	3.47
	Z_2	6.67	45.48	47.85	16.23	56.68	27.09
	Z_3	0.00	24.81	75.19	1.75	42.60	55.64
BT_2	Z_1	26.16	56.46	17.38	39.14	52.15	8.52
	Z_2	7.88	47.02	45.10	15.93	54.36	29.71
	Z_3	14.94	45.54	39.52	20.98	56.70	22.32
BT_3	Z_1	13.89	70.54	15.58	36.37	59.17	4.46
	Z_2	5.50	52.97	41.53	13.33	65.75	20.92
	Z_3	7.65	51.54	41.82	20.66	56.04	23.30

When the distribution of carrot diameters was examined in terms of pressure wheels, it was determined that the distribution between 25-45 mm diameters varied between 24.81% and 70.54% and the distribution of diameters <24.99 mm varied between 0 and 26.16%. The distribution of carrot diameters > 45.01 changed between 14.29% and 75.19%. Variance analysis for the diameter range 25-45 mm showed a statistical significance for the parameters of pressure wheel (F = 6.75) and row distance (F = 16.00). When the pressure-only averages were examined, it is clear that in the BT_2 and BT_3 press wheel, when considering the distances between the rows, the maximum values of the distances between 25-45 mm were obtained at the Z_1 sowing distance (Table 7).

Table 7. LSD test applied to average of 25-45 mm diameter ranges

Pressure Wheel Average	Average Row Spacing
BT_1 43.86_b	Z_1 62.76_a
BT_2 49.67_{ab}	Z_2 48.49_b
BT_3 58.34_a	Z_3 40.63_b
LSD ($p<0.01$)=11.42	LSD ($p<0.01$)=11.42

Results of measurements of mass values in the study, found the change of rates of carrot fractions with masses of 50-200 g were between 52.84% and 60.32%. It was determined that the mass of carrots lighter than 49.99 g is between 18.42% and 25.35% and the mass of carrots weightier than 200 g is between 16.22% and 28.73%. Analysis of variance applied to carrot fractions with masses between 50 and 200 g did not reveal a statistically significant relationship between pressure wheel (F = 1.30), row distance (F = 1.13) and pressure wheel and row spacing interactions (F = 0.96).

It can be said that the carrot diameter and mass values were generally evaluated, but the distance of the row had a significant impact. It is evident that the proportion of carrots with diameters <24.99 mm and between 25-45 mm in diameter decreases while the ratio of those

with diameters >45.01mm increases. In general, it can be said that similar relations are also seen in the diameter range values of 25-45 mm.

The moisture, brix, total phenolic material and antioxidant activity values of the harvested black carrots are given in Table 8. The obtained moisture values ranged from 83.54% to 88.11% and the brixs values ranged from 9.87% to 11.57%. Total phenolic values of black carrots ranged from 349.80 to 745.37 mg GAE (100 ml) $^{-1}$. The highest total amount of phenolic material was obtained in the BT_2 press wheel [505.68 mg GAE (100 ml) $^{-1}$] and Z_2 planting distance [619 mg GAE (100 ml) $^{-1}$] based on the distance averages over the printing press averages. Antioxidant activity values ranged from 28.71% to 69.48%. The highest antioxidant activity values were found in the BT_2 press wheel (45.50%) and at the Z_2 planting distance (55.66%) when the average of the pressurized and row spacing were examined. In studies using black carrots, the antioxidant effect value of the black carrot concentrate was found to be 10.98% in unpasteurized black carrot juice [16], 187.8 in the amount of phenolic substance in the Antonina variety and 492 89 mg GAE (100 g) $^{-1}$ in the Purple Haze variety [17]. Reports also stated that the antioxidant effect of black carrot concentrate was 89.71% [18]. The reported high antioxidant value may be due to the different antioxidant contents of black carrot varieties. The results obtained generally correspond to the data obtained in the investigations.

Table 8. Phenolic substance and antioxidant activity values

Pressure Wheel	Row Spacing	Moisture (%)	Brixs (%)	Total Phenolic Matter [mg GAE (100 ml) $^{-1}$]	Antioxidant Activity (%) DPPH
BT_1	Z_1	85.22	11.57	388.43	28.71
	Z_2	83.54	11.27	745.37	54.80
	Z_3	88.11	10.33	349.80	30.01
BT_2	Z_1	83.63	10.37	510.47	40.30
	Z_2	85.18	9.87	633.30	69.48
	Z_3	85.03	10.67	373.26	26.71
BT_3	Z_1	83.61	11.37	507.01	41.81
	Z_2	85.91	11.00	478.00	42.70
	Z_3	84.21	11.00	490.67	42.21

4. DISCUSSION

Black carrot farming in the Eregli Region uses seeds that are of a local population. In addition, these seeds are not classified according to quality class. Therefore, the planting quality is deteriorating. According to the results of the experiment, planting should be done on small rows at planting distances in order to obtain the desired plant density in the sowing of black carrots. Field tests suggested to plant with pressure wheels (BT_2), which are front and rear press wheels, due to the lowest increase in the row, the lowest variation coefficient value and the highest average yield values. In terms of quality criteria, total phenolic substance and antioxidant activity values containing mass values between 50 and 200 g, determined Z_2 should be planted at the planting site.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Physical and Chemical Properties of a Type of Almond Called "Akbadem" Grown in the Aegean Region in Turkey

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Abstract: A type of almond, called "Akbadem", grown in the Aegean region in Turkey were evaluated in terms of several physical and chemicial properties of nut and kernel. The average length, width, thickness, arithmetic mean diameter, geometric mean diameter, particle size and surface area of nuts were 39.04±2.94 mm, 23.56±1.88 mm, 15.60±1.15 mm, 26.07±1.19 mm, 24.29±1.75 mm, 5053.05±1100.12 mm^3 and 1565.14±225.63 mm^2 respectively. Corresponding values for kernel were 28.25±1.92 mm, 14.28±1.17 mm, 6.87±0.53 mm, 16.46±1.05 mm, 14.03±0.87 mm, 887.05 ± 167.28 mm^3 and 533.90±65.86 mm^2 respectively. "Akbadem" nut shell has a significant impact on the dimensional properties. Dimensional properties were decreased significantly than the almond nut to almonds kernel. The average almond nut shell thickness was determined as 3.35±0.34 mm. Akbadem nut gravimetric properties; thousand seed weight, seed density and bulk density were determined 4950±0.01 g, 1140±0.001 kg/m³ and 375±5.00 kg/m³ respectively. Corresponding values for "Akbadem" kernel were determined 1430±0.08 g, 1080±0.003 kg/m³ ve 485±5.00 kg/m³ respectively. Internal efficiency of Akbadem was determined as 30±0.50%. The "Akbadem" kernel was determined *L*-value 59.56±1.98, *a*-value 5.68±0.79 and *b*-value 16.74±0.54. "Akbadem" kernel shell is quite dark as shown in Hunter color values. Chemical composition of Akbadem kernel; moisture, total oil, total ash, protein, oleic acid, linoleic acid, palmitic acid and palmitholeic acid were 3.57±0.15%, 52.32±1.21%, 3.15±0.01%, 20.57±0.07%, 76.11±1.18%, 17.71±1.14%, 6.14±0.05% and 0.04±0.01%. "Akbadem" is seen that fatty acids and the relatively high amount of protein.

Keywords: Almond, "Akbadem", Physical Properties, Chemicial Properties

1. INTRODUCTION

Dental Almonds (*Prunus amygdalis var. dulcis*) are members of the family Rosaceae and the fruit is classified as a drupe in which the edible seed or kernel is the commercial product [1]. The almond is a nutritionally important and valuable specialty crop grown in many temperate and sub-tropical regions in the world [2]. Almonds originated in the Middle East and have been cultivated for 4000 years [3-6]. It is believed to have originated in Middle East but is now grown more widely, including in southern Europe, Africa, Southern Australia, and California [7].

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Almond fruit consists of the hull, shell, and kernel (nut) [6, 8]. The edible kernel or nut is separated and collected for commercial uses [9]. Almond also grows well in different regions of Turkey. Almond is grown in inner Anatolia, the Mediterranean and the Marmara regions of Turkey [10-12]. Almond cultivation in Turkey is concentrated in the Aegean region [13, 14]. Datça Peninsula, cultivated radiant, white, row almond, etc. as varieties, is the most widely grown in the Aegean region [15]. Aegean region in terms of number of trees and almond production takes first place in Turkey. Akbadem variety meets 30% of the production of almonds Aegean region of Turkey [16-18]. According to 2013 data Turkey ranks seventh in the world almond production with 69.838.00 tons [19]. In recent years, production of almonds in Turkey has increased considerably, where cultivation has increased, due to the improvement of agricultural techniques and selection of new almond cultivars [20].

Almonds and other tree nuts, are nutrientdense foods that can be a valuable plant source of lipids and protein in the diet. Researchers have identified that the contents of dietary fiber, vitamin E, phytosterols and several key micronutrients found in almonds and other nuts contribute to a healthy nutrient profile [21, 22].

Kernel size, and the closely correlated kernel weight, is variable from year to year, though less variable in almond than in other Prunus species. In addition to overall kernel size, its linear dimensions of length, width, and thickness are also important for certain commercial applications [1, 8]. Kernel size is commercially important, and larger sizes generally confer greater value [6]. In addition to kernel characteristics, shell and hull characteristics are also important determinants of a variety's market acceptance. For these reasons, for optimum threshing performance, processes of pneumatic conveying, storing and other processes of almond nut, its physical properties must be known. The physical parameters of different European and Californian almond cultivars are readily measured.

2. MATERIAL and METHODS

Freshly harvested raw almonds in shell (Akbadem) were supplied from the ''Sındı Village Agricultural Development Cooperatives'' (Datça/Muğla/Turkey).

Examples of almonds (40 g) were finely ground for color analysis in the grinder. Color analyzes were performed as triplicate. The color of the samples was measured using a Color Flex CX2733 Hunter Lab (Hunter Associates Laboratory, USA). The *L*, *a* and *b*-values are the three dimensions of the measured color which gives specific color value of the material [23, 24]. Moisture was determined by gravimetrically using moisture analyzer (OHAUS MB45, USA) at 105°C, ash amount was performed according to AOAC [25] method using burning in a furnace (Nuve MF 110, Turkey) at 650±25°C. Total protein was determined by nitrogen determination according to Dumas method (combustion) using nitrogen analyzer (NDA 701, Italy) [26, 27]. Total oil was performed according to AOAC [28] method using Soxhlet extraction systems (Gerhart Soxtherm Multistat, UK).

Measurement of the three major perpendicular dimensions of the seed was carried out with a digital compass (Mitotoyo, Japan) with an accuracy of 0.01 mm. The arithmetic mean diameter (D_a), geometric mean diameter (D_g) and and sphericity ($\emptyset$) were calculated by using the aquations (1), (2), (3), respectively (29).

$$D_a = \frac{L+W+T}{3} \quad (1)$$

$$D_g = \sqrt[3]{L \times W \times T} \quad (2)$$

$$\emptyset = \frac{D_g}{L} \quad (3)$$

Where, D_a, arithmetical mean diameter, L is length (mm), W is width (mm), T is thickness (mm) D_g, geometric mean diameter and ∅, sphericity.

The one volume (V) and surface area (S) were calculated by using the aquations (4), (5), (6), respectively (30).

$$V = \frac{\pi B^2 L^2}{6(2L-B)} \quad (4)$$

$$S = \frac{\pi B W L^2}{2L-B} \quad (5)$$

$$B = \sqrt{W \times T} \quad (6)$$

Where V is volume, S is surface area.

The bulk density is the ratio of the mass of a sample of seed to its total volume. The bulk density was determined with a weight per hectolitre tester which was calibrated in kg per hectolitre. The nuts and kernels were poured in the calibrated bucket up to the top from a height of about 15 cm and excess amount was removed by strike off stick [31]. Seeding density was determined using a gas pycnometer (Mikromeritics Accupyc II 1340 Gas Pycnometer, USA) [32]. Thousand grain weight of the almond samples were measured with electronic precision balance with 0.001 g sensitivity [31].

Gas chromatography standard method of AOAC [33] International was used for the determination of fatty acid composition.

3. RESULTS and DISCUSSIONS

3.1. Chemical Composition of Almonds

The Oils and proteins are the most intensive components of almonds. Variability in oil content and fatty acid composition, as well as tocopherol (vitamin E) content, depends mainly on the almond genotype. Chemical composition of Akbadem kernel; moisture, total oil, total ash, protein, oleic acid, linoleic acid, palmitic acid and palmitoleic acid were 3.57±0.15%, 52.32±1.21%, 3.15±0.01%, 20.57±0.07%, 76.11±1.18%, 17.71±1.14%, 6.14±0.05% and 0.04±0.01% (Table 1). The chemical properties of Akbadem samples shows similarity to the literature. Moisture, protein, fat, and ash content of the major almonds maketing in the US as was reported as between 4.35% - 5.86%, 16.42% - 22.17%, 53.59% - 56.05%, and 2.69 - 2.93%, respectively by Sathe [34]. In addition Yildirim et al. [35] reported that total oil, protein, ash, humidity content of the 14 almond genotypes (province of sparta/Turkey) was between 44.25 - 54.68%, 21.23 - 35.2%, 2.75 - 3.81%, 3.41 - 4.52%, respectively. Dimensional characteristics of the Akbadem seed were found higher than other almond seed. Fruit weight with shell, kernel weight and kernel ratio of 5 almond types were reported between 0.67 to 2.07 g, 0.44 to 1.18 g and 44.44% to 59.29 %, respectively by Simsek et al. [36].

Table 1. The chemical composition of the Akbadem samples

Moisture (%)	3.57±0.15
Total oil (%)	52.32±1.21
Total ash (%)	3.15±0.01
Protein (%)	20.57±0.07
Oleic acid (%)	76.11±1.18
Linoleic acid (%)	17.71±1.14
Palmitic acid (%)	6.14±0.05
Palmitoleic acid (%)	0.04±0.01

3.2. Physical Composition of Almonds

Different almond varieties of is kernel size highly variable. Their particular of almond kernels has characteristic dimensions, shapes, appearances, membrane thickness and flavors. Akbadem shell has a significant impact on the dimensional properties. There are significant differences between nut almonds with almonds kernel example.

Table 2 shows the size distribution of the almond nuts and kernels. The almond nuts have a length ranging from 31.76 to 46.52 mm, width ranging from 19.13 to 27.58 mm, and thickness ranging from 13.22 to 19.13 mm. The almond kernels have a length ranging from 22.82 to 32.52 mm, width ranging from 11.60 to 17.15 mm, and thickness ranging from 5,87 to 8.77 mm. The average values of geometric mean diameter, arithmetic mean diameter and sphericity of almond nuts and almond kernels were calculated as 26.07, 16.46 mm 24.29, 14.03 mm and 0.62, 0.50 mm respectively.

Table 2. The dimensional characteristics of the Akbadem nuts and kaernels

	Almond Nut			Almond Kernel		
	Average	Minimum	Maximum	Average	Minimum	Maximum
Length (mm)	39.04 ± 2.94	31.76	46.52	28.25 ± 1.92	22.82	32.52
Width (mm)	23.56 ± 1.88	19.13	27.58	14.28 ± 1.17	11.60	17.15
Thickness (mm)	15.60 ± 1.15	13.22	19.13	6.87 ± 0.53	5.87	8.77
Arithmetic mean diameter (mm)	26.07 ± 1.19	21.88	30.30	16.46 ± 1.05	13.43	18.80
Geometric mean diameter (mm)	24.29 ± 1.75	20.34	28.11	14.03 ± 0.87	11.58	15.99
Sphericity	0.62 ± 0.02	0.58	0.67	0.50 ± 0.02	0.46	0.58
Volume (mm^3)	5053.05 ± 1100.12	2895.15	7864.19	887.05 ± 167.28	496.58	1371.32
Surface area(mm^2)	1565.14 ± 225.63	1090.32	2085.54	533.90 ± 65.86	361.07	682.73
Compact	Almond Nut			Almond Kernel		
Bladed	56			-		
Elongated	44			-		
Very bladed	-			66		
Very elongated	-			33		

Individual measured values were projected on to triangular diagrams by using the tri-plot spread sheet method. As can be seen from Fig 1 and Table 2, shape indices of ungraded Akbadem nut were dimensions were classified in bladed (56%) and elongated (44%) depends on their perpendicular. Shape indices of ungraded Akbadem kernel dimensions were classified in very bladed (66%) and very elongated (33%) depends on their perpendicular.

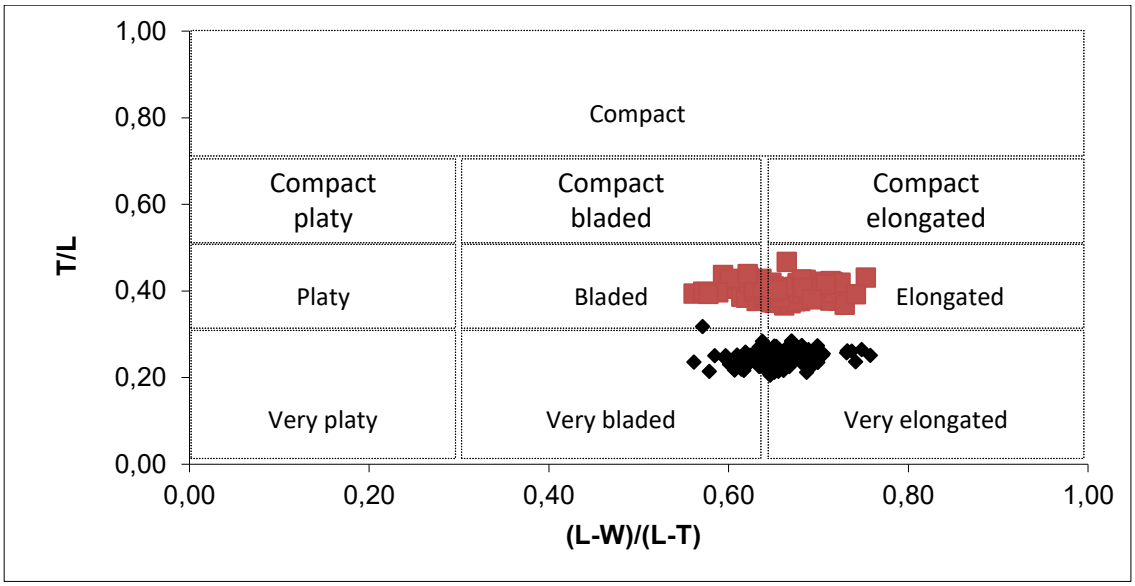

Figure 3. Sneed and Folk descriptive particle shape classes of ungraded almond nut and kernel.

Aydın [37] average length, width, thickness, geometric mean diameter, unit mass and volume of almond nuts and kernel were reported as 25.49, 21.19 mm 17.03, 14.34 mm 13.12, 6.38 mm 18.13, 11.42 mm, 2.64, 0.69 g and 2.61, 0.71 cm^3 respectively.

A summary of the results for all the measured parameters that related with gravimetric of Akbadem nut and kernel is given in Table 3. The mean one-thousand seed weight was 4950g and 1430 g for Akbadem nut and kernel, respectively. A reduction of 72% was determined in shelled grain according to the internal grain weight. Dimensional properties of almond nut were decreased significantly than the almond kernels. The average thickness and internal efficiency of almond nut shell were determined as 3.35 mm, 30% respectively.

Table 3. Gravimetric, efficiency and color properties of Akbadem nut and kernel examples.

Properties	Almond Nut	Almond Kernel
Thousand seed weight (g)	4950±0.01	1430±0.08
Seed density (kg/m³)	1140±0.001	1080±0.003
Bulk density (kg/m³)	375±5.00	485±5.00
Efficiency (kg)/(%)	300±5 / %30±0.50	
Color values		Ground almond Kernel
L		59.56±1.98
a		5.68±0.79
b		16.74±0.54

The mean seed densities and was mean bulk densities of Akbadem nut and kernel were 1140, 375 kg/m³ and 1080, 485 kg/m³ respectively. The seed density of Akbadem nut was found to be higher than that of Akbadem kernel while the bulk density of Akbadem kernel was higher than that of Akbadem nut. Aydın [37] reported a decreement from 655 to 525 kg/m^3 and an increement from 1015 to 1115 kg/m^3 for bulk density and true density in almond nut respectively. For the kernel, the corresponding values changed from 595 to 475 kg/m^3, 900 to 995 kg/m^3.

The *L*-value, indicative of the brightness, of Akbadem kernel is given in Table 3. The *L*, *a* and *b*-values of Akbadem kernel were determined as 59.56, 5.68, 16.74, respectively. According to the *L*, *a* and b-values color of Akbadem kernel shell is quite dark.

Mexis et al. [38] investigated the effect of active and modified atmosphere packaging, container oxygen barrier and storage conditions on quality retention of raw ground almonds finding a decrease at L, parameter and increase a and b values after 12 mounth of storage. The most apparent color change was determined in samples of PET//LDPE pouches stored at 20°C.

4. CONCLUSION

This study deals with the physical properties of Akbadem nut and kernel, providing useful data for its postharvest handling and industrial processing. It is recommended that making to efforts encouraging of cultivation and improving the quality of agriculture for an important kind of almond "Akbadem".

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Antioxidant Properties of Some Herbal Teas (Green tea, Senna, Corn Silk, Rosemary) Brewed at Different Temperatures

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Abstract: Some non-wood forest products are brewed and consumed as tea. Among the reasons for the consumption of herbal tea, digestive problems are located in the first row. Antioxidants help to human body for arranging digestive and immune system. Herbal tea is brewed in various ways such as boiling at different durations or waiting in hot water at different temperatures etc. Type of brewing can affect to bioactive properties of herbal tea. In this study, it was investigated the bioactive properties (total phenolic content, total flavonoid content, condensed tannin content and antioxidant properties) of some herbals brewed (Green tea / *Camellia sinensis.*, senna / *Cassia* sp., corn silk / *Zea mays*, rosemary / *Rosmarinus officinalis*) at different temperature. These herbs were brewed for 10 minutes at 60ºC, 80 ºC and 100 ºC temperatures. After cooling, total phenolic, flavonoid content, total condensed tannin content and antioxidant properties of these herbs were determined. Consistently, the highest results were found in the tea brewed at 100ºC. The highest total flavonoid (0.305 ± 0.005 mg QE/g) and ferric reducing ability (670.150 ± 2.121 µmol $FeSO_47H_2O$/g**)** was in *Rosmarinus officinalis.* The highest condensed tannin (9.443 ± 0.524 mg CE/g) and the highest total phenolic content (4.872 ± 0.005 mg GAE/g) was in *Camellia sinensis* and *Cassia* sp., respectively.

Keywords: Antioxidant, corn silk, green tea, herbal tea, rosemary, senna

1. INTRODUCTION

The tendency towards natural products to live a healthy life is increasing day by day. One of the practical prepared and most consumed of these natural products is herbal teas [1]. Thanks to the climate and soil, Turkey has a very wide range of plant that can be consumed as tea [2]. Plants have many phytochemicals that are potential sources of natural antioxidants such as phenolic diterpenes, flavonoids, tannins and phenolic acids [3]. These phytochemicals were reported that have bioactive properties e.g. antioxidant, anti-inflammatory, antitumoral, anti-cancer and immuno-modulatory characteristics [4]. It has been advocated that there is a direct correlation between the increase of antioxidant-rich foods and the decrease in the number of human diseases [5].

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Natural antioxidants, especially phenolics and flavonoids, are safe and bioactive [6]. The natural antioxidants help to clear away harmful free radicals from our bodies. A free radical is any species that has the ability to independently exist, containing one or more unpaired electrons that react with the other molecule by taking or donating electrons [7]. Free radicals have been associated with an increased risk of cardiovascular disease, cancer and other chronic diseases [8]. Therefore, antioxidant research is important for the formula of healthy life.

Many people are using medicinal herbs to alleviate and cure their illnesses due to their mild characteristics and low side effects [9]. In Turkey, there are a variety of plant species brewed and consumed for various reasons, such as strengthen the immune system and digestive system. Some of these herbal teas are *Camellia sinensis* (green tea), *Zea mays* (corn silk), / *Cassia* sp. (senna), *Rosmarinus officinalis* (rosemary). The teas of herbals can be brewed at different temperatures, but the effect of temperature to antioxidant properties is not yet clearly defined. Therefore, the aim this work to compare the antioxidant activity of some herbals such as *Camellia sinensis* (green tea), Zea *mays* (corn silk), / *Cassia* sp. (senna), *Rosmarinus officinalis* (rosemary) brewed at different water temperature.

2. MATERIAL and METHODS

2.1. Plant Material

The four different commercial, pre-packaged, dry herbs were purchased from a local medicinal herbs market in Trabzon / Turkey 2016**.** Scientific name, common name of studied herbs and the months of preparation of the herbs are presented in Table 1.

Table 1. Scientific name and common name of studied herbs

Scientific name	Common name	Year	The months of preparation of the herbs
Camellia sinensis	Green tea	2016	August-November
Cassia sp.	Senna	2016	August-September
Zea mays	Corn silk	2016	August-November
Rosmarinus officinalis	Rosemary	2016	September-November

2.2. Preparation of the Extracts for Determination of Antioxidant Activity

Approximately 5 g of samples were placed into a falcon tube with additional 100 mL water. Each herbal tea was brewed at 3 different temperatures: 60 °C, 80 °C and 100°C. The mixture was stirred continuously with a shaker (Heidolph Promax 2020, Schwabach, Germany) for 10 minutes. Particles were removed using Whatman No. 4 filter paper pore size 20-25 µm. Then solutions were filtrated from hydrophilic polyvinylidene fluoride (PVDF) 0.45 µm for sterilization. The finally volume of the solution was adjusted by the level of water.

2.3. Determination of Polyphenolic Contents

The polyphenolic contents of the samples were evaluated three different ways; total phenolic contents (TPC), total flavonoids (TF) and condensed tannin (CT). For the determination of the total phenolic contents, the Folin-Ciocalteau procedure was employed and gallic acid was used as standard [10]. Shortly, 20 µL of various concentrations of gallic acid and samples, 400 µL of 0.5 N Folin-Ciocalteu reagent and 680 µL of distilled water were mixed and vortexed. After 3 min incubation, 400 µL of Na_2CO_3 (10%) solution was added and vortexed. Then the mixture was incubated for 2 h at 20 °C with interrupted shaking. Absorbance measurement was carried out at 760 nm at the end of the incubation period. A standard curve was prepared using gallic acid as a standard with different concentrations of gallic acid, and the results were expressed as mg (GAE) per g extracts.

The concentration of total flavonoid present in the water extracts was measured using a spectrometric assay. Briefly, 0.5 mL samples, 0.1 mL of 10% $Al(NO_3)_3$ and 0.1 mL of 1 M $NH_4.CH_3COO$ were added to a test tube and incubated at room temperature for 40 min. Then the absorbance was measured against a blank at 415 nm. Quercetin was used for the standard calibration curve. The total flavonoid concentration was expressed as mg of quercetin equivalents per g sample [11].

Condensed tannins were determined according to the method by Julkunen-Tıtto [12]. For each sample, various concentrations of 25 µL from extracts of herbs were mixed with 750 µl of 4% vanillin (prepared with MeOH) and then 375 µL of concentrated HCl was added. The well-mixed solution was incubated at room temperature in darkness for 20 mins. The absorbance against the blank read at 500 nm. (+)-Catechin was used to help make the standard curve (0.05–1 mg/ml). The results were expressed as mg catechin equivalent to (CE)/g sample.

2.4. Determination of Antioxidant Capacity

The antioxidant capacity was determined using ferric reducing antioxidant power.

2.4.1. Ferric Reducing Antioxidant Assay (FRAP)

FRAP assay was also tested to determine the total antioxidant capacity of the samples. This method is based on the reduction of tripyridyltriazine complex (Fe (TPTZ) $^{3+}$) to blue colored $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium [13]. The preparation of working FRAP reagent was carried out by mixing 25 mL of 0.3 M acetate buffer pH 3.6 with 2.5 mL of 10 mM 2,4,6-tripyridylstriazine (TPTZ) solution in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution. The reaction mixture consisting of 1 mL of the sample and 3 mL of freshly prepared FRAP reagent was incubated at 37 °C for 4 min. Then, the absorbance was determined at 593 nm against blank prepared with distilled water. A calibration curve prepared with an aqueous solution of ferrous sulfate $FeSO_4.7H_2O$ in the range of 100-1000 µM was used. FRAP values were expressed in wet weight of the samples as µmol of ferrous equivalent Fe (II) per g sample.

2.5. Statiscal Analysis

All assays were performed in triplicate. The data were recorded as means ± standard deviations and analyzed by using Statistical Package for Social Sciences (SPSS version 23.0). The obtained data were analyzed by ANOVA and tests of significance were carried out using Duncan's multiple range tests.

3. RESULTS and DISCUSSIONS

3.1. Total Phenolic Content

Total polyphenol content of herbal teas brewed at different temperature are presented in Fig. 1. The herbal teas are very important because of containing a high amount of phenol in human diets. It is reported that rich in phenolic compounds significantly affect human health [6]. In this study, the highest total phenolic content was determined in water extract of senna tea brewed at 100°C with 4.872±0.005 mg GAE/g (Fig 1) and the lowest in rosemary tea brewed at 60°C with 0.313±0.008 mg GAE/g. The highest total phenolic content in all variations was found in herbal samples brewed at 100°C (Fig. 1) The total phenol content of herbal teas brewed at different temperatures is statistically significantly different from each other ($p<0.05$). In a previous study, phenolic content of extracts of 27 culinary herbs and 12 medicinal herbs were reported arrange of 0.43±0.08 between 17.51±0.22 mg of GAE/g of fresh weigh [14]. Therefore, it can be said that the total amount of phenolic content may change depending on the plant species.

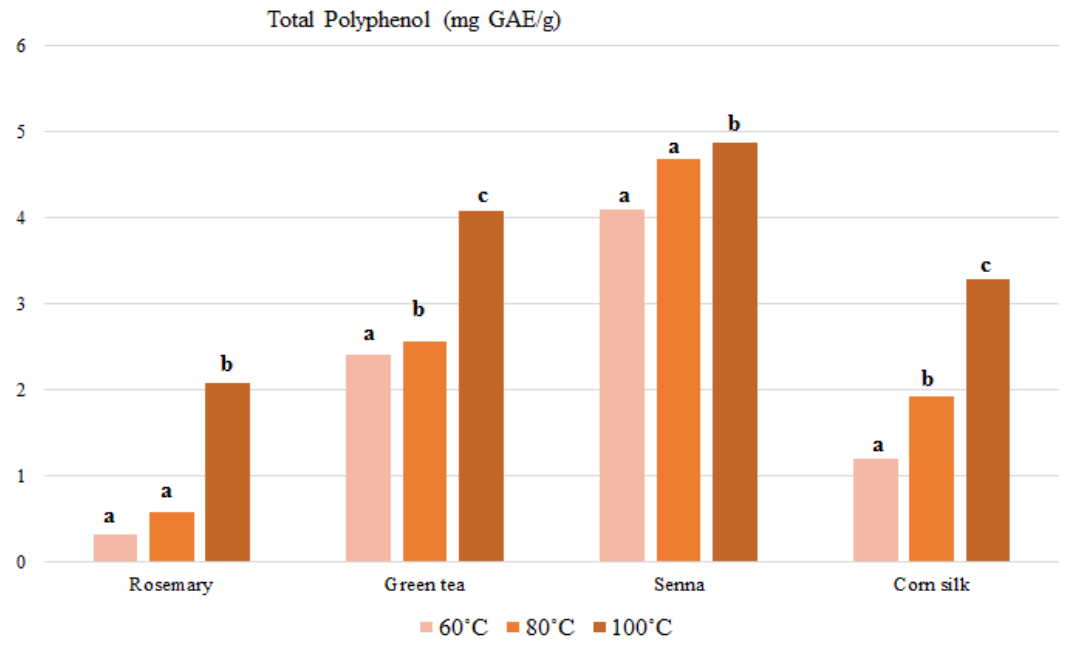

Figure 1. Total polyphenol content of herbal teas at different temperature. *Same letter(s) are not significantly different (p>0.05) by Duncan's multiple range test; n=3.

3.2. Total Flavonoid Content

Total flavonoid content of herbal teas brewed at different temperature are presented in Fig. 2. Flavonoids, one of the leading antioxidant compounds in plants that giving color to fruit and leaves, generally show bioactive properties such as exhibiting therapeutic functions for enzyme inhibition, free radical cleaning and cofactor activity for antioxidant C vitamins [15]. The total amount of phenol in this study ranged from 0.004±0.002 to 0.305±0.005 mg QE/g (Fig 2). The highest total flavonoid content were determined in extract of rosemary tea brewed at 100°C. The flavonoid content could not be determined in rosemary tea brewed at 60°C. As can be seen in Fig 2., there is not significantly difference between senna tea brewed 60°C and 80°C (p>0.05). In generally; the total flavonid contents are quite low especially in low temperatures. In this case; it can be said that 60°C and 80°C temperatures are not sufficient degrees to achieve the full benefits from the herbal plants.

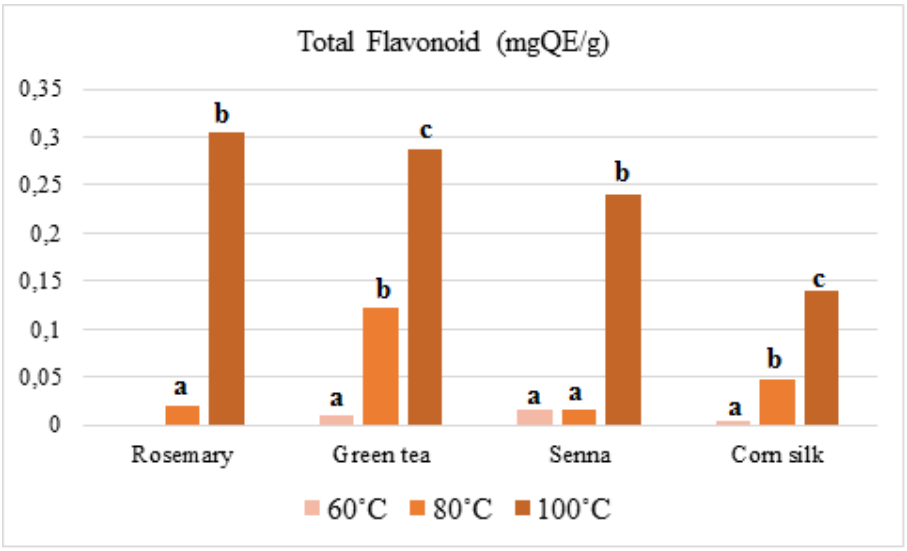

Figure 3. Total flavonoid content of herbal teas brewed at different temperature *Same letter(s) are not significantly different (p>0.05) by Duncan's multiple range test; n=3.

Some researches were reported that total flavonoid levels of 11 herbs include rosemary and green tea methanol extracts were ranged between 23.7 and 225 mg CE/g of extract [16]. So the type of solvent also affects the total flavonoid content.

3.3. Condensed Tannin Content

The condensed tannin content of herbal teas brewed at different temperature are presented in Fig. 3. Condensed tannins are structurally more complex, and more widely spread among the plants than hydrolysable tannins [17]. Some tannin molecules (e.g., tea polyphenols) have been reported that they have anticancer or anticarcinogenic or antimutagenic activity [18, 19]. So, tannin of herbal teas is important for healthy life. In this study, condensed tannin content could not be determined in rosemary teas brewed at 60°C and 80°C temperatures. The highest and the lowest condensed tannin content was found in extract of green tea brewed at 100°C (9.443±0.524 mg CE/g) and rosemary tea brewed at 100°C (0.105±0.006 mg CE/g), respectively. The amount of condensed tannins in the green tea was found to be 9 times higher from rosemary especially in the treatment at 100°C. In a previous study, condensed tannin content could not be determined rosemary tea, too. Also, the condensed tannin content of balm, mint, black tea, sage and common verbena was reported between 0.02 ± 0.00 and 2.11 ± 0.18 mg CEs/ml [20].

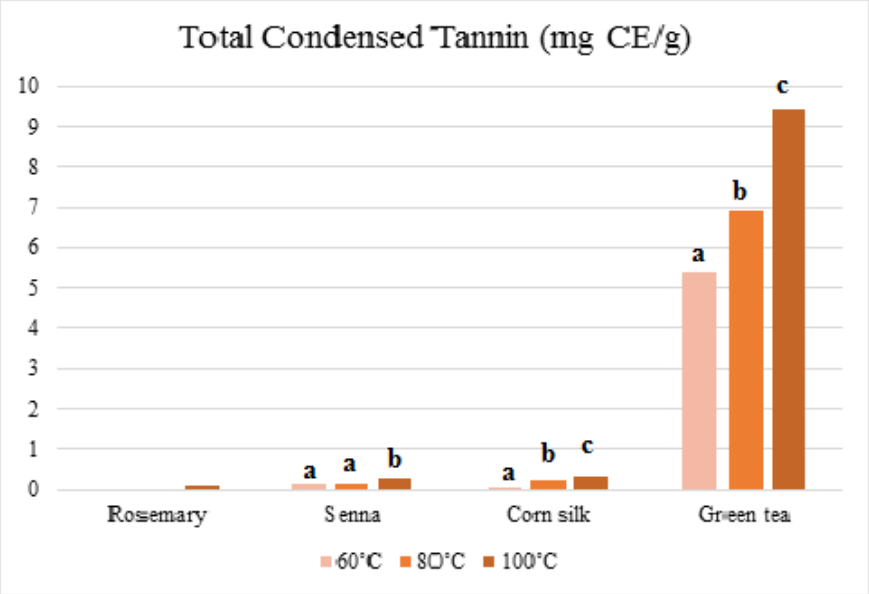

Figure 4. Total condensed tannin content of herbal teas brewed at different temperature*Same letter(s) are not significantly different (p>0.05) by Duncan's multiple range test; n=3.

3.4. Ferric Reducing Antioxidant (FRAP) Activity

Ferric reducing antioxidant (FRAP) activity of herbal teas brewed at different temperatures are presented in Fig. 4. The FRAP assay (Ferric Reducing Ability of Plasma), a simple test of the total antioxidant power have been chosen to assess the presumable effects of some kind of tea and medicinal plant [21]. In this study, FRAP activities of herbal teas can be listed that rosemary > green tea > senna > corn silk. The highest FRAP activity was determined in rosemary tea brewed at 100°C with 670.150±2.121 µmol $FeSO_47H_2O$/g and this value was 77 times higher than the lowest FRAP activity (8.763±0.226 µmol $FeSO_47H_2O$/g); in corn silk herbal tea brewed at 60°C. FRAP activities of all herbal teas brewed at different temperatures were found significantly different (p<0.05) from each other by Duncan's multiple range test. In a previous study; FRAP activity of ethanol-based lyophilized hydrophilic extracts prepared

from herbal teas from Eastern Anatolia have been reported, ranging from 390.8 ± 13.5 to 1130.8 ± 48.2 µmol Fe^{2+}/g dried weight [22].

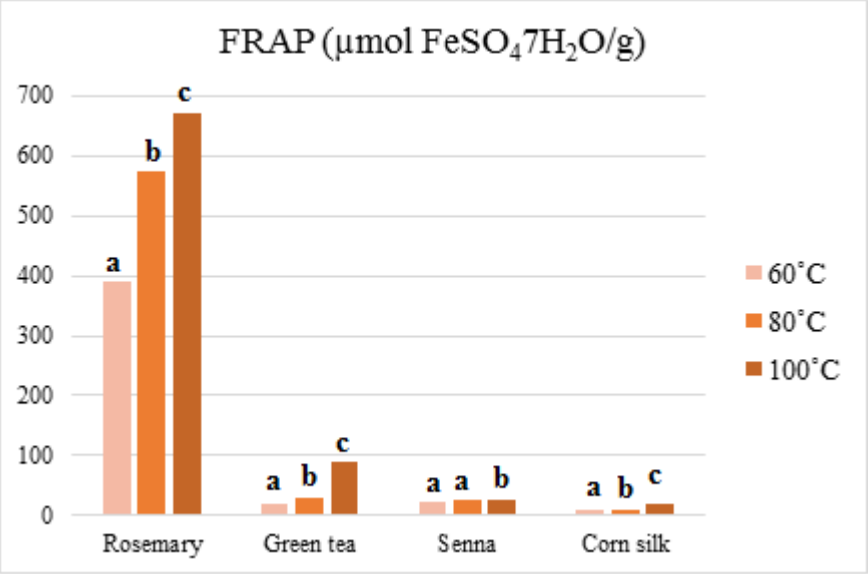

Figure 3. Ferric reducing antioxidant (FRAP) activity of herbal teas brewed at different temperatures.
*Same letter(s) are not significantly different (p>0.05) by Duncan's multiple range test; n=3

4. CONCLUSIONS

In this study, antioxidant activities of *Camellia sinensis* (green tea), Zea *mays* (corn silk), *Cassia* sp. (senna), *Rosmarinus officinalis* (rosemary) herbal teas brewed at different water temperatures (60°C, 80°C and 100°C) were determined and compared with each other. The highest total phenolic content was determined in senna tea; the highest total flavonoid content and the highest ferric reducing ability in rosemary tea, the highest condensed tannin content in green tea. The highest values were found in brewed at 100 °C temperatures in among all herbal teas. Consequently, it can be stated that plant species, solvent types, boiling temperatures can affect the total phenolic content, total flavonoid content, condensed tannin content and ferric reducing antioxidant activity. In order to make more comparison; similar analysis can be made with different herbal species and different temperatures and solvent types.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Determination of Effect of Nitrogen Fertilization on Some Quality Properties of Salep Orchid (*Orchis sancta* L.) Cultivated in Field Conditions in Turkey

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Abstract: Turkey has rich biodiversity due to located at the intersection of Europe-Siberia, Mediterranean and Iran-Turan flora regions. Orchidaceae family has a distinct place within this rich biodiversity. It has been reported total 204 orchid species those belongs to the 24 genera are grown in Turkey. The exports of the salep orchids were banned in 1974 by the Ministry of Agriculture due to high destruction of natural distribution areas of the plant. Despite the fact that nowadays the salep plants are protected by laws, the tubers of salep orchids still have been collected by people. Washing, boiling in water or milk, washing in cold water and drying stages are used to prevent growing activity of salep tubers. After this, tubers of salep are grind and prepared to use as salep powder. All of salep production is provided by collection of salep tubers from nature. For one kilogram of salep, 1000-4000 tubers is used. And it was assumed that our country produces 45 tons of tubers per year.This study was carried out to determine the effect of nitrogen fertilization on some quality characteristics of *Orchis sancta* L. grown in field conditions, in order to take part in agricultural cultivation of salep orchids. In the study, the effect of four fertilizer doses (0, 5, 10 and 15 kg/da) was investigated on starch ratio (%), mucilage ratio (%), protein ratio (%) and ash ratio (%). Mucilage ratio (salep mannia) was varied between 14% and 26% according to nitrogen fertilizer doses.

Keywords: Salep, *Orchis sancta* L., Cultivation, Nitrogen fertilizer, Quality.

1. INTRODUCTION

Turkey has rich biodiversity due to located at the intersection of Europe-Siberia, Mediterranean and Iran-Turan flora regions. Orchidaceae family has a distinct place within this rich biodiversity. It has been reported total 204 orchid species those belongs to the 24 genera are grown in Turkey. Forty-nine of these 204 species were hybrid species. Seventeen species of Turkish orchids contain tubers. Excludes six of them (*Coeologlossum*, *Gymnadenia*, *Listera*, *Spiranthes*, *Steveniella*, *Traunsteinera*), others (*Aceras*, *Anacamptis*, *Barlia*, *Comperia*, *Dactylorhiza*, *Himantoglossum*, *Neotinea*, *Ophrys*, *Orchis*, *Platanthera*, *Serapias*) are benefited from the use of salep [1-3]. Salep has been obtained as a result of the natural collection of tubers of Orchidaceae family. Many endemic orchid species have been collected from nature for many years and now they are almost about to extinction [4].

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The exports of the salep orchids were banned in 1974 by the Ministry of Agriculture due to high destruction of natural distribution areas of the plant [5]. Despite the fact that nowadays the salep plants are protected by laws, the tubers of salep orchids still have been collected by people. Washing, boiling in water or milk, washing in cold water and drying stages are used to prevent growing activity of salep tubers. After this, tubers of salep are grinded and prepared to use as salep powder. salep powder has starch, sugar, mucilage and nitrogenous compounds in its composition. salep is a plant-derived polysaccharide, contain 7-6% glucomannan, 8-19% starch, 0.5-1.5% nitrogenous substances, 0.2-6% ash (dry matter), 1-4% sugar and 6-12% moisture [6-8].

salep is put in the final product as flavor purpose [9]. The salep has darkening effect when added any formulation so it does increase viscosity that provides higher product quality, also glucomannan provides stabilization for ice cream. Salep provides unique structural taste and aroma to Maraş-type ice cream [10,11]. Salep drinks that consumed fondly in the winter are prepared in two ways, with milk or plain. According to the conventional method, salep powder and starch are put together in water or milk, and it is boiled by mixing slowly. When it gets thick, it is cooked little more by putting sugar into it [12]. All of salep production is provided by collection of salep tubers from nature. For one kilogram of salep, 1000-4000 tubers is used. And it was assumed that our country produces 45 tons of tubers for per year [13,14].

Cultivation of highly demanding plants are necessary for conservation and sustainable of natural resources. Orchis sancta L. is one of the most commonly collected species from nature in the Aegean Region. This study was carried out to determine the effect of nitrogen fertilization on some quality characteristics of *Orchis sancta* L. grown in field conditions, in order to take part in agricultural cultivation of salep orchids.

2. MATERIAL and METHODS

Orchis sancta L. is a perennial plant that called locally pürin flower or piriç flower. It is a non-endemic species of Eastern Mediterranean that spreads in grassland and calcareous soil between 0-450m altitudes in Western and Southern Anatolia. It is also located in Greece, Aegean, Cyprus and Western Syria [15]. The experiment material of this study is *Orchis sancta* L. seedlings that taken from another research that conducted at Aegean Agricultural Research Institute. This study was carried out during the period of 2012-2014 in Adnan Menderes University (ADU), Faculty of Agriculture Research, Application and Production field.

The province of Aydin where the experiment was conducted resides in the Mediterranean region and it was under the influence of temperate Mediterranean climate. Due to the Mediterranean climate, the plants in the country are beginning to grow in late winter and early spring.

In this study, commercial nitrogen fertilizer with 4 different doses (0, 5, 10, 15 kg/da) was conducted in three replications according to randomized blocks trial design. The seedlings of *Orchis sancta* L. were planted to 1 m width and 25 cm height planting sites with 20x20 cm distance in first year 21/12/2012 and second-year 30/11/2013. Thirty-six seedlings were used for each parcel and total 432 seedlings were used in the experiment. Half of the nitrogen fertilizer doses and 10 kg/da raw P and K fertilizer were given as basic fertilizer before planting. Other half of the nitrogen fertilizer doses in the study was used during the rosette period of the plants.

The removal of weeds was made with the hoe when it is necessary during the growth of the plants. Plants have benefited from rainfall due to good rainfall during the vegetation process so there was not needed for irrigation. The harvest was carried out on 17/05/2013 of the first year and on 07/05/2014 of the second year. Quality analyses were carried out in Aegean Agricultural Research Institute Technology Laboratory. Preliminary preparations before the

analysis were carried out at the Medical Plants Laboratory of the Field Crops Department of ADU Agriculture Faculty. In the study, the effects of four fertilizer doses (0, 5, 10 and 15 kg/da) on the starch ratio (%), mucilage ratio (%), protein ratio (%) and ash ratio (%) were investigated.

Starch ratio (%): It was performed enzymatically and spectrophotometrically according to Amyloglucosidase/alfa amylase method (AOAC METHOD 996.11, AACC METHOD 76-13.01) [16].

Mucilage ratio (%): Samples were weighed for 1'g and placed in a 25 ml graduated cylinders. The samples in the graduated cylinders were soaked with 1 ml of 96% ethanol and completed to 25 ml with purified water. The samples were shaken every 1-hour intervals for 10 minutes and this process was repeated for 3 times [17].

Protein ratio (%): It was carried out by DUMAS method [18]

Ash ratio (%): 1g of the sample was placed in porcelain crucibles and were weighed, then the porcelain crucibles were burnt in an ash oven at 600 °C. The samples were weighed again and the ash amounts were determined [17].

Due to a high number of quality analyses, trial replications were combined and analyses were carried out in parallel. For this reason, the data have not subjected to statistical analysis and were interpreted in a table form for information purposes..

3. RESULTS

A two-year study of nitrogen fertilizer and quality measures of *Orchid sancta* L. was given in Table 1. The effect of nitrogen fertilizer on the starch ratio was determined in the experiment. It was observed that the values changed between 12.946-24.822% in the first year and between 17.229-28.391% in the second year. The biennial mean values varied between 17.134-25.099% and the highest value was obtained at 5 kg/da nitrogen application (Table 1, Figure 1.).

Table 1. Effects of nitrogen fertilizer on some quality characteristics of *Orchis sancta* L.

Nitrogen Fertilization	Starch Ratio (%)			Mucilage Ratio (%)		
	1. Year	2. Year	Mean	1. Year	2. Year	Mean
0 kg/da	16.965	17.303	17.134	22.000	26.000	24.000
5 kg/da	21.807	28.391	25.099	14.000	18.000	16.000
10 kg/da	24.822	17.229	21.025	18.000	25.000	21.500
15 kg/da	12.946	21.347	17.146	19.000	23.000	21.000
Mean	19.135	21.068	20.101	18.300	23.000	19.600
Nitrogen Fertilization	Protein Ratio (%)			Ash Ratio (%)		
	1. Year	2. Year	Mean	1. Year	2. Year	Mean
0 kg/da	11.892	14.568	13.230	19.95	8.04	13.99
5 kg/da	10.371	13.737	12.054	16.18	6.60	11.39
10 kg/da	11.966	14.885	13.426	19.78	7.12	13.45
15 kg/da	14.991	10.323	12.657	13.86	5.77	9.82
Mean	12.305	13.378	12.842	17.44	6.88	12.16

Figure 1. Effects of nitrogen fertilizer on starch, mucilage, protein and ash ratio of *Orchis sancta* L.

It was determined that the protein ratio was changed according to doses of nitrogen in the experiment. Protein ratio was varied between 10.371-14.991% in the first year, 10.323-14.885% in the second year and 12.054-13.426% for a mean of both years. According to the results of two years, the highest protein value was reached 13.426% at 10 kg/da nitrogen application (Table 1., Figure 1.).

The ash ratio was found 17.44% in the first year, 6.88% in the second year and 12.16% in mean of both years. According to mean of both years, control has the highest value of ash ratio that 13.99%, it was followed by 10 kg/da nitrogen application with 13.45% (Table 1, Figure 1).

4. DISCUSSION

According to study that was carried out in 1865 by Dragendorf indicates that salep contains 48% mucilage, 27% starch, 5% protein, 2% ash and 1% oz (sugar) [19]. It was reported that there were differences in chemical content of salep orchids depending on different species [7]. It has been determined that mucilage ratio of different genus and species collected from Muğla region was varied between 6.82-61.05%, starch ratio 0.45-36.04%, total nitrogen ratio 0.57-0.95% and ash ratio 0.27-5.98% [6]. In the same study, the researcher determined that mucilage ratio and starch ratio of *Orchis sancta* L. was 15.70% and 10.64% respectively [6].

It was determined that commercial Maraş salep has 55.17% glucomannan and 2.70% starch content [20]. It was also reported that salep should contain > 40% of glucomannan and <8% of starch for production of Maras-type ice cream. It was reported that the salep contents (mucilage, starch, reducing sugar, non-reducing sugar, total nitrogen, water and ash ratios) were

changed between (6-61%), (0.6-36%), (0.4-4.5%), (0,1-2,3%), (0.4-1.2%), (6-12%) and (0.2-9%) respectively [21].

Our study aim for cultivation, it is very important because the previous studies do not contain cultivation knowledge and they only give general information about their region that they work. When we compare the values obtained by Sezik (1967) with our values in *Orchis sancta* L., it was determined that the mucilage and starch ratios in our study were higher than researcher's findings [6]. On the other hand, another study's mucilage value was higher than our finding and our starch value was higher than their finding [17]. Our mucilage and starch findings were similar with Akgül (1993) however protein and ash values higher than his values [18].

5. CONCLUSION

The effect of nitrogen fertilization on some quality characteristics of *Orchis sancta* L. was determined in Aydin ecological conditions. Nitrogen fertilizer applications have a positive effect on the starch and protein ratios of *Orchis sancta* L. and have a negative effect on the mucilage ratio. The starch ratio and protein ratio was determined between 17.134-25.099% and 12.054-13.426% respectively. The highest values were obtained with 5 kg/da and 10 kg/da nitrogen applications, respectively. On the other hand, the mucilage ratio was determined between 16-24% and the highest values obtained from control application. In the same experiment, the ash content was found in the range of 9.82-13.99% and the lowest values were determined with 15 kg/da nitrogen application.

This study was a unique study due to the first study in the field conditions for salep. The results are promising for the growth of salep orchids in field conditions. 5 kg/da nitrogen fertilization can recommend for starch ratio, 0 kg/da for mucilage ratio, 10 kg/da for protein and 15 kg/da for ash ratio. Due to mucilage ratio is used as darkening for maras-type icecream, 0 kg/da nitrogen fertilization recommended for cultivation of *Orchis sancta* L.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Investigating of Phytochemicals, Antioxidant, Antimicrobial and Proliferative Properties of Different Extracts of *Thymus spathulifolius* Hausskn. and Velen. Endemic Medicinal Plant from Sivas, Turkey

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Abstract: *Thymus* species has been used for antioxidant, antiseptic, antitussive, carminative, anti-inflammatory and antimicrobial activities as well as tonic and herbal teas. The present study was conducted to evaluate antioxidant, antimicrobial and proliferative properties of ethanol crude extract and fractions of *Thymus spathulifolius* (Hausskn. and Velen.) herbes. The antioxidant properties of ethanol extracts and fractions of *Thymus spathulifolius* were investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, ferrous chelating activity, total flavonoid and total phenolic content analysis. Antimicrobial activity of the plant extracts were tested using the microdilution method, while proliferative activity were evaluated by MTT assay. Results showed that IC_{50} of *T. spathulifolius* extracts that scavenged 50% of the DPPH radical was found to be ranged from 62.39 to 1000 µg/mL. Hexane extract possessed moderate antimicrobial activity towards gram-positive bacteria of *S. aureus* and fungi of *C. albicans*. *S. aureus* was the most sensitive bacteria than other tested microorganisms. The hexane and water extracts exhibited a good proliferative activity with ED_{50} of 3.28 µg/mL and 2.77 µg/mL, respectively. The antioxidant and antimicrobial activities, together with the ability of proliferation, provide some support for the *T.spathulifolius*'s traditional use.

Keywords: *Thymus spathulifolius*, antioxidant, antimicrobial, proliferative activity

1. INTRODUCTION

Herbal medicine has been used for the effective treatment of various disorders in various forms including decoction, maceration, powdered sample, oleoresins, crud extracts, fixed oil, essential oil etc. [1]. It is well recognized that free radicals are critically involved in various pathological conditions such as cancer, cardiovascular disorders, arthritis, inflammation and liver diseases [2]. Many studies have shown that natural antioxidants in medicinal plants are closely related the prevention or suppression of aging and many diseases associated with oxidative stress; cancer, cardiovascular diseases, rheumatoid arthritis, autoimmune diseases [3]. These beneficial effects have been partly attributed to antioxidants, which may play important roles in inhibition of free radicals and oxidative chain-reactions within tissues and membranes

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[4]. The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins [5].

The genus *Thymus L.*, belonging to Lamiaceae family, comprises approximately 300 evergreen species of herbaceous perennial and subshrubs, native to southern Europe and Asia [6]. This genus is represented by 38 species and 64 taxa, 24 of which are endemic in Turkey and the East Aegean Islands [7-8]. Various species of *Thymus* is used all over the world as condiments, ornamentals and sources of essential oil [9]. The genus are used in traditional medicine as tonic, antiseptic, antitussive and carminative as well as for treating colds and in pharmaceutical, cosmetic and perfume industry for preservation of several food products or as condiments [10]. Members of this genus are locally known as ''kekik'' in Turkish and are used as herbal tea and condiments either in the form of fresh or dry [11].

Thymus spathulifolius Hausskn. and Velen. is a dwarf shrub, up to 10 cm high, endemic in inner Anatolia, especially distributed in Sivas, growing in wild in open steppe on gypsous or poor fertile calcareous soils [12]. In terms of Pharmacological and phytochemical studies, many *Thymus* species have been reported for antioxidant, antimicrobial and other biological activities [13-15]. In addition, in the search for phytochemicals, plant parts are usually screened for phytochemicals that may be present. The presence of a phytochemical of interest may lead to its further isolation, purification and characterization. Then it can be used as the basis for a new pharmaceutical product. Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction process. This therefore underscore the need to try as much solvent as possible in screening plant parts for phytochemicals [16-17]. Nevertheless, to the best of our knowledge, no data on phytochemical studies of the *Thymus* species are available to now. This study aims to evaluate the antioxidant activity by different methods such as DPPH, metal chelating, FRAP, ferric reducing and ABTS tests, the antimicrobial activity agains five gram-positive and gram-negative bacteria and fungi, and proliferative effect of different extracts prepared from *T. spathulifolius* . For pharmaceutical application and naturaceutical industry, the present study may supply important information on the phytochemical properties of the *Thymus* species as well as biological activities.

2. MATERIAL and METHODS

2.1. Chemicals

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), 2,2-azino-bis(3-ethylbenz- thiazoline-6-sulfonic acid (ABTS), quercetin, gallic acid, 3,5-Di-tert-4-butylhydroxytoluene (BHT), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), and trichloroacetic acid (TCA) were obtained from Sigma Aldrich Co., St. Louis, USA. All other chemicals used were of analytical grade.

2.2. Plant Materials

Test plants were collected during the flowering period from natural populations in Sivas province of Turkey. Collected locality is: B6 Sivas: Zara-Divriği road, between Bolucan-Sincan. Plant materials were prepared as herbarum vouchers. Thes were registered under collector number as M.Tekin 1744. Voucher specimens are conseved at the Cumhuriyet University, Faculty of Science Herbarium (CUFH), Department of Biology, Sivas, Turkey.

2.3. Preparation of the extract

The dried plant materials were powderd using a grinder. The extraction was done at room temperature. 100g of dried and grounded herbs of *T. spathulifolius* were soaked in 80% ethanol (1000 mL) for 48h with intermittent shaking. Then extracts were filtered through Whatman filter paper No.1. In order to increase the yiled of extract, the procedure was repeated for three times. The filtrates combined together and concentrated under vaccum on a rotary evaporator

(Buchi R-100 equipped with Vacuum Pump V-300 and Control unit I-300) at 40°C to dryness and dissolved in distilled water. The aqueous extract was fractioned by successive solvent extraction with hexane, chloroform, n-butanol (pre-water-saturated butanol solution). All of the fraction obtained through solvent extractions were then evaporated to dryness and stored at -20ºC for further use.

2.4. Antioxidant Activities

2.4.1. *DPPH free radical scavenging activity*

The antioxidant activity of the plant extracts and the standard was evaluated on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by the method of Blois [18]. The stock solution of crude extracts (2 mg/mL) was prepared by dissolving a known amount of dry extract in 10% DMSO of methanol. The working solution (1, 10, 50, 100, 250, 500, 1000 µg/mL) of the extracts were prepared from the stock solution using suitable dilution. Ascorbic acid was used as standard in 1-100 µg/mL solution. 0.1mM of DPPH was prepared in methanol and 1 mL of this solution was mixed with 3 mL of sample solution and standard solution in test tubes separately in triplicates. These solution mixtures shanked vigorously, then were allowed to stand at dark for 30 min and optical density was measured at 517 nm using UV-VIS Spectrophotometer. Methanol (3 mL) with DPPH solution (0.1mM, 1 mL) was used as blank. Methanol was used for base line corrections in absorbansce of sample. The effective concentration of sample required to scavenge DPPH radical by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations. The optical density was recorded and % inhibition was calculated by the formula given below:

Percent (%) inhibition of DPPH activity = (Absorbans of Blank-Absorbans of Test) / Absorbans of Blank × 100

2.4.2. *Ferrous ion chelating activity*

Ferrozine can form complex by chelates with $Fe^{2+.}$ This reaction is restricted in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{2+} complexes. Measurement of the color reduction determines the chelating activity to compete with ferrozine for the ferrous ions .The chelation of ferrous ions is estimated using the method of Dinis et al. [19]. 1 mL of the extract is added to a mixture of 3.7mL methanol and a solution of 0.1 mL ferrous chloride (2 mM). The reaction is started by the addition of 0.2 mL of ferrozine (5 mM) and incubated at room temperature for 10 min and then the absorbance is measured at 562 nm. EDTA was used as a positive control.

2.4.3. *ABTS radical cation decolorization assay*

In this method, measure the loss of color when an antioxidant is added to the blue–green chromophore ABTS+. The antioxidant reduces ABTS.+ to ABTS and decolorize it. Antioxidant activity can be measured as described by Ree et al. [20]. ABTS radical cations are generated with 2.45 mM potassium persulphate and a 7 mM aqueous ABTS stok solutions. The ABTS cation working solution was obtained by mixing the two stok solutions in equal volumes and incubate them to react for 16h at 25°C in the dark. Before using, this solution was dilute with methanol and adjustified the absorbance 0.70 ± 0.2 units at 734nm by spectrophotometer. Frech solvent was prepared for each assay. Trolox, a water-soluble analog of vitamin E, used as an antioxidant standard. A standard calibration curve is constructed for Trolox at 0, 50, 100, 150, 200, 250, and 500 µM concentrations. 1mL of diluted samples are mixed with 1 mL of ABTS+ radical cation solution in test tubes, and absorbance is read (at 734 nm) after 7 min. TEAC values can be calculated from the Trolox standard curve and expressed as Trolox equivalents (in µM).

2.4.4. *FRAP Assay*

This assay was used to measure the Fe^{3+} ion's reducing power according to the method described by Aksu et al. [21] with slight modifications. 1 mL of samples, BHT and Ascorbic acid at different concentrations in appropriate solvent were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%) and incubated at 50 ◦C for 20 min. Then, 2.5 mL trichloroacetic acid (10%) was added to the mixture to stop the reaction. 2.5 mL of pure water and 0.5 mL of $FeCl_3$ (0.1%) were added to 2.5 mL of the reaction mixture, then allowed to stand for 30 min. The increases in the absorbance were spectrophotometrically measured at 700 nm as an indication of reducing capacity.

2.4.5. *Determination of total phenolics contents*

The determination of total phenolic content was performed by the Foline-Ciocalteu method [22] with slight modifications. The samples were read at 730 nm in spectrophotometer. The total phenolics content was expressed in milligrams equivalents of gallic acid (GAE) per gram of each fraction. The equation obtained for the calibration curve of gallic acid in the range of 0.1–1 mg. mL^{-1} was Y =0.7846X + 0.0764 (r = 0.9995).

2.4.6. *Determination of total flavonoids contents*

The determination of flavonoids was performed according to the method [23] with slight modifications. The absorbance was determined by spectrophotometer at 415 nm. Ethanol was used as a blank. The equation obtained for the calibration curve of quercetin in the range of 0.0625–1.0 mg. mL^{-1} was Y = 3.1965X + 0.2828 (r = 0.9933). The content of flavonoids was established as quercetin mg/g dry extract. The experiments were conducted in triplicate.

2.4.7. *Reducing power*

Reducing activity was carried out by using the method of Oyaizu [24]. Different concentration (1000, 500, 250,100, 10, 1 mg/mL) of extracts and fractions were prepared with methanol and taken in test tube as triplicates. To the test tubes 2.5 mL of sodium phosphate buffer and 2.5 mLof 1% potassium ferric cyanide solution was added. These contents were mixed well and were incubated at 50°C for 20 minutes. After incubation 2.5 mL of 10% TCA was added and were kept for centrifugation at 3000 rpm for 10 minutes. After centrifugation, 5mL of supernatant were taken and to this 5 mL of distilled water was added. To this about 1mL of 1% ferric chloride was added and was incubated at 35 °C for 20 minutes. The absorbans measured at 700 nm and the blank was prepared by adding every other solution but without extract and ferric chloride (0.1%) and the control was prepared by adding all other solution but without extract.

2.5. Antimicrobial Activities

2.5.1. *Micro-well dilution assay*

In order to determine the minimal inhibitory concentration (MIC) of *T. spathulifolius* extracts the broth microdilution method was used [25]. The antimicrobial activity of the plant extracts were tested against bacterial and fungal strains: Gram positive (*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)), Gram-negative (*Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922)) and fungal strain (*Candida albicans* (ATCC 10231)). Plant extracts were dissolved in 8% DMSO (20 mg/mL). 50 µL sterile distilled water was added in each well of 96-well microtiter plate. 50 µL of plant extract was added into the first well and a serial 2-fold dilution was performed by transferring 50 µL of the suspension to the subsequent wells up till the 9th well; the final 50 µL of the suspension was discarded. 10th well was added Gentamicin for bacteria and Flucanazole for *Candida*. 11th well was added 50 µL 2% DMSO and 12th well was added 50 µL Mueller Hinton Broth

(sterility control). Concentration of plant extract in wells ranged from 5.00 to 0.02 µg/mL. Final inoculum size was 5 $\times 10^5$ CFU/mL at bacteria and 0.5-2.5 $\times 10^3$ CFU/mL at *Candida* in each well [26-27]. Mueller Hinton Broth and Saboraud Dekstroz Broth was used for dilution bacteria and *Candida* culture's, respectively. Microtiter plates were incubated at 37 °C for bacteria and 35 °C for *Candida* between 16-24 hours. Afterwards, 50 µL 2 mg/mL 2,3,5-Triphenyltetrazolium chloride (TTC) (Meck, Germany) was added to each well to indicate microbial growth. The microtitre plates were further incubated at 37 °C for 2 h. Reduction in density of formazan's red color after incubation was accepted MIC value. The experiment was performed in duplicate and the standard deviation was zero.

2.6. Assessment of *In Vitro* proliferative activity

The *In vitro* proliferative activity was evaluated in the colorimetric MTT assay [28]. Exponential growing L929 Mouse fibroblast cells were plated in 96-well micropates at a density of 5 × 10^3 cells per well in 100 µL of culture medium and were allowed to adhere for 16 h, cultured in a humidified atmosphere at 37 °C in 5% CO_2 before treatment. Increasing concentrations of extract (1–1000 µg/mL) in their respective extraction solvent were then added. The final concentration of ethanol in the culture medium was maintained at 0.5% (volume/volume) in order to avoid solvent toxicity. The cells were incubated for 24 h in the presence or absence of extract or fractions. After incubation, 100 µL of MTT [5 mg/ml in PBS: medium (1:3)] was added per well, and the plate incubated for 4 h to allow reaction of MTT by cellular mitochondrial dehydrogenases. The excess MTT was aspirated and the formazan crystals formed were dissolved with 100 ml of dimethyl sulfoxide (DMSO). The absorbance of purple formazan, proportional to the number of viable cells, was measured at 595 nm using a microplate reader (Epoch, USA). The experiments were carried out in triplicate. The potency of cell proliferation for each extract was expressed as ED_{50} value, defined as the concentration that caused a 50% of maximum proliferation of cells.

2.7. Qualitative phytochemical screening

Chemical test for the preliminary phytochemical screening and identification of bioactive chemical constituents in the different extracts obtained from *T. spathulifolious* were performed using the standard procedures as described by Trease and Evans [29], Sofowara [30], and Ugochukwu [31].

3. RESULTS and DISCUSSIONS

The yields of crude ethanol extract (TSE), and n-hexane (TSH), chloroform (TSC), n-butanol (TSB) and water (TSW) fractions obtained from crude extract were calculated according to dry weight basis. The highest yield was obtained with TSW (25.0%), followed by TSB (23.3%), TSC (12.31%), TSE (11.88%) and TSH (10.27%) fractions, respectively.

Scavenging activity for free radicals of DPPH has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources. Free radical scavenging activity of ethanolic extract and fractions prepared from the herbs of *T .spathulifolius* was quantitatively determined using DPPH assay. Figure 1 shows the results of DPPH radical scavenging assay.

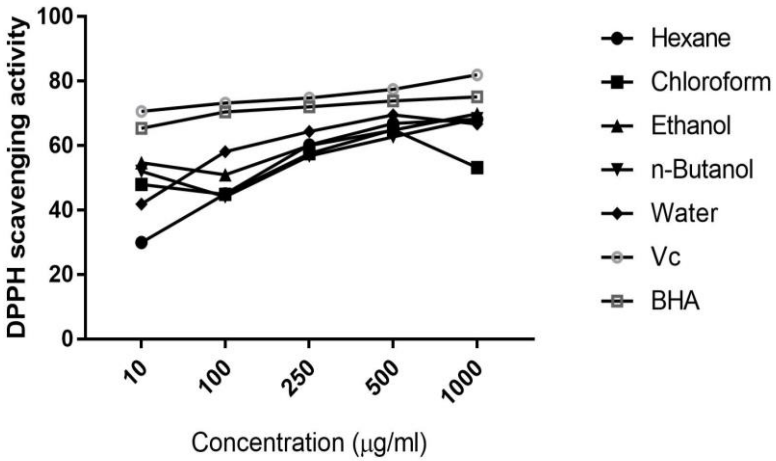

Figure 1. DPPH free radical scavenging activity of different extracts from herbs of T. *spathulifolius*

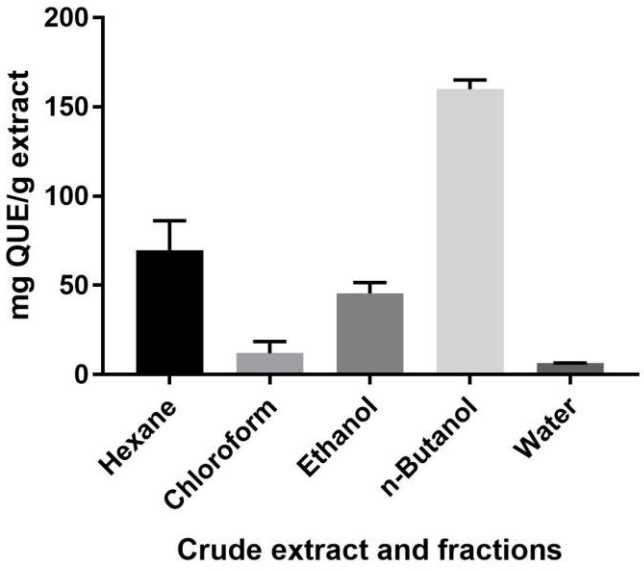

Figure 2. Total flavonoid content of crude ethanol extract and fractions from herbs of T. *spathulifolius*

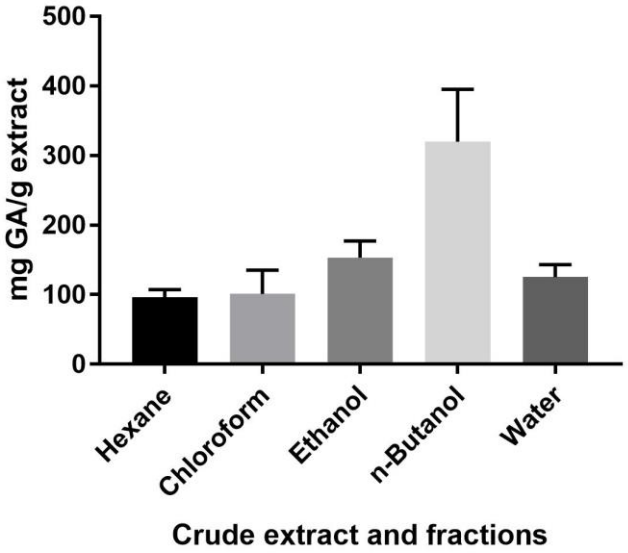

Figure 3. Total phenolic content of extract and fractions from herbs of T. *spathulifolius*

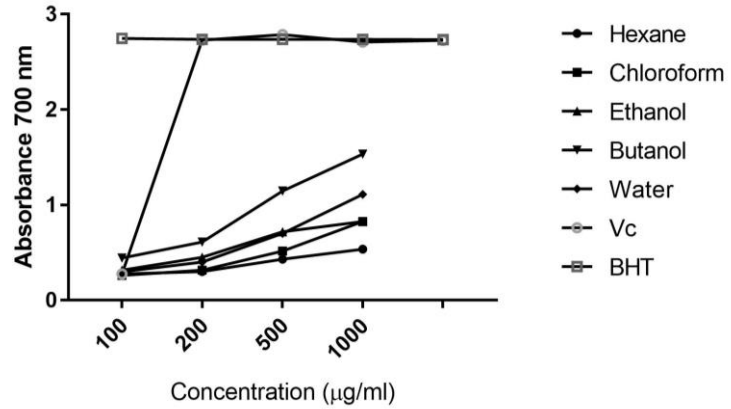

Figure 4. Ferric reducing power of different extracts from herbs of T. *spathulifolius.*

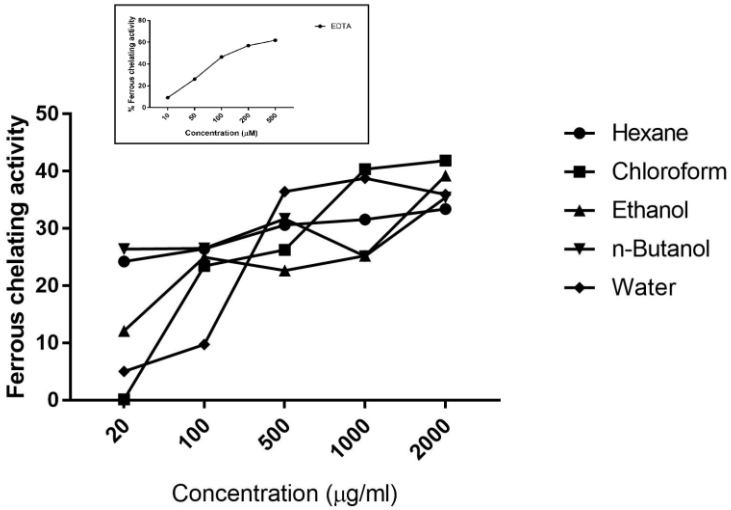

Figure 5. Metal chelating activity of various extracts from herbs of T. *spathulifolius*

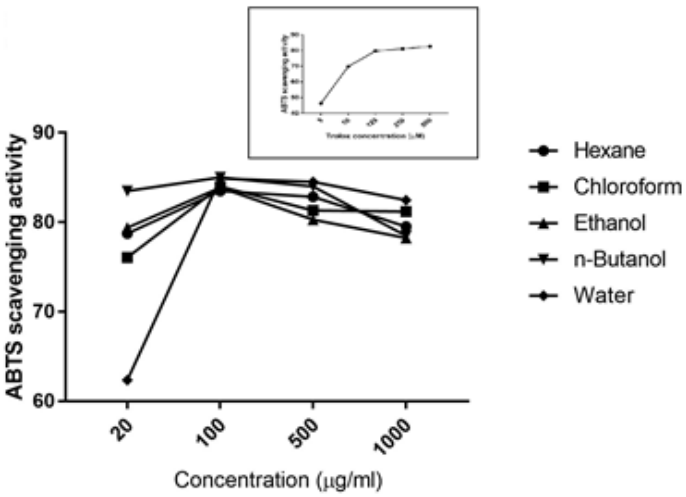

Figure 6. ABTS radical scavenging activity of different extracts from herbs of *T. Spathulifolius*

The total phenolic and total flavonoid contents in different extracts of *T. spathulifolius* are given in Figure 2 and Figure 3. Among studied *T. spathulifolius* extracts, total phenolic content was highest in the butanol extract and lowest in the hexane extract. Total phenolic content in the extracts ranged from 98.31 to 320.29 mg/g GAE. Total flavonoid content was highest in the butanol extract and lowest in water extract, ranged between 6.55 - 160.0 mg

quercetin equivalent flavonoid in g dry weight of extracts. Though the bioactivity of flavonoids appears to be mediated through a variety of mechanisms, particular attention has been focused on their direct and indirect antioxidant actions. The antioxidant properties are conferred on flavonoids by the phenolic hydroxyl groups attached to ring structures and they can act as free radical scavenger, reducing agents and metal chelators [32].

In general, the ABTS radical scavenging activity of the extracts is higher than the DPPH scavenging effect. It was reported that, the capacity of extracts in different test system was effected by solubility of the extract and stereoselectivity of radicals [33]. In this work, all of the tested extracts of *T. spathulifolius* in different polarity showed strong scavenging activities against ABTS radicals. Therefore, they may be valuable therapeutical agents in the treatment of some pathological damage due to free radicals.

3.1. *Antimicrobial activity*

MIC values of the different extracts of *T. spathulifolius* were detected with broth microdilution assay. As can be seen from the Table 1, plant extracts showed different antimicrobial activity against the test microorganisms. MIC values of the extracts in the range between >5–0.31 mg/mL. According to the results, the most sensitive microorganisms against the Hexane extract of *T. spathulifolius* were *S. aureus* and C. *albicans* that have the lowest MIC values 0.31 and 0.62 mg/mL, respectively.

Table 1. Minimum inhibitory concentrations of different extracts of *T. spathulifolius* herbs

		Ethanol extract and partitioned fractions fromethanol extract of *T.spathulifolius* herbs				
		TSH	TSC	TSE	TSB	TSW
		Concentration (mg/ml)				
S/No.	Microorganisms					
1	*E. coli*	2.5	>5	2.5	2.5	5
2	*S. aureus*	0.31	0.62	2.5	1.25	5
3	*P. aeruginosa*	5	>5	5	2.5	>5
4	*E. faecalis*	5	2.5	5	5	>5
5	*C. albicans*	0.62	2.5	5	5	5

3.2. *Proliferative assay*

The proliferative effect of plant extracts on cell growth was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan by the action of mitochondrial enzyme succinate dehydrogenase in viable cells. As can be seen from Table 2, the proliferative activities hexane and aqueous fractions with ED_{50} of 3.28 µg/mL and 2.77 µg/mL, respectively, while ethanolic crude extract showing antiproliferative activity with 12.0 µg/mL. The results in this study suggest that the compounds responsible for proliferative effects may be non-polar or polar compounds. The crude extract has an antiproliferative effect, when fractionated, has the opposite effect, this may be due to the synergistic effect between the compounds in the extract. As can be seen from the study results,

it is worth to evaluating of the active extracts in terms of wound healing activity due to the proliferative effect.

Table 2. Proliferative effects of various fractions of *Thymus spathulifolius* measured using MTT assay and the determined ED_{50}

Extract	ED_{50} (µg/mL)
Crude Ethanol extract	-12.0
Hexane fraction	3.28
Chloroform fraction	1056.44
n-Butanol fraction	58.36
Aqueous fraction	2.77

3.3. *Phytochemical screening*

The screening of chemical constituents was carried out with different extracts by using standard chemical methods according to the methodology, results were summarized in Table 3.

Table 2. Results of phytochemical screening of various extracts of *T. spathulifolius* herbs

Phytochemicals	Test	TSH	TSC	TSE	TSB	TSW
Alkaloids	Dragendorff's	–	–	–	–	–
	Mayer's	–	–	–	–	–
	Wagner's	–	–	–	–	–
Cardiac glycosides	Legal's	–	+	–	–	–
	Keller-Kiliani's	–	+	–	–	–
Carbonhydrate	Molisch's	+	+	+	+	+
	Barfoed's	+	+	+	+	+
	Fehling's	+	+	+	+	+
Flavonoids	Shinoda's	–	+	+	+	+
	$FeCI_3$	–	+	+	+	–
	NaOH	–	+	+	+	–
Phenols	$FeCI_3$	–	+	+	+	+
Proteins	Ninhydrine's	–	–	–	–	–
Saponins	Foam	–	–	–	–	–
Steroids	L-Buchard's	–	–	–	–	–
Tanins	$FeCI_3$	–	+	+	+	+
	Lead acetate	–	+	+	+	+
	Jelatin	–	+	+	+	+
Antraquinones	Borntrager's	–	–	–	–	–
Volatile oil	Sudan III	+	+	+	+	–

4. DISCUSSION AND CONCLUSION

The results of our investigation confirm the use of the studied plants in Turkish ethnomedicine. The results from this study suggest that TSB fractions have stronger antioxidant properties than other fractions, which can be attributed to its high content of total phenolics and flavonoids. Previous study on the antioxidant activities of other species of the genus *Thymus* measured by DPPH, showed an IC_{50} value of 16.15 µg/mL for methanol extract of *T. spathulifolius* [34], 38.2 and 44.5 µg/mL for methanol and hexane fractions of *T. capitatus* [35].

The results of the antimicrobial activity evaluation of different extracts of *T. spathulifolius* against four bacteria and one fungi are summarized in Table 1. Results demonstrated that TSH fractions displayed moderate antibacterial activities against *S. aureus*

and *C.albicans* with MIC value of 0.31 and 0.62 mg/mL, while TSC shows moderate antibacterial activities against *S.aureus* with MIC value of 0.62 mg/mL. The MIC value of other extracts against all of the tested microorganisms were higher than 1.25 mg/mL. In prious study, the MIC value of *T. capitatus* [35] methanol fractions against *S. aureus* was found to be 0.25 mg/mL, hexane fractions was higher than 1 mg/mL, which are close to our results. Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce MIC in the range of 100 to 1000 ug/mL [36]. Activity is considered to be significant if MIC values are below 100 µg/mL for crude extracts and moderate when $100 < MIC < 625$ µg/mL [37]. Therefore, none of the studied samples could be considered as promising source of antimicrobial activities.

The results of the present study show that the ethanol extract and fractions of T. *spathulifolius* contained a high total phenolics level, and can be a promising source of antioxidant as well as antibacterial agents; therefore, it can be considered potentially useful for medicinal application. However, more detailed *in vivo* studies are required to make firm the safety, bioavailability and quality control of *T. spathulifolius* as well as phytochemical characterization and identification of responsible bioactive compounds.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Volatile Compounds in the Leaf of Plane Tree (*Platanus orientalis*) with Solid Phase Microextraction (SPME) Technique

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Abstract: Plane tree belongs to Platanaceae family. There is a widespread belief that *Platanus orientalis* leaves (POLs) have beneficial effects on joint disorders. Therefore, many people consume POLs as tea. To our knowledge, as there is no study on volatile compounds (VCs) of POLs, we objected to determine the VCs in POLs obtained from *Platanus orientalis* trees grown in Hatay province, Turkey. The VCs were extracted using solid phase micro-extraction (SPME) and analyzed by gas chromatography-mass spectrometry (GC-MS). A total of 140 VCs were found in POLs. Aldehydes, alcohols, ketones, terpenes and alkenes were determined in POLs as major VCs groups, which accounted for 32.40 %, 23.51 %, 18.08 %, 10.24 % and 4.82 % of total VCs identified, respectively. *Trans*, *trans*-2,4-heptadienal (6.62 %), nonanal (6.46 %), benzaldehyde (6.42 %), *cis*-3-hexen-1-ol (6.32 %), benzenemethanol (6.13 %) were the most abundant VCs identified in POLs. *Trans*-2-hexenal (3.46 %), 3-phenyl-2-butanone (2.87 %), *trans*-3,5-dimethyl-1,6-octadiene (2.80 %), 6-methyl-5-hepten-2-one (2.56 %), octan-1-ol (2.43 %), *trans*-geranyl acetone (2.17 %), *trans*-4,8-dimethyl-1,3,7-nonatriene (1.98 %), phenyl methyl ketone (1.69 %), 6-methyl-3,5-heptadiene-2-one (1.57 %) were the second most plentiful compounds found in POLs. 11H-dibenzo[b,e][1,4]diazepin-11-one,5,10-dihydro-5-[3-(methylamino)propyl] (1.38 %), benzeneethanol (1.36 %) and β-ionone (1.02 %) were found as the third most abundant VCs. The above-mentioned VCs were accounted for about 57 % of total VCs identified in POLs. The remaining VCs were below 1.00 % that is, found at trace levels. According to the VCs profiles of POL, its beneficial effects on health may be due to aldehydes, alcohols, ketones and terpenes.

Keywords: *Platanus orientalis* leaf, Volatile compounds, SPME

1. INTRODUCTION

Plane tree belonging to Platanaceae family is known for its grandeur and longevity. *Platanus orientalis* is one of the most common plane varieties in the world and it is the most widespread plane tree growing in Turkey. The common name of this variety is oriental plane. Among plane trees, it was the first variety to be discovered. The oriental plane is widely used as an ornamental, especially in urban areas and also is cultivated sometimes for timber. Plane tree roots have been used as a hemostatic agent and antivenom for snake bite [1]. The pollens of plane tree are an important source of airborne allergens such as asthma, allergic rhinitis and allergic conjunctivitis [2, 3] and also systemic reactions [4]. Nevertheless, plane leaves are used

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in traditional medicine, especially traditional Persian folk medicine, to treat several disorders such as dermatological, gastrointestinal, rheumatic and inflammatory [5-6]. Recently, the tea of plane tree leaf has widely been used for treating joint pains in the folk medicine in Turkey. It has indicated that the extract of leaves of plane tree resulted in a favorable effect on the removing the symptoms of food allergy in dogs [6]. According to Persian scientists and hakims, people suffer from joint pains such as rheumatism and arthritis or from teeth pain can cope with these pains with methods that are completely natural and herbal [1]. However, it is not known that what compounds are responsible for removing the symptoms of food allergy.

Previous chemical investigations of American sycamore (*Platanus occidentalis* L.) have shown the presence of triterpenoids or flavonoids which are probably responsible for its anti-inflammatory and antinociceptive activity [8]. It is known very-well that treating effect of plants is related to their chemical or biochemical composition. Some volatile compounds such as terpenes, aldehydes, alcohols, ketones occur in plants as a result of the mostly biochemical metabolic pathways. For example, volatile terpene compounds (citronellal, linalool, β-cubebene, β-pinene, myrcene, limonene, γ-terpinene, ρ-cymene, terpinolene, copaene, caryophyllene, citronellyl acetate, citronellol, geranyl acetate and δ-cadinene) isolated from lime leaf were found to be effective in inhibiting tumors in the digestive tract [9] and had good antioxidant properties [10]. On the other hand, mono- and sesquiterpenes have been used in studies of geographic variation within the species in wild populations [11]. In general, fragrant and volatile compounds from plants have been used in food industry as additives, medicines and aromatherapy [12].

Based on the common uses of *Platanus orientalis* leaves in traditional folk medicine, in the present study we were objected to determine the volatile compounds profile of *Platanus orientalis* leaf.

2. MATERIAL and METHODS

The leaves were collected from plane trees about twenty years in Harbiye region of Hatay province. The leaves dried under normal atmospheric condition in summer. VCs analyze of leaves was performed according to the procedures described by Guler [13] with minor modification. Dried samples were cut into in small pieces in cold mortar and taken into headspace vials (Agilent, Palo Alto, CA, USA). Three mL NaCl solution (3 % concentration) was added in samples. The adsorption of VCs was carried out using Solid Phase Micro-extraction Technique (SPME) by a divinylbenzene/carboxen/polydimetylsilioxan fiber (Supelco, Bellefonte, PA, USA). The extraction of VCs was done in water bath at 60 ^{o}C. The samples were hold in water bath for 45 min than fiber was inserted to headspace vial and kept at 30 min. For desorption of VCs, fiber hold in injection port at 250 ^{o}C for 5 min. The VCs were analyzed by gas chromatography-mass spectrometry (GC-MS) using HP-Innowax capillary column (60 m x 0.25 mm id x 0.25 μm film thickness). The oven temperature program was initially held at 50 ^{o}C for 5 min and then programmed from 50 ^{o}C by a ramp of 5 ^{o}C min^{-1} up to 230 ^{o}C, which was held for 5 min. Peak identification was carried out by comparing the mass spectra with the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) library, version 0.2 L. Peak areas (arbitrary units) were calculated from the total ion current. The results from the VCs analyses were expressed as the percentage composition of each compound.

3. RESULTS and DISCUSSIONS

A total of 140 volatile compounds including aldehydes (25), ketones (25), alcohols (21), terpenes (21), phenyls and phenols (15), alkenes (9), alkanes (8), esters (6), furans (5), others (5; alkyne, acid, compound with sulfur, lactone and unknown) were identified in the leaf of

Platanus orientalis tree. As shown in Table 1. and Figure 1., aldehydes were the most abundant compounds accounting for 32.4 % of total volatile compounds identified in *Platanus orientalis* leaf, followed by alcohols (23.5 %), ketones (18 %), terpenes (10 %), alkenes (4.8 %), alkanes (2.8 %), esters (2.7 %), phenyls and phenols (2.0 %) and furans (1.8 %). A total of 17 volatile compounds (Figure 2) including *trans,trans*-2,4-heptadienal (6.6 %), nonanal (6.5 %), benzaldehyde (6.4 %), cis-3-hexen-1-ol (6.3 %) and benzenmethanol (6.2 %), *trans*-2-hexenal (3.5 %), 3-phenyl-2-butanone (2.9 %), *trans*-3,5-dimethyl-1,6-octadiene (2.8 %), 6-methyl-5-hepten-2-one (2.7 %), octan-1-ol (2.4 %), *trans*-geranyl acetone (2.17 %), *trans*-4,8-dimethyl-1,3,7-nonatriene (2.0 %), phenyl methyl ketone (1.7 %), 6-methyl-3,5-heptadiene-2-one (1.6 %), 11H-dibenzo[b,e][1,4]diazepin-11-one-5,10-dihydro-5-[3-(methylamino) propyl] (1.4 %) benzenethanol (phenylethyl alcohol) (1.4 %) and β-ionone (1.0 %) were constituted the majority of volatile compounds, which accounted for 57 % of total volatiles identified in head space of the leaf. These VCs are the mainly products derived from unsaturated fatty acids, carotenoids, phenylpropanoid/benzenoid and terpenes.

The major volatiles, *trans, trans*-2,4-heptadienal has a fatty and green odor. It retards or prevents the development of 'off-flavors' in autoxidizing fats and oils [14]. Nonanal has a fatty and citrus-like flavor note. It is one of the main constituents in the oils of citrus fruits and also reported in over 200 food and beverages. *Cis*-3-hexen-1-ol and *trans*-2-hexenal with an intense grassy-green odor note are known as leaf alcohol and aldehyde, respectively. They act as an attractant to many predatory insects. It was observed that *cis*-3-hexen-1-ol had a less neurotoxicity effect in rat, compared with nonanal [12]. It is safer than nonanal.

Table 1. The percentage values of volatile compounds identified in *Platanus orientalis* leaf.

Volatile Compounds (140)	**RT**	**RI**	**MEAN±SD**
Aldehydes (25)			
Hexanal	10.55	842	0.12±0.02
2-Pentenal	12.37	1202	0.01±0.01
trans-Citral	13.71	1262	0.82±0.08
Heptenal	14.39	1291	0.12±0.03
trans-2-Hexenal	15.65	1353	3.46±0.40
Octanal	18.00	1484	0.75±0.11
trans-2-Heptenal	19.15	1561	0.67±0.32
Nonanal	21.19	1715	6.46±0.85
trans,trans-2,4-Hexadienal	21.64	1754	0.97±0.14
2-Octenal	22.30	1811	0.81±0.15
trans,trans-2,4-Heptadienal	23.33	1905	6.62±0.48
Benzaldehyde	25.09	2072	6.42±0.78
trans,cis-2,6-Nonadienal	26.51	>2100	0.43±0.02
trans-2-Decenal	27.88	>2100	0.59±0.15
Safranal	28.12	>2100	0.62±0.08
cis-Citral	28.84	>2100	0.14±0.03
2-Hydroxy-benzaldehyde	29.04	>2100	0.18±0.05
trans,trans-2,4-Nonadienal	29.35	>2100	0.97±0.14
4-Ethyl-benzaldehyde	29.67	>2100	0.19±0.07
2-Undecenal	30.42	>2100	0.59±0.14
trans,cis-2,4-Decadienal	30.76	>2100	0.34±0.03
Tridecanal	31.74	>2100	0.34±0.04
Myrtenal	32.71	>2100	0.14±0.03
Tetradecanal	36.17	>2100	0.54±0.15
4-Methoxy-benzenaldehyde	36.62	>2100	0.06±0.01
TOTAL			32.40±2.73
Ketones (25)			
3-Octanone	16.81	1413	0.29±0.07
2-Octanone	17.76	1470	0.13±0.03
6-Methyl-5-hepten-2-one	19.51	1586	2.56±0.10
Camphenilone	19.97	1619	0.30±0.09
Octamethyl-cyclotetrasiloxane	23.05	1879	0.12±0.04
11H-Dibenzo[b,e][1,4]diazepin-11-one, 5,10-Dihydro-5-[3-(methylamino)propyl]	23.42	1913	1.38±0.24
4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-,(R)-2-Butanone	23.49	1919	0.21±0.03
1,3-Dihydroxy-6-methoxy-1,2,3,4,-tetrahydroquinolin-2-one	23.94	1960	0.19±0.02
3,5-Octadien-2-one	24.77	2042	0.24±0.07
4,8-Dimethyl-nona-3,8-dien-2-one	25.66	>2100	0.15±0.04
3,5-Octadiene-2-one	26.13	>2100	0.14±0.02
6-Methyl-3,5-heptadiene-2-one	26.70	>2100	1.57±0.25
Phenyl methyl ketone	28.31	>2100	1.69±0.50
Glycocyanidine	29.27	>2100	0.17±0.03
2-Hydroxy acetophenone	31.86	>2100	0.10±0.01
β-Damascenone	32.09	>2100	0.10±0.04
trans-Geranyl acetone	32.63	>2100	2.17±0.11
6-Methyl-5-hepten-2-one	32.80	>2100	0.34±0.17
3-Phenyl-2-butanone	33.01	>2100	2.87±0.07
6,10-Dimethyl-9-undecen-2-one	34.42	>2100	0.22±0.02
β-Ionone	34.62	>2100	1.02±0.29
2,3-Epoxy-β-ıonone	35.78	>2100	0.67±0.09
6,10,14-Trimethyl-2-pentadecanone	38.00	>2100	0.70±0.10
3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	41.01	>2100	0.24±0.04
5,6,7,7a-Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	43.03	>2100	0.50±0.10
TOTAL			18.08±0.20

Table 1. (Continued).

Alcohols (21)			
Hexen-1-ol	19.79	1605	0.62±0.12
cis-3-Hexen-1-ol	20.81	1684	6.32±0.77
1-Octen-3-ol	22.60	1838	0.95±0.07
2,6-Dimethyl-7-octen-2-ol	23.09	1883	0.13±0.03
cis-1,5-Octadien-3-ol	23.62	1931	0.56±0.09
Octan-1-ol	25.46	>2100	2.43±0.17
trans-2-Octen-1-ol	26.97	>2100	0.68±0.08
Nonan-1-ol	27.99	>2100	0.56±0.19
cis-Geraniol	31.34	>2100	0.59±0.28
α-Methyl-benzene-methanol	31.81	>2100	0.15±0.02
Cuminol	32.54	>2100	0.22±0.02
cis-Carveol	32.88	>2100	0.34±0.17
Benzene-methanol	33.25	>2100	6.13±1.46
Benzene-ethanol	34.02	>2100	1.36±0.31
cis-Farnesol	34.22	>2100	0.47±0.07
4-Phenyl-2-butanol	35.61	>2100	0.12±0.03
Nerolidol	36.28	>2100	0.98±0.04
2-Methoxy-benzene-ethanol	36.45	>2100	0.14±0.03
Octadecan-1-ol	38.79	>2100	0.16±0.07
(1S*,6S*,7S*)-Tricyclo[5.3.2.0(1,6)]dodecan-7-ol	38.96	>2100	0.55±0.07
D11-Dodecene-1-ol	39.31	>2100	0.07±0.01
TOTAL			23.51±1.19
Terpenes (21)			
Monoterpenes (10)			
dL-Limonene	14.77	1309	0.76±0.07
γ-Terpinene	16.55	1398	0.81±0.10
Styrene	16.98	1423	0.65±0.10
trans-4,8-Dimethyl-1,3,7-nonatriene	18.42	1510	1.98±0.05
Theaspirane A	24.32	1995	0.30±0.04
9-Hydroxy theaspiran A	25.32	2094	0.13±0.04
cis-α-Bisabolene	28.91	>2100	0.11±0.01
Camphene	29.09	>2100	0.34±0.10
p-Cymene	33.39	>2100	0.48±0.15
Allo ocimene	33.68	>2100	0.14±0.02
TOTAL			5.70±0.24
Sesquiterpenes (11)			
α-Ylangene	22.98	1873	0.28±0.10
trans-Caryophyllene	26.88	>2100	0.40±0.11
β-Cyclocitral	27.50	>2100	0.85±0.05
α-Humulene	28.65	>2100	0.94±0.10
Zingiberene	29.57	>2100	0.21±0.08
β-Bisabolene	29.75	>2100	0.39±0.18
trans,trans-α-Farnesene	30.13	>2100	0.24±0.03
Δ-Cadinene	30.54	>2100	0.79±0.16
trans-β-Farnesene	31.01	>2100	0.08±0.04
1 S-*cis*-Calamenene	32.32	>2100	0.26±0.10
cis-α-Bisabolene epoxide	37.50	>2100	0.08±0.02
TOTAL			4.53±0.52
Phenyls and Phenols (15)			
1,2,3,4-Tetramethyl-benzene	17.34	1445	0.25±0.10
2-Methyl-1,4-benzene-diol	25.98	>2100	0.13±0.04
trans-Anethole	32.23	>2100	0.26±0.12
2-Methyl-naphthalene	33.83	>2100	0.18±0.03
Phenol	35.90	>2100	0.15±0.05
5-Ethyl-m-xylene	36.08	>2100	0.05±0.00
2-Propenyl-benzene	36.71	>2100	0.17±0.02
2,5-Dimethyl-phenol	37.10	>2100	0.04±0.01

Table 1. (Continued).

1-Methyl-5,8-dimethoxy-1,2,3,4-tetrahydro-1,4-ımino-naphthalene	37.20	>2100	0.07±0.01
2,6-Dimethyl-phenol	37.31	>2100	0.23±0.03
m-Cresol	37.55	>2100	0.12±0.04
trans-Stilbene	38.64	>2100	0.14±0.01
Tymol	39.80	>2100	0.06±0.01
Biphenylene	40.06	>2100	0.10±0.01
3-Ethyl-1-naphthol	40.46	>2100	0.09±0.04
TOTAL			2.04±0.20
Alkenes (9)			
4-Methyl-2,7-octadiene	19.39	1577	0.31±0.07
7-Methyl-3,4-octadiene	20.17	1635	0.31±0.13
trans-3,5-Dimethyl-1,6-octadiene	21.46	1738	2.80±0.41
cis-3-Ethyl-2-methyl-1,3-hexadiene	21.93	1779	0.12±0.02
2,5-Dimethyl-2,4-hexadiene	24.54	2020	0.08±0.00
4,8-Dimethyl-1,7-nonadiene	25.88	>2100	0.13±0.02
4-Methyl-2,6-octadien	27.06	>2100	0.70±0.26
1,2,4,4-Tetramethyl-cyclopentene	27.62	>2100	0.26±0.06
trans- 6-(2-Butenyl)-1,5,5-trimethyl-cyclohexene	33.54	>2100	0.10±0.02
TOTAL			4.82±0.51
Alkanes (8)			
Tetradecane	21.06	1704	0.41±0.05
9-Methyl-nonadecane	22.16	1798	0.57±0.16
Octadecane	22.75	1852	0.25±0.07
Pentadecane	23.82	1949	0.17±0.07
Hexadecane	26.38	>2100	0.21±0.01
Heptadecane	28.79	>2100	0.71±0.04
cis-1,2-Divinylcyclohexane	30.23	>2100	0.32±0.09
Cyclododecane	34.76	>2100	0.15±0.04
TOTAL			2.78±0.37
Esters (6)			
Benzoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	13.44	1250	0.42±0.07
Tiglic acid, *cis*-3-hexenyl ester	28.38	>2100	0.28±0.04
Acetic acid, phenyl methyl ester	30.00	>2100	0.75±0.16
Benzoic acid, 2-hydroxy-, methyl ester	31.27	>2100	0.74±0.41
Benzoic acid, *cis*-3-hexen-1-ol ester	38.35	>2100	0.42±0.08
Phthalic acid, ethyl ester	42.95	>2100	0.12±0.03
TOTAL			2.72±0.54
Furans (5)			
2-Ethyl-furan	5.95	914	0.01±0.00
2-Pentyl-furan	15.94	1367	1.00±0.05
2-(2-Propenyl)-furan	22.37	1817	0.07±0.02
Dihydro-β-agarofuran	29.20	>2100	0.28±0.04
2-Methyl-5-(1,1,5-trimethyl-5-hexenyl)-furan	34.88	>2100	0.43±0.11
TOTAL			1.80±0.11
Others (5)			
Unknown	18.92	1545	0.86±0.14
4-Nonyne	22.04	1788	0.25±0.09
Ethyl-dimethyl-thiophene	31.07	>2100	0.22±0.06
Mınt furanone	34.15	>2100	0.07±0.01
trans,trans-2,4-Hexadienoic acid	38.53	>2100	0.21±0.02
TOTAL			1.61±0.02

RI: retention index based on the identified VCs retention time (RT) and calculated from a linear equation between each pair of straight alkanes (C5-C25).

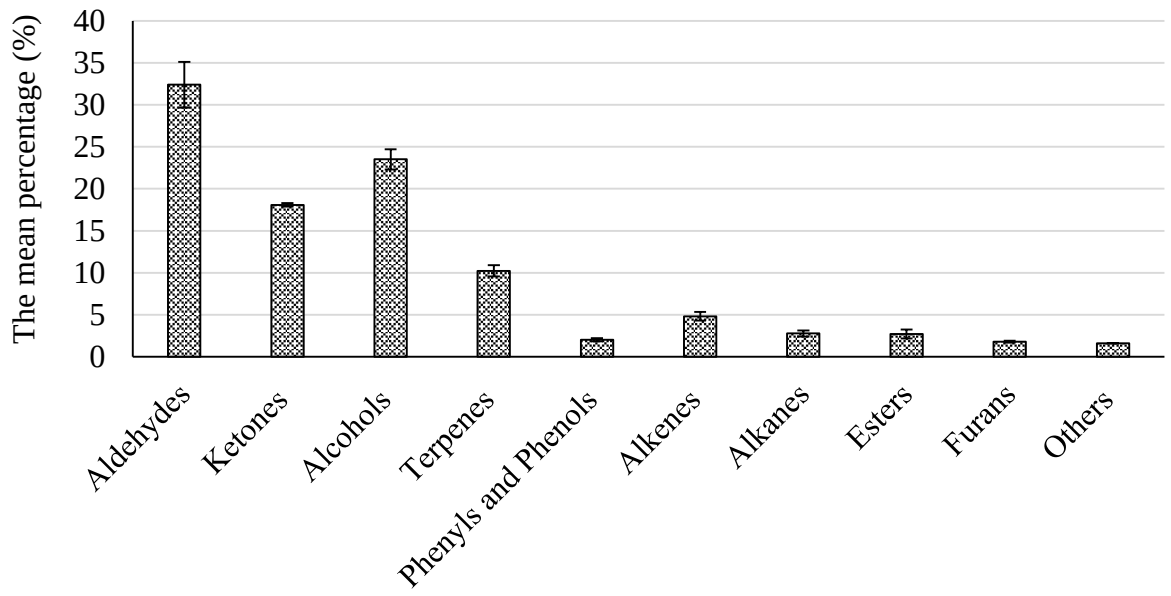

Figure 1. The percentage of volatile compounds identified in *Platanus orientalis* leaf according to the chemical groups.

Aldehydes, alcohols and also ketone containing six, seven, eight and nine carbons such as *cis*-3-hexen-1-ol, *trans*-2-hexenal, *trans, trans*-2,4-heptadienal, 2-octanone, 1-octanol and nonanal are formed either from linoleic acid or linolenic by oxidative degradation or from β-scission reactions via unsaturated fatty acids [15]. Glycolipids and phospholipids are rich in linolenic acid and linoleic acids [16]. Octanol or volatiles compounds containing octane give a typical fungal odor. β-Ionone, benzaldehyde, benzenethanol, *cis*-3-hexen-1-ol and nonanal were identified in various tea types (white, green e.g.) as main volatile compounds.

Another major VC benzenemethanol is used in perfumes and flavors, dyes for nylon, textiles, and plastics, hair products, plastic packaging and cosmetic industry. But, it is listed as a liver toxicant, neurotoxicant, and immunotoxicant [17]. Benzaldehyde, simplest aromatic aldehyde, is accepted in the European Union as a flavoring agent since it occurs naturally in many foods and is the primary component of bitter almond oil. Benzaldehyde having carcinostatic (anti-cancer) properties does not accumulate in any specific tissues and is metabolized, and then excreted in urine [18].

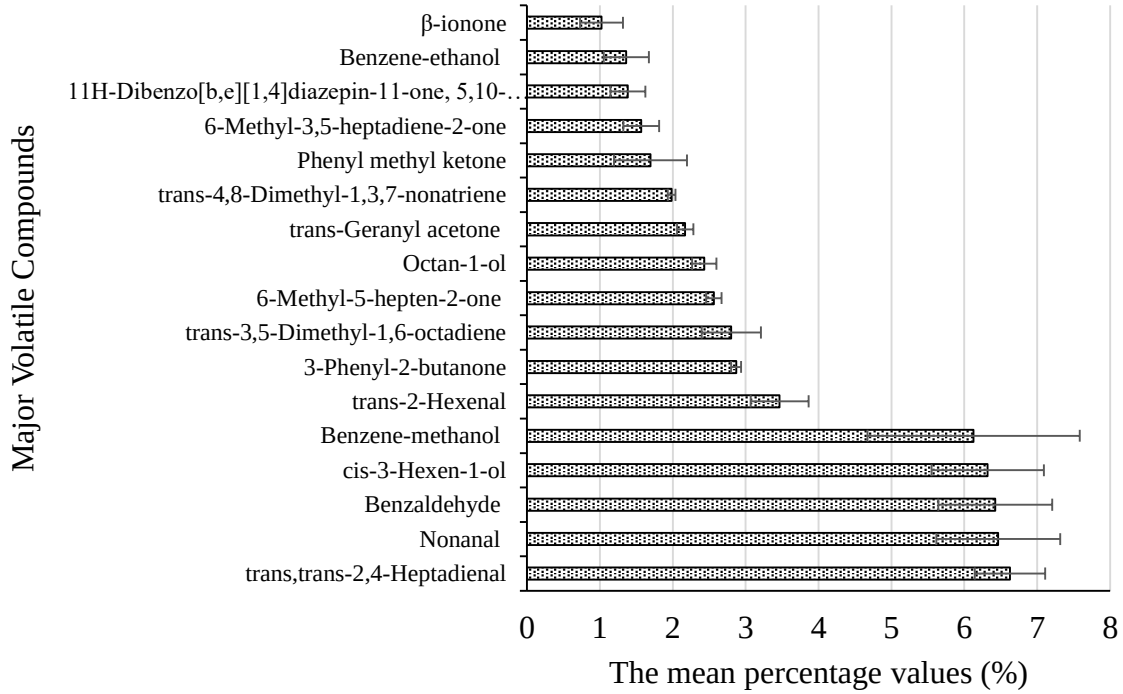

Figure 2. Major volatile compounds identified in *Platanus orientalis* leaf.

Actually, benzaldehyde, benzenmethanol, 3-phenyl-2-butanone and benzenethanol are phenyl propanoid/benzenoid derivatives [19]. These compounds and also VCs derived from unsaturated fatty acids were more abundant over the other VCs, in terms of their percentages.

Trans-4,8-dimethyl-1,3,7-nonatriene was the most abundant terpene identified in leaf. This compound was found to be in bergamot oil. It was reported that plants synthesize and emit to volatile compounds such as mainly *trans*-4,8-dimethyl-1,3,7-nonatriene and *cis*-3-hexen-1-ol in response to insect herbivory [20]. Geranyl acetate is a natural constituent of most essential oils and is used in cosmetic industry as a flavoring agent. β-Ionone is a product obtained from oxidative degradation of β-carotene [19]. Ketone 6-methyl-3,5-heptadiene-2-one is a product of lycopene degradation, which is one of main constituents of watermelon flavor [21]. Actually, both β-carotene and lycopene are terpene. It is well-known that terpenes have therapeutic properties such as antimicrobial and anticancerogenic.

4. CONCLUSION

A complex volatile compounds profile including the carotenoid, the unsaturated fatty acid, the terpene, the phenylpropanoid/benzenoid derivatives, the glycoside hydrolysis and the Maillard reaction products (furans) was identified in *Platanus orientalis* leaves. The diversity of VCs identified indicates that POLs may have a complex chemical and also biochemical composition with the probably majority of phenylpropanoid/benzenoid and unsaturated fatty acids. It is probably that the degradation products derived from phenylpropanoid/benzenoid and unsaturated fatty acids or themselves may be mainly related to the treating effects of Plane tree leaf in folk medicine.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Protein contents and antioxidant properties of p*leurotus ostreatus* cultivated on tea and espresso wastes

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Abstract: In this study, *Pleurotus ostreatus* was cultivated on tea (*Camellia sinensis*) and espresso wastes. Tea wastes were used in two forms; sterilized or non-sterilized. Then, total phenolic, flavonoid, condensed tannin contents, ferric reducing/antioxidant capacity (FRAP) and 2,2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging were used as antioxidant determinants and also protein content were investigated in these mushrooms' methanolic extracts. Same measurements were determined in mushrooms' growing medium except protein content. The highest protein content (20.89%) was found in non-sterilized tea wastes. The highest total phenolic (1.460±0.012 mg GAE/g), total flavonoid (0.120±0.005 mg QE/g), condensed tannin (0.877±0.011 mg CE/g) and the lowest scavenging of free radical activity (17.190±0.001 mg/mL) were determined in sterilized tea wastes. The highest ferric reducing antioxidant power (8.498±0.089 µmol$FeSO_4.7H_2O$/g) were determined in espresso wastes. Additionally, there was no statistically significant difference between the sterilized and non-sterilized substrates for the total yield and biological efficiencies. In this case, it can be said that the kinds of substrates and their usage forms are very important in terms of energy savings especially does not require sterilization like tea wastes. Consequently, tea and espresso wastes can be used as a beneficial source of substrate material for *Pleurotus ostreatus* mushroom cultivation.

Keywords: Antioxidant, espresso wastes, mushroom, tea wastes, total phenolic

1. INTRODUCTION

The consumption of mushroom and therefore the production of mushroom is increasing day by day. It was reported that average of 300 mushroom species can be edible and only 30 of them can be cultivated [1]. Among the cultivated mushrooms, *Agaricus bisporus* is in the first place and *Pleurotus osteatus* follows this sequence [2]. Mushrooms contain various secondary metabolites such as polyketides, phenolic compounds, steroids and terpenes [3]. It was reported that mushroom have many medical values such as anti-oxidant, antimicrobial [4], antitumor [5], antidiabetic [6] etc.

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The production of mushroom is very important not only in terms of nutritional and pharmacological properties but also in the evaluation of agro–industrial waste. Mushrooms, thanks to their enzymes, they can degrade lignocellulosic materials [7]. Especially developing countries will contribute to the country's economy when they use their agricultural waste as substrate in the production of mushroom. On this count, both the high protein content of the mushrooms will be produced and the environmental pollution will be reduced [8, 9]. Many materials such as tea waste [10] banana waste [11], bean, corn, straws and coffee husk [12] have been used as substrates in mushroom cultivation. Tea is the second most popular non-alcoholic drink after the water, consumed extensively by the world's population [13]. This is true also for our country. In addition, Coffee (*Coffea* sp.) is one of the most important agricultural products in the world [14]. The amount of waste left over from tea and coffee consumption every day is quite high. Therefore, the residue left behind after the consumed tea and espresso can also be evaluated in the production of mushrooms.

It was reported that the chemical composition of fruiting bodies is directly affected by chemical composition of substrates [15]. There are very few studies examining the content of the substrates and the mushroom cultivated in that substrate. The aim of this study is (i) to determine the using possibility of sterilized and non-sterilized tea (*Camellia sinensis*) wastes and espresso wastes as substrates on *Pleurotus ostreatus* cultivation (ii) to determine protein contents of cultivated mushrooms and (iii) to evaluate total polyphenolic contents (total phenolic contents, total flavonoids, total condensed tannin) and antioxidant properties of mushrooms and their substrates.

2. MATERIAL and METHODS

2.1. Materials

Pleurotus ostreatus spawn was obtained a commercial firm located in Denizli province, in Turkey. Tea wastes were obtained from canteen of Department of Forest Industry Engineering, Karadeniz Technical University. Espresso wastes obtained from a famous cafe preferred by people in Trabzon.

2.2. Mushroom Cultivation

Tea wastes were used in two forms; sterilized and non-sterilized. In non-sterilization method, tea wastes were used directly without any treatment. It was presumed that tea was self-sterilizing because it was always in hot water while was brewing and because it was exposed to hot water vapor. Other tea wastes and espresso wastes were moistened with water until %70-80 and sterilized in an autoclave at 121˚C for 1.5 h. After cooling the all substrates to 20˚C, they were placed in nylon bags of 1 kg and inoculated by spreading spawn on the surface of the substrate with a weight percentage of about 3% of the wet weight of compost. Substrate was carried out in four replications. Each nylon bags were transported to the mushroom growing laboratory (at 15-25˚C, %70-80 relative humidity). Harvesting was started in fifth week and the fruit bodies' stipe and cap was measured and weighed.

2.3. Yield and Biological Efficiency

Mushroom yield was calculated as total fresh weight of mushrooms obtained from 3 or 4 flushes in the harvest period [16]. Biological efficiencies were defined as the percentage ratio of the fresh weight of harvested mushrooms over the dry weight of substrates [17].

2.4. Determination of Protein Content

Each mushroom was dried at 40 ˚C before analysis. Dried mushroom samples were crushed and powdered for passing a 40 mm mesh sieve. Protein contents of mushrooms were determined by Dumas method. Briefly, 0.500 - 0.700 mg dried mushroom samples were

weighed and placed on 5 mm x 9 mm tin capsules. Capsules were placed into Costech ECS 4010 elemental analysis instrument and burned. Ratio of carbon, hydrogen and nitrogen were determined using Costech ECS 4010 program. Protein contents were determined by multiplicationing (%) carbon results with conversion factor (4.38) [18].

2.5. Preparation of the Extract for Determination of Polyphenolic Contents and Antioxidant Capacity

Harvested mushrooms were sliced and dried in a food dryer 8 hours at 60 ^{o}C (Profilo, PFD1350W, Turkey). Dried mushroom was ground in a basic micro fine grinder and passed through 1 millimeter sieve (IKA, WERKE MF10, Germany). Approximately 5 g of powder samples in were placed into a falcon tube 50 mL 99% with additional methanol. The mixture was stirred continuously with a shaker (Heidolph Promax 2020, Schwabach, Germany) at room temperature for a total of 24 hours. Particles were removed using Whatman No. 4 filter paper pore size 20-25 μm. Then solutions were filtrated from hydrophilic polyvinylidene fluoride (PVDF) 0.45 μm for sterilization. The finally volume of the solution was adjusted by the level of methanol.

2.6. Determination of Polyphenolic Contents

The polyphenolic contents of the methanolic samples were evaluated three different ways; total phenolic contents (TPC), total flavonoids (TF) and total condensed tannin (TCT). For the determination of the total phenolic contents, the Folin-Ciocalteau procedure was employed and gallic acid was used as standard [19]. Shortly, 20 μL of various concentrations of gallic acid and samples, 400 μL of 0.5 N Folin-Ciocalteu reagent and 680 μL of distilled water were mixed and vortexed. After 3 min incubation, 400 μL of Na_2CO_3 (10%) solution was added and vortexed. Then the mixture was incubated for 2 h at 20 °C with interrupted shaking. Absorbance measurement was carried out at 760 nm at the end of the incubation period. A standard curve was prepared using gallic acid as a standard with different concentrations of gallic acid, and the results were expressed as mg (GAE) per g methanolic extracts.

The concentration of total flavonoid present in the methanolic extracts was measured using a spectrometric assay. Briefly, 0.5 mL samples, 0.1 mL of 10% $Al(NO_3)_3$ and 0.1 mL of 1 M $NH_4.CH_3COO$ were added to a test tube and incubated at room temperature for 40 min. Then the absorbance was measured against a blank at 415 nm. Quercetin was used for the standard calibration curve. The total flavonoid concentration was expressed as mg of quercetin equivalents per g sample [20].

Condensed tannins were determined according to the method by Julkunen-Tıtto [21]. For each sample, various concentrations of 25 μL from extracts of plant were mixed with 750 μl of 4% vanillin (prepared with MeOH) and then 375 μL of concentrated HCl was added. The well-mixed solution was incubated at room temperature in darkness for 20 mins. The absorbance against the blank read at 500 nm. (+)- Catechin was used to help make the standard curve (0.05–1 mg/ml). The results were expressed as mg catechin equivalent to (CE)/g sample.

2.7. Determination of Antioxidant Capacity

The antioxidant capacity was determined using ferric reducing antioxidant power, free radical scavenging activity of DPPH•.

2.7.1. Ferric Reducing Antioxidant Assay (FRAP)

FRAP assay was also tested to determine the total antioxidant capacity of the samples. This method is based on the reduction of tripyridyltriazine complex (Fe (TPTZ)$^{3+}$) to blue colored Fe(TPTZ)$^{2+}$ by antioxidants in acidic medium [22]. The preparation of working FRAP reagent was carried out by mixing 25 mL of 0.3 M acetate buffer pH 3.6 with 2.5 mL of 10 mM

2,4,6-tripyridylstriazine (TPTZ) solution in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution. The reaction mixture consisting of 1mL of the sample and 3 mL of freshly prepared FRAP reagent was incubated at 37 °C for 4 min. Then, the absorbance was determined at 593 nm against blank prepared with distilled water. A calibration curve prepared with an aqueous solution of ferrous sulfate $FeSO_4.7H_2O$ in the range of 100-1000 µM was used. Trolox was also tested under the same conditions as a standard antioxidant compound. FRAP values were expressed in wet weight of the samples as µmol of ferrous equivalent Fe (II) per g sample.

2.7.2. Scavenging of Free Radical (DPPH) Assay

The DPPH assay was applied using [23] to determine the radical scavenging capacity of the methanolic extracts of the plant. The simple method is based on scavenging the DPPH radicals with an antioxidant substance of the investigated solution. For each sample, six different concentrations of 0.75 mL of the extracts of the samples were mixed with 0.75 mL of 0.1 mM of DPPH in methanol, and the absorbance was read at 517 nm. The values were expressed as SC_{50} (mg sample per mL), the concentration of the samples causing 50% scavenging DPPH radicals.

2.8. Statistical Analysis

All assays were performed in triplicate. The data were recorded as means ± standard deviations and analyzed by using Statistical Package for Social Sciences (SPSS version 23.0). The obtained data were analyzed by ANOVA and tests of significance were carried out using Duncan's multiple range tests.

3. RESULTS and DISCUSSIONS

3.1. Total Yield and Biological Efficiency

Total yield and biological efficiency of cultivated mushrooms on sawdust are presented in Table 1.

Table 1. Total yield (g/100g substrates) and biological efficiency (%) of cultivated mushroom

	Yield (g/100g substrates)			Biological efficiency (%)		
Form and type of substrates	$\bar{X}$	SD	H.G.	$\bar{X}$	SD*	H.G.**
Tea wastes (non-sterilized)	18.9	1.2	a	65.0	4.2	a
Tea wastes (sterilized)	19.6	1.7	a	67.6	5.1	a
Espresso wastes	21.3	2.8	b	74.6	8.6	b

*SD: Standard division. **: Homogeneity groups means having the same letter(s) are not significantly different ($p>0.05$) by Duncan's multiple range test.

The highest yield (21.3 g/100g substrates) and highest biological efficiency (74.6%) were obtained from the mushroom cultivated on espresso wastes. There was no statistically significant difference between the form of tea wastes substrates. In this case, it can be said that the tea wastes, which were the non-sterilized can be spontaneously sterilized during the brewing. On the other hand, it is necessary to produce larger quantities in order to prove its correctness. These kinds of raw materials are very important in terms of energy savings especially does not require the sterilization, like tea wastes.

3.2. Protein Content

Protein content of cultivated mushroom is presented in Table 2. It has been reported that amino acid configurations of mushrooms are comparable to some animal protein to some animal proteins [24]. Therefore, the mushrooms' proteins are important for nutrition. In this

study, the highest protein content was found *P. ostreatus* cultivated on non-sterilized tea wastes with 20.89%. Other protein content of mushroom were similar to each other (13.16% and 13.05%). High temperature treatment applied during the sterilization for the other variations may be caused a decrease in protein content. These results can be compared with different mushroom fruit bodies' protein content that varied between 8.6% and 42.5% [25]. It can be said that the substrate types and treating forms can affect the amount of protein content of mushrooms.

Table 2. Protein content of cultivated mushroom.

Mushroom' substrate	H (%)	C (%)	N (%)	Protein (%)
Tea wastes (non-sterilized)	6.70	41.22	4.77	20.89
Tea wastes (sterilized)	6.85	38.27	3.15	13.16
Espresso wastes	6.79	39.68	2.98	13.05

3.3. Polyphenolic Contents and Antioxidant Properties

Polyphenolic contents and antioxidant properties of cultivated mushroom are presented in Figure 1 and polyphenolic contents and antioxidant properties of substrates are presented in Table 3 and Table 4.

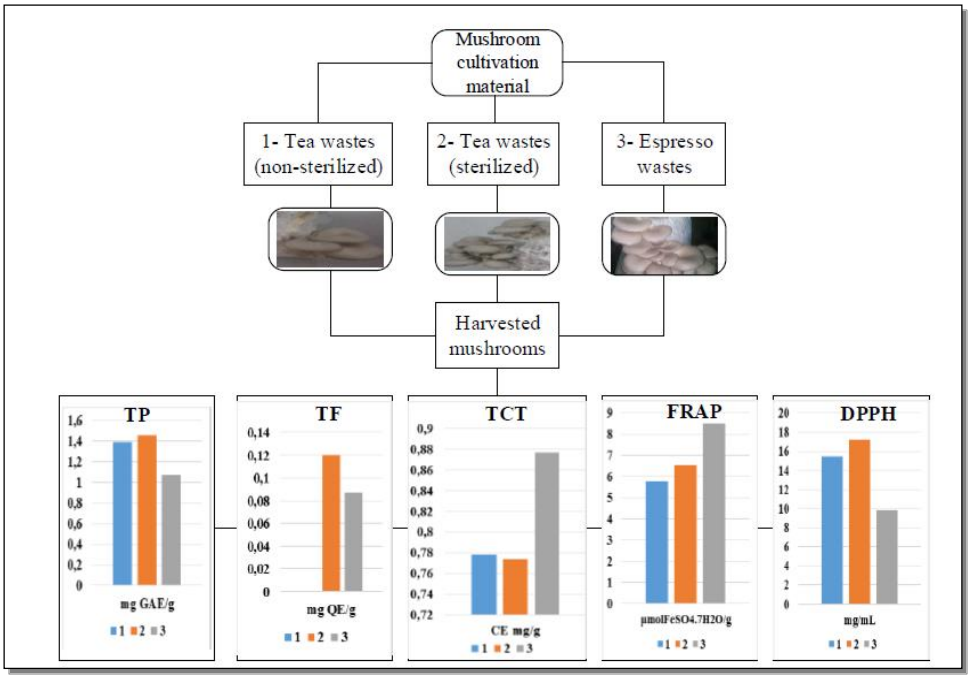

Figure 1. Polyphenolic contents and antioxidant properties of cultivated mushroom (1: Cultivated on non-sterilized tea waste, 2: Cultivated on sterilized tea waste, 3: Cultivated on espresso waste)

3.3.1. Total Phenolic Content

It has been reported that there is a strong correlation between the phenolic components and antioxidant capacity [26]. Therefore, it can be said that as the total amount of phenol in the mushroom increases, it will be more effective as antioxidant. In this study, the highest total phenolic content (1.460±0.012 mg GAE/g) was found in *P. ostreatus* cultivated on sterilized tea wastes (Fig 1). It was observed that this result was close to total phenolic content of mushroom cultivated on non-sterilized tea (1.393±0.060 mg GAE/g). Our total phenolic content values are lower than previously reported values of some wild mushrooms *Leucopaxillus giganteus, Sarcodon imbricatus* and *Agaricus arvensis* (2.83±0.09- 6.29±0.20 mg GAE/g) [27] and also seven *Morchella* species (12.36±1.21- 25.38±0.70 mg GAE/g) [28]. Among the

substrates, the highest total phenolic content was determined in sterilized tea wastes with 4.439±0.062 mg GAE/g. Total phenolic content of substrates was found significantly different (p>0.05) from each other by Duncan's multiple range test.

Table 3. Total polyphenol (mg GAE/g), total flavonoid (mg QE/g) and total condensed tannin (mg CE/g) contents of mushrooms' growth mediums.

Growth medium	Total Polyphenol (mg GAE/g)			Total Flavonoid (mg QE/g)			Condensed Tannin (mg CE/g)		
	$\bar{X}$	SD*	H.G.	$\bar{X}$	SD	H.G.	$\bar{X}$	SD	H.G. **
Tea wastes (sterilized)	4.439	0.062	c	0.932	0.023	c	0.889	0.038	a
Tea wastes (non-sterilized)	4.134	0.187	b	0.670	0.016	b	1.941	0.024	b
Espresso wastes	1.254	0.016	a	0.143	0.007	a	5.418	0.001	c

*SD: Standard Division. **: Homogeneity groups means having the same letter(s) are not significantly different (p>0.05) by Duncan's multiple range test, n=3.

3.3.2. Total flavonoid content

Flavonoids have been shown to exhibit a wide range of pharmacological and biochemical effects such as antimicrobial, antithrombotic, antimutagenic and antigenic activities [29]. In this study, total flavonoid content of *P. ostreatus* cultivated on non-sterilized tea wastes could not be determined. Total flavonoid content of *P. ostreatus* cultivated on sterilized tea wastes and espresso wastes was found 0.120±0.005 and 0.087±0.008 mg QE/g, respectively. These results are higher than total flavonoid content of seven *Morchella* species (0.15±0.02 -0.59±0.01 mg QE/g) [28]. Among the substrates, highest total flavonoid content was determined in sterilized tea wastes (0.932±0.023 mg QE/g) like total phenolic content.

3.3.3. Total condensed tannin content

Tannins are generally defined as naturally occurring polyphenolic compounds of high molecular weight to form complexes with proteins [30]. In this study, highest total condensed tannin was found in *P. ostreatus* cultivated on espresso wastes with 0.877±0.011 mg CE/g (Fig 1). Also, the highest total condensed tannin content was observed in espresso wastes with 5.418±0.001 mg CE/g, too (Table 3). It has been reported that the spent coffee waste contains large amounts of organic compounds such as fatty acids, lignin, cellulose, hemicellulose and other polysaccharides [31]. In this study, it was found that the content of condensed tannin of spent coffee waste is high, also. There are only a few studies about condensed tannin of mushrooms. In our previous study, condensed tannin of *P. ostreatus* and *P. citrinopileatus* cultivated on various sawdust was found in the range of 0.618±0.062 -3.674±0.009 mg CE/g [32]. So, it is possible to say that mushrooms' condensed tannin content is affected by substrates composition.

3.3.4. Ferric reducing antioxidant (FRAP) activity

Some researchers have been reported that the FRAP technique show high reproducibility, is simple and show the highest correlation with both ascorbic acid as a high antioxidant power and total phenolic [33]. In this study, among the mushrooms, the highest ferric reducing antioxidant activity was observed in *P. ostreatus* cultivated on espresso wastes with 8.498±0.089 μmol$FeSO_4.7H_2O$/g (Fig 1). This value was followed by *P. ostreatus* cultivated on sterilized tea wastes and non-sterilized tea wastes values respectively (6.548±0.019 and 5.762±0.095 μmol$FeSO_4.7H_2O$/g). FRAP activities of mushrooms were found higher than some fresh wild edible mushrooms (*Lactarius deliciosus, Lactarius sanguifluus, Lactarius*

semisanguifluus, Russula delica, Suillus bellinii) growing in the island of Lesvos, Greece (0.271±4.3 – 0.523±2.8 μmol Fe^{2+}/g, respectively) [34].

Table 4. The antioxidant activity of cultivated mushrooms' growth mediums.

Mushroom' substrate	FRAP (μmol $FeSO_4.7H_2O$/g)			DPPH-SC50 (mg/mL)		
	$\bar{X}$	SD	H.G.	$\bar{X}$	SD	H.G.*
Tea wastes (non-sterilized)	59.290	0.051	c	2.922	0.002	b
Tea wastes (sterilized)	57.787	0.068	b	0.806	0.001	a
Espresso wastes	19.515	0.046	a	4.045	0.002	c

*: Homogenity groups means having the same letter(s) are not significantly different (p>0.05) by Duncan's multiple range test, n=3.

Among the substrates (Table 4), it was found that ferric reducing antioxidant (FRAP) activity of non-sterilized tea wastes (59.290±0.051 μmol $FeSO_4.7H_2O$/g) and sterilized tea wastes(57.787±0.068 μmol $FeSO_4.7H_2O$/g) were close to each other and 3.5 times more than espresso wastes value (19.515±0.046 μmol $FeSO_4.7H_2O$/g). It can be concluded that tea wastes can be re-evaluated as a natural antioxidant source.

3.3.5. Scavenging of Free Radical (DPPH) Activity

DPPH radical dot analysis is routinely performed to assess the free radical scavenging potential of an antioxidant molecule and it is considered to be one of the standard and easy colorimetric methods for evaluating the antioxidant properties of pure compounds [35]. The higher FRAP values indicate high antioxidant capacity, while smaller DPPH values are indicative of higher antioxidant capacity. So, in this study, the lowest scavenging of free radical (DPPH) activity was found in *P. ostreatus* cultivated on sterilized tea wastes. Among the substrates, non-sterilized tea wastes show the highest DPPH activity. In addition, scavenging of free radical (DPPH) activity of substrates was found significantly different (p>0.05) by Duncan's multiple range test (Table 4).

4. CONCLUSION

The most consumed drinks after water are tea and coffee. Therefore, a lot of waste is left behind. This situation is especially true for tea and with the evaluation of these wastes, many benefits can be achieved. In this study, the possibilities of utilization tea and espresso wastes as substrate for *P. ostreatus* mushroom cultivation were investigated. The highest protein content (20.89%) was found in non-sterilized tea wastes. The highest total phenolic (1.460 ± 0.012 mg GAE/g), total flavonoid (0.120 ± 0.005 mg QE/g) and the lowest free radical scavenging activity of DPPH (17.190 ± 0.001 mg/mL) were determined in sterilized tea wastes. The highest condensed tannin (0.877 ± 0.011 mg CE/g) and ferric reducing antioxidant power (8.498 ± 0.089 μmol$FeSO_4.7H_2O$/g) were determined in espresso wastes. Additionally, there was no statistically significant difference between the sterilized and non-sterilized substrate types for the total yield and biological efficiencies. In this case, it can be said that the kinds of substrates and their usage forms are very important in terms of energy savings especially does not require sterilization like tea wastes. Consequently, tea and espresso wastes can be used as a beneficial source of substrate material for *Pleurotus ostreatus* mushroom cultivation. In order to make more comparison; similar analysis can be made with different mushroom species and different wastes which may contribute to energy saving.

Conflict of Interests

Authors declare that there is no conflict of interests.

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The Effects of Cultivation Area and Altitude Variation on the Composition of Essential Oil of *Laurus nobilis* l. Grown in Eastern, Western and Central Karadeniz Region

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Abstract: *Laurus nobilis* L. is one of the most valuable non-wood forest products on world export market and Turkey. Turkey is the biggest provider country for *Laurus nobilis* in the world. Therefore, laurel is an important commercial product for our country. In this study, the effects of cultivation area and altitude variation on essential oil content and quantity of laurel leaves were examined which grown in Trabzon, Bartın and Samsun. It was aimed to determine chemical composition of laurel's leaves grown in Karadeniz region. *Laurus nobilis* L. leaves were collected in three different height ranges. These were 0-100 m, 100-300 m, 300-600 m. Leaves were shade-dried and crushed. A device called ''Clevenger'' was used for getting volatile oil and their yields were calculated according to dry weight. The yields of essential oils ranged between 0.91% to 1.66 %. These essential oils were obtained from Bartin (B_2) (100-300 m) and Artvin (A_1) (0-100 m) respectively. The major components of these essential oils were 1,8- cineole (19.71%-35.63%), α-terpinyl acetate (12.86%-21.24%), sabinene (5.98%-9.40%), α- pinene (3.67%-8.45%) and β- pinene (2.91%-5.87%) were the most abundant volatile compounds in the leaves of bay.

Keywords: Laurel, essential oils, GC-MS

1. INTRODUCTION

Laurel has an important product in the trade of non-wood forest products in terms of Turkey and this respect laurel plant is one of the high value-added products for our country. According to foreign trade statistics of Turkish Statistical Institute (TSI) the year of 2015, 2,207,550 kg of bay leaf were exported from Turkey and the financial provision corresponds to 6,365,257 $.

The bay, known as *Laurus nobilis* L., is a peculiar plant of Mediterranean Region. It belongs to Magnoliophyta (closed seeds) branch and it is in the Magnoliopsida class. *Laurus nobilis* which is a member of the Lauraceae family of Laurales, is a genus of Laurus [1]. The leaves are short and thick. The fresh leaves are thin and they have light green vein and they have red tinged yellow color, then their color turns into light green, with little aromatic odor. The fresh shoots are green, the next is red, black and hairless. Their maximum length is 2 cm [2]. The place of use of *Laurus nobilis* L. is, thanks to its phenolic compounds which are taking

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place in substances of laurel leaves, in food industry as natural antioxidants with antioxidant properties [3]. In particular, thanks to its volatile compounds in dried leaves, they are used in meat, soup, candy and sauce making as a flavoring [4-7]. Bay leaves and volatile oils of leaves have an effects of antiepileptic, anticonvulsive [8-10], antimicrobial [10], antibacterial [11,12].

The antibacterial effect which are found in bay leaf essential oil, thanks to the high ratio of methyl eugenol and 1,8-cineole in the bay volatile oil. Thus, they have antibacterial properties against bacteria that cause foodborne diseases such as, *Staphylococcus aureus, Staphylococcus intermedius, Klebsiella neumonia, Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella typhimurium and Staphylococcus aureus* [12, 13].

The antimicrobial effect is due to the presence of compounds such as 1,8-cineole, camphor, myrcene, β-caryophyllene, eugenol, α-pinene, β-pinene and p-cymene in the content of volatile oil in bay leaf [2, 14]. Apart from beneficial properties, there have lots of unique properties of bay leaf volatile oil, such as having thermal stability, does not showing phototoxic effect, positive effects against migraine, headache, high blood sugar, bacterial and fungal infections [9, 15]. Leaf volatile oil is advised to use as a mucolytic agent (antipyretic) in advanced asthmatic disorders, upper and lower respiratory tract disorders since it has a 1,8-cineole (volatile compound) in it. So it has an anti-inflammatory effect (pain, fever cutter) which is from oxygenated monoterpenes and also it is a high concentration in bay volatile oil [16]. Recent studies have shown that bay leaf and its' essential oil have a positive effect against colitis which causes flatulence in stomach [17].

In recent years, it is indicated that the high rate of volatile oil of the leaves in the southern regions of our country makes bitter taste in the bay leaves. Thus, tendency towards the bay leaves in the EU countries is shifted progress to the laurel leaves grown in Russia. These leaves have less essential oils than Mediterranean Regions. Russia is on the shore to the black sea. Therefore, the Karadeniz Region may also be a suitable region for bay exports. Based on this,

Laurel leaves were collected from Artvin, Trabzon, Samsun and Bartın from Karadeniz Region and then essential oils were obtained and they were compared in oil yields and essential oil compositions.

2. MATERIAL and METHODS

2.1. Plant Material

The areas where the bay leaves were collected are given in Table 1. Laurel leaves were collected at three altitudes. These were 0-100 m, 100-300 m, 300-600 m but in Artvin (A_3) (300-600m) plant material were not collected because this plant were not encountered.

Dried by leaves (11 samples of 75 gr each) were subjected to the hydro distillation for 3 hours on a Clevenger-type apparatus. The essential oils were removed from the water and stored at 4ºC until gas chromatography-mass spectrometry (GC-MS) analysis.

Table 1. Locations and altitudes of collected laurel leaves.

Location	*Altitude (m)*		
	0-100	100-300	300-600
Artvin	90	230	-
Trabzon	20	200	600
Samsun	77	186	380
Bartın	10	200	400

2.2. GC-MS Analysis

GC-MS analysis was carried out with a 5975 Agilent apparatus equipped with İnowax FSC column (60 m long x 0.25 mm i.d. x 0.25 μm film thickness). The column temperature program was 60 ºC during 10 min, with 4 °C/min increases to 220 °C, then wait 10 min in 220 °C and 1°C/min increases to 240 °C. The carrier gas was helium at a flow-rate of 0.8 ml/min. Split mode injection (ratio 1:50) was employed. Injector temperature was 250 °C. Mass spectra were taken over the *m/z* 35–450 range with an ionizing voltage of 70 eV. The relative delay times for defining the essential oil components were determined by comparing the mass spectrum profiles of the materials using Wiley GC/MS Library, Adams Library, and Mass Finder 2.1 Library mass spectrum libraries on the computer to which the device was connected [18].

3. RESULTS and DISCUSSIONS

3.1. Extraction Yield

In our study, volatile oil yield which was obtained by Hydro distillation method are shown in Table 2. The highest yield of volatile oil in our work was 1.66 % Artvin (0-100 m) and the lowest amount was 0,91% Bartın (100-300 m) was obtained. Our volatile oil yield results vary between 0.91% and 1.66%.

Fiorini et al., investigated laurel leaf which were collected from France. They reported that the yield of laurel leaf essential was 0.57% [19]. Our results are much higher than this research.

Table 2. Avarage yield of *Laurus nobilis* L. essential oil.

Location	***Avarage Yield of Essential Oil (ml/100gr)***		
	0-100 m	***100-300 m***	***300-600 m***
Trabzon, (T_1,T_2,T_3)	1,33±0,0070	1,58±0,0141	0,99±0,1272
Bartın, (B_1,B_2,B_3)	1,47±0,0170	0,91±0,1484	1,44±0,1767
Samsun, (S_1,S_2,S_3)	0,92±0,0212	1,17±0,0848	1,33±0,6997
Artvin, (A_1,A_2)	1,66±0,0212	1,60±0,0001	-

3.2. GC-MS Analysis

Fifty-six compounds accounting for 78.07%–98.78% of the essential oils were identified by capillary GC-MS. Components are listed in Table 3 according to their retention times. There are lots of factors affecting the production of secondary metabolites in plants. It can be explained as an environmental, geographical, physiological, genetic, political and social factors [20]. The percentage of volatile compounds also depends on climate conditions and edaphic factors.

Table 3. The chemical composition of essential oils of leaves from laurel (*Laurus nobilis* L.).

C. N.*	Compound	R.T.**	Peak Area (%)										
			Ar_1	Ar_2	T_1	T_2	T_3	S_1	S_2	S_3	B_1	B_2	B_3
1	α- Pinene	8.861	3.68	4.05	5.96	5.75	3.67	8.02	5.98	8.45	6.0	7.33	6.15
2	α-Thujene	8.950	-	-	-	-	0.37	-	0.76	-	-	-	-
3	Camphene	10.568	-	-	-	-		1.31	1.97	1.17	0.98	1.72	0.97
4	β- Pinene	12.463	3.18	3.65	4.28	4.01	2.91	5.87	5.60	4.98	4.52	5.27	4.68
5	Sabinene	13.138	8.0	9.40	8.53	7.52	7.44	6.10	5.98	6.40	7.31	7.67	8.56
6	Myrcene	15.109	-	-	0.96	1.11	0.72	-	-	0.59	0.97	1.04	1.21
7	Phellandrene	15.156	2.8	2.73	-	-	-	-	-	-	-	-	-
8	α-Terpinene	15.867	-	-	-	0.82	0.61	1.05	0.97	1.14	1.02	0.78	1.03
9	Cineol (dehidro-1,8)	16.288	-	-	-	-	-	-	0.54	0.75	-	-	-
10	Limonene	16.873	-	-	2.33	2.37	2.03	2.44	-	-	2.25	2.62	-
11	1,8- Cineole	17.387	26.54	34.78	26.42	19.71	25.92	35.63	30.90	28.53	28.67	24.62	28.71
12	γ-Terpinene	18.968	0.99	1.32	1.44	1.45	1.09	1.93	1.77	1.79	1.64	1.37	1.69
13	O-Cymene	20.100	-	-	0.83	0.98	1.62	2.55	3.11	1.50	0.84	0.85	0.59
14	P-Cymene	20.109	1.19	1.06	-	-	-	-	-	-	-	-	-
15	Cis-Sabinene Hydrate	27.602	0.32	0.45	-	-	-	-	-	0.48	-	-	-
16	α- Terpinolene	20.608	0.40	0.50	-	0.53	-	-	0.54	0.57	0.58	0.51	0.66
17	Linalool	30.430	0.99	2.66	1.45	2.38	2.98	-	1.35	1.38	3.06	3.83	3.38
18	Pinocarvone	31.439	-	-	-	-	-	-	0.84	-	-	-	-
19	Bornyl Acetate	31.744	0.50	0.47	-	0.52	0.44	2.38	3.44	2.07	1.24	1.97	1.77
20	β-Elemene	32.106	1.89	0.45	1.56	1.11	0.73	-	-	0.36	0.47	0.73	0.58
21	2-Undecacone	32.220	-	-	-	-	-	-	-	0.43	-	-	-
22	Terpinene-4-ol	32.389	4.83	5.27	5.33	5.84	4.54	5.03	3.74	3.46	4.18	3.29	3.65
23	Myrtenal	33.374	-	-	-	-	-	-	0.92	0.63	-	-	-
24	δ-Patchhoulene	33.754	-	-	0.76	-	-	-	-	-	-	-	-
25	α-Gurjunene	33.765	-	-	-	-	0.58	-	-	-	-	-	-
26	δ-Terpinyl Acetate	33.917	2.49	2.52	1.52	2.53	1.84	1.56	1.70	1.57	1.44	1.45	1.87
27	Trans-Pinocarveol	34.060	-	-	-	-	-	-	0.87	0.65	-	-	-
28	α- Humulene	34.728	0.52	-	-	0.58	-	-	-	-	-	-	-
29	α-Terpinyl Acetate	35.440	20.73	20.36	21.24	18.1	20.86	20.26	12.86	17.20	18.28	18.72	18.03
30	Germacrene-D	35.886	-	-	1.03		0.49	-	-	-	-	0.46	-
31	Neryl Acetate	36.043	-	1.06	-	-	-	-	-	-	-	-	-
32	Lavanduly 2 Methyl Butanoate	36.046	1.97	1.06	-	-	-	-	-	-	0.85	-	-
33	Neryl Propanote	36.050	-	-	0.9	1.51	0.84	-	-	-	-	-	1.14

C.N.*:Compound number; RT.**:Retention time.

Tablo 3. (Continued).

C. N.*	Compound	R.T.**	Peak Area (%)										
			Ar_1	Ar_2	T_1	T_2	T_3	S_1	S_2	S_3	B_1	B_2	B_3
34	β- Selinene	36.210	1.12	-	1.34	0.79	0.80	-	1.04	-	-	-	0.93
35	Bicyclogermacrene	36.553	0.79	-	-	-	-	-	-	-	0.96	1.24	1.00
36	δ- Cadinene	37.159	0.33	-	-	-	-	-	-	-	0.40	0.72	0.63
37	Naphthalene	37.256	0.54	-	0.66	-	0.88	-	-	-	-	-	-
38	γ - Cadinene	37.299	0.54	-	-	-	-	-	-	-	-	-	-
39	Cis-α- Bisabolene	37.504	0.51	-	1.13	1.42	0.94	-	-	-	-	-	-
40	Myrtenol	38.007	-	-	-	-	-	-	0.68	0.69	-	-	-
41	Geraniol	38.073	0.54	0.39	-	-	-	-	-	-	-	-	-
41	Nerol	38.114	-	-	-	-	-	-	0.68	0.65	-	-	0.73
42	Muuroladien-8 Beta-ol		-	-	-	-	0.90	-	-	-	-	-	-
43	Caryopyllene Oxide	43.369	0.84	0.64	1.86	2.63	1.88	-	1.45	0.74	0.53	0.73	0.46
44	Methyl Eugenol	43.832	0.49	2.73	6.00	3.06	2.58	2.96	4.55	2.88	4.72	4.54	3.39
45	Juneol	45.238	-	-	-	0.69	-	-	-	-	-	-	-
46	Spathulenol	46.600	0.81	-	-	0.34	0.58	-	-	-	1.09	2.89	1.07
47	Trans-Cinnamyl Acetate	47.292	-	-	-	0.50		-	-	-	-	-	-
48	Cinnamyl Acetate	47.309	-	-	-	-	-	-	-	2,47	-	-	-
49	Eugenol	47.668	5.27	3.38	1.94	2.47	1.02	1.42	2.31	1.38	2.54	2.84	2.69
50	Methyl Isoeugenol	47.904	-	-	-	0.92	1.46	-	-	-	-	-	-
51	Elemicin	48.886	-	-	0.80			-	-	-	-	-	-
52	α- Eudesmol	48.941	0.47	-	-	0.73	0.62	-	-	0.32	-	-	0.37
53	β- Eudesmol	49.131	0.64	-	1.17	1.19	1.91	0.83	1.03	0.92	0.62	0.63	0.58
54	Intermedeol	49.255	-	-	-	-	0.50	-	-	-	-	-	-
55	Caryophylla-4(12),8(13)-dien-5.beta.-ol	50.635	-	-	-	1.16	0.91	-	0.75	0.50	-	-	-
56	Chavibetol Acetate	51.864	-	-	-	0.64	-	-	-	-	-	-	-
	Total		93.98	98.64	98.78	91.60	92.11	96.79	96.33	92.46	78.07	97.82	96.15

C.N.*: Compound number; RT.**: Retention time.

Our results show high similarity in Karadeniz region. The highest volatile compounds were 1,8-cineole (19.71%-35.63%), α-terpinyl acetate (12.86%-21.24%), sabinene (5.98%-9.40%). Yilmaz et al., reported that 1.8-cineole (51.8 %), α-terpinyl acetate (11.2 %) and sabinene (10.1 %) as the major compounds in their research which is smilar with the results of our study but results is much higher than our results [21].

GC-MS analysis of volatile oils shows that the majority of volatile oil compounds come from monoterpenes, and the majority of these monoterpenes are oxygenated monoterpenes and also Peris and Blazquez reported that oxygenated monoterpenes represented quantitatively the highest concentration of bay leaf volatile oils [22]. 1,8 cineole (19.71% -35.63%), α-terpinyl acetate (12.86% -21.24%), terpinene-4-ol (3.29% -5.84%), δ-terpinyl acetate (1.44%-2.53%) and linalool (0.99%-3.83%) were the most abundant volatile compounds from oxygenated monoterpenes of our research. 1,8-cineole and α-terpinyl acetate were found to be the most abundant volatile compounds among the oxygenated monoterpenes. The results obtained in this respect are similar to the work done by Yalçın et al., but the amount of 1,8-cineol (58.59%) was found to be higher in the relevant study [23].

The other main compounds α-terpinyl acetate (12.86%-21.24%) is a monocyclic monoterpene. It has an ester in its structure [23]. It was reported by Chericoni et al., as a (6.0%), Bouzouita et al., as a (11.20%), Dadalioglu and Evrendilek, as a (16.87%) and Ozcan and Chalcat, as a (4.04-9.87%) [13, 24-26]. On the other hand, the second most common compound among bicyclic monoterpenes are sabinene (5.98%-9.40%), α- pinene (3.67%-8.45%) and β-pinene (2.91%-5.87%). These were the most abundant compound as well for our research. These compounds were reported in these articles as well: Derwich et al., sabinene (6.13%), α-pinene (3.72%) and β-pinene (3.14%) [12], Verdian-rizi, sabinene (5.8% -6.5%), α-pinene (2.6%-3.2%), β-pinene (2.4%-2.9%) [27], Chalchat et al., sabinene (7.07%), β-pinene (2.84%), α-pinene (3.17%) [28] and Moghtader and Salari, α-pinene (%5.25) and β-pinene (%3.99) [29] and also linalool is a bicyclic monoterpene, was found abundant in Bartin province (3.06%-3.83%). It has a significant effects against several symptoms especially in convulsions [30].

Therefore, we detected some sesquiterpenes in lower amounts of our research. These are β-elemene (0.36%-1.89%), caryopyllene oxide (%0.46-2.63%), β- eudesmol (0.58%-1.91%). These compounds have been detected in several essential oils and the other oils have not been contained this compounds. These compounds have inhibitory effect on ethanol absorption [31]. Methyl eugenol (0.49% -6.00%) from the phenylpraponoids was a compound that we have detected in our analysis as a high amounts. Methyl eugenol has an anesthetic and relaxing effect on muscles [23].

4. CONCLUSION

In this study, volatile compounds which were in the content of volatile oil in the leaves of the *Laurus nobilis* L. that grow in Karadeniz Region (Eastern, Western and Central), were revealed and we also aimed to determine if there is any similarity in the essential oils of laurel leaves which are grown in Karadeniz Region. We determined similarities in substances of essential oils. 1,8 cineole is the major compound but the amount of 1.8 cineole is considerably less than most of the previous studies. Since 1.8-cineole is a flavoring compound, if the compound is found in a small amount in volatile oil, it promotes that the taste of the oil can be much softer. The results of our research support, exporting laurel leaf to the EU countries from Karadeniz Region.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Identification of Volatile Compounds (VCs) in the Leaves Collected from 'Gemlik', 'Halhalı' and 'Sarı Hasebi' Olive Tree Varieties

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Abstract: There is a considerably interest on some fruits and leaves extracts such as olive leaf, due to their beneficial health effects. Olive leaf has been consumed as tea for many years. However, the studies on volatile compounds (VCs) of leaves are scarce. Therefore, this study was aimed to evaluate of VCs in the leaves collected from 'Gemlik', 'Halhalı' and 'Sarı Hasebi' olive trees varieties grown in Hatay province. The VCs were analyzed by gas chromatography-mass spectrometry (GC-MS) using solid phase micro-extraction (SPME). The 97 out of 127 VCs identified were found common in all the olive leaves. Terpenes, aldehydes, alcohols and ketones were identified in the olive leaves as major VCs groups, which accounted for about 36-60%, 20-28%, 6-14% and 4-8% of total VCs identified in leaves, respectively. The relative proportions of these chemical groups showed considerably differences among olive leaves. α-Cubebene was found as major VC followed by *trans*-caryophyllene, α-farnesene, *trans*-2-hexenal, benzeneethanol, nonanal, *trans*,*trans*-2,4-heptadienal, cycloisosativene, *trans*-4,8-dimethyl-1,3,7-nonatriene, 2,4-heptadienal, α-humulene, α-muurolene and benzaldehyde. These compounds accounted for 56-75% of total VCs identified in the olive leaves. While 'Halhalı' olive leaf had highest ($p<0.01$) levels of α-cubebene (31.79%), cycloisosativene (7.69%) and α-muurolene (4.05%), 'Sarı Hasebi' had *trans*-caryophyllene (23.16%), *trans*-4,8-dimethyl-1,3,7-nonatriene (4.65%), α-humulene (3.64%) and 'Gemlik' had benzeneethanol (6.93%), nonanal (5.07%), and benzaldehyde (2.17%) at the highest levels. This study has showed that olive leaves from each variety are a good terpene source that makes them important in terms of beneficial effects on health.

Keywords: Olive tree leaves, Volatile compounds, Gemlik, Terpenes

1. INTRODUCTION

Turkey is the fourth biggest olive producing country after Spain, Italy and Greece with 1 768 000 tons in an 826.092 ha area [1]. These Mediterranean countries are producing approximately 65% of the world's total olive production since olive tree belongs to a member of Oleaceae family that is well adapted to Mediterranean basin. Olive is important in context of religion since it has also been praised as a blessed tree and fruit in the Holy Quran. Olive is not used as a natural fruit because of its extremely bitter taste but is rather consumed either as olive oil or as table olive. Olive leaves are known as olive by-products, which are remained during oil extraction, fruit harvesting and olive tree pruning. They are burned, grounded or thrown in away as a by-product that is resulted in environmental pollution or wasting a resource [2].

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According to Herraro et al. [3], olive leaves represent approximately 10% of the weight of olives collected for oil extraction. But, recently olive leaves have been used in folk medicine in Mediterranean regions for arthritic pains, decreasing blood pressure, antimicrobial and antiviral effects, supporting immune system, relax and benefits to the cardiovascular system [4-5-6]. These beneficial effects could be related to phenolic or volatile compounds. According to Talhaoui et al. [2], due to phenolic compounds olive leaf has a matrix rich in antioxidants and also shows anti-inflammatory property. Kubo et al. [7] have reported that olive leaves are the main sources of glycosides, oleuropein and ligstrosides. The degradation of these compounds by a β-glucosidase enzyme resulted in the releasing of volatile compounds such as aldehydes. They may show antimicrobial property. However, detailed studies on the volatile compounds of olive leaves are scarce [8]. Therefore, the specific objects of the present study were; to identify the volatile compounds of olive leaves from different varieties such as 'Gemlik', 'Halhalı' and 'Sarı Hasebi and also if the olive varieties can be grouped based on the volatile compounds identified in the olive leaves.

2. MATERIAL and METHODS

The leaf samples of three olive tree varieties ('Gemlik', 'Halhalı' and 'Sarı Hasebi') were collected from Hatay province located in the Eastern Mediterranean Region of Turkey. 'Gemlik' trees were 10 years old and, 'Halhalı' and 'Sarı Hasebi' were 45 years old. No pesticide was used for pest control in olives. The leaves were randomly collected from each olive tree variety in December month by hand. They were dried in an oven at 30-35 °C for 3 days. Thereafter, VCs analyses of leaves from each variety were carried out according to the procedure described by Guler [9] with minor modification. For VCs analyses, samples were prepared in triplicate from each cultivar. For this purpose, 3 g of dried leaves was separately transferred to a 20 mL head-space vial (Agilent, Palo Alto, CA, USA), adding 3 mL NaCl solution (3% w/v). The vials were sealed using crimp-top caps with TFE-silicone headspace septa (Agilent). The VCs were adsorbed on a fibre consisting of divinylbenzene/ carboxen/polydimetylsilioxan (Supelco, Bellefonte, PA, USA) using Solid Phase Micro-extraction (SPME) technique. For extraction of VCs, the vials were kept at 60°C for 45 min in a water bath then SPME fiber was inserted to the headspace vial and held for 30 min at constant temperature. Desorption of VCs from fiber was performed in the injection port at 250°C for 5 min. The VCs were separated on a HP-Innowax capillary column (60 m x 0.25 mm id x 0.25 μm film thickness; Agilent, USA). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature program was initially held at 50°C for 5 min and then programmed from 50°C by a ramp of 5°C min^{-1} up to 230°C, which was held for 5 min. Identification of VCs was carried out by a computer-matching of their mass spectral data with those of known compounds from the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) library, version 02.L. Based on the peak resolution, their areas were estimated from the integrations performed on selected target ions. Relative percent qualitative recovery per compound was expressed as the target ion response divided by the total target response from the integrated suite of compounds per sample.

All data was subjected to ANOVA and means were compared using Duncan's multiple range test (SPSS Version 17.0; SPSS Inc., Chicago, IL, USA) to determine statistically differences at $p<0.05$ level. All the data on VCs obtained from olive leaves were used for discriminant function analysis based on Eigenvalues.

3. RESULTS and DISCUSSIONS

To our knowledge, this study is the first report on VCs identified in the leaves of the different olive varieties. Thus, it would be very difficult to compare them now. As shown in Table 1., a total of 127 VCs were identified in experimental olive leaves. VCs were grouped

according to their chemical classes and the profile was composed of 27 terpenes (21 sesquiterpenes and 6 monoterpenes), 22 aldehydes, 17 ketones, 15 alcohols, 14 phenyls and phenols, 11 esters, 8 alkenes, 7 alkanes, 3 furans and 3 the others. 'Gemlik' and 'Sarı Hasebi' leaves had a similar VC number whereas 'Halhalı' olive leaf had the less VC number. Twenty-six VCs are listed in Table 2, which constituted the majority of volatile compounds accounting for 75%, 69% and 56% of total VCs identified in 'Halhalı', 'Sarı Hasebi' and 'Gemlik' olive leaves, respectively. 'Gemlik' variety was considerably different from 'Sarı Hasebi' and 'Halhalı', in terms of the percent proportions of volatile compounds identified in the leaves. As shown in Tables 1 and 2., and in Fig. 1, terpenes, especially sesquiterpenes, were the major chemical group found in all the olive leaves, in terms of their number and their percentage composition. A similar tendency was obtained for *Ficus carica* leaves which were rich in potential health-promoting phytochemicals [10].

While the main terpene and also VC identified in the headspace of 'Gemlik' and 'Sarı Hasebi' olive leaves was *trans*-caryophyllene, it was α-cubebene in 'Halhalı' leaf. *Trans*-caryophyllene (TC) is an important constituent of the essential oils derived from several species of medicinal plants such as *Cordia verbenacea.* The recent studies have indicated that TC had multiple pharmacological effects including anti-inflammation, anti-apoptosis and neuroprotection [11]. When compared with 'Halhalı' and 'Gemlik' olive leaves, 'Sarı Hasebi' leaf is one of important tools for the management and/or treatment of especially inflammatory diseases due to its considerably high *trans*-caryophyllene and also α-humulene (α-caryophyllene) percentages [11].

Tablo 1. The volatile compounds identified in olive leaves

Volatile Compounds (127)	RT	RI	Gemlik	Halhalı	Sarı Hasebi
Terpenes (27)					
Monoterpenes (6)					
γ-Terpinene	16.55	1398	+	+	+
Styrene	16.98	1423	+	+	+
trans-4,8-Dimethyl-1,3,7-nonatriene	18.43	1511	+	+	+
cis-α-Bisabolene	28.92	>2100	+	+	-
Camphene	29.09	>2100	+	+	+
Allo ocimene	33.68	>2100	+	+	+
Sesquiterpenes (21)					
α-Ylangene	22.98	1873	+	+	+
Cycloisosativene	23.75	1943	+	+	+
α-Cubebene	23.99	1965	+	+	+
β-Bourbonene	24.56	2022	+	-	+
β-Gurjunene	26.18	>2100	+	+	+
β-Cubebene	26.66	>2100	-	+	+
trans-Caryophyllene	26.86	>2100	+	+	+
α-Gurjunene	27.25	>2100	+	+	+
Caryophyllene	28.49	>2100	+	-	+
α-Humulene	28.65	>2100	+	+	+
α-Amorphene	28.99	>2100	+	+	+
Zingiberene	29.57	>2100	+	+	+
α-Copaene	29.72	>2100	+	-	+
α-Muurolene	29.80	>2100	+	+	+
α-Farnesene	30.14	>2100	+	+	+
Δ-Cadinene	30.56	>2100	+	+	+
α-Amorphene	30.68	>2100	+	-	+
trans-β-Farnesene	31.01	>2100	+	+	+
γ-Muurolene	31.41	>2100	+	-	+
Epi-ligulyl oxide	31.50	>2100	+	+	+
cis-Calamenene	32.33	>2100	+	+	+

Table 1. (Continued)

Aldehydes (22)					
cis-2-Butenal	8.95	1083	+	+	+
Hexanal	10.43	1138	+	+	+
trans-Citral	13.71	1262	+	+	+
Heptenal	14.39	1291	+	+	+
trans-2-Hexenal	15.65	1353	+	+	+
Octanal	18.00	1484	+	+	+
trans-2-Heptenal	19.16	1561	+	+	+
Nonanal	21.18	1800	+	+	+
trans,trans-2,4-Hexadienal	21.64	1754	+	+	+
2-Octenal	22.30	1811	+	+	+
trans,trans-2,4-Heptadienal	23.33	1905	+	+	+
2,4-Heptadienal	24.15	1979	+	+	+
Benzaldehyde	25.09	2072	+	+	+
trans,trans-2,6-Nonadienal	26.51	>2100	+	+	+
2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde	27.49	>2100	+	+	+
trans-2-Decanal	27.88	>2100	+	+	+
Safranal	28.12	>2100	+	+	+
trans,trans-2,4-Nonadienal	29.35	>2100	+	+	+
2-Undecenal	30.42	>2100	+	+	+
trans,cis-2,4-Decadienal	30.75	>2100	+	+	+
Tridecanal	31.76	>2100	+	-	+
4-Methoxy-benzenaldehyde	36.62	>2100	+	+	+
Ketones (17)					
6-Methyl-5-hepten-2-one	19.51	1586	+	+	+
11H-Dibenzo[b,e][1,4]diazepin-11-one, 5,10-dihydro-5-[3-(methylamino)propyl]-	23.44	1915	+	+	+
3,5-Octadien-2-one	24.58	2024	+	+	+
4,8-Dimethyl-nona-3,8-dien-2-one	25.65	>2100	+	-	-
3,5-Octadiene-2-one	26.13	>2100	+	-	-
6-Methyl-3,5-heptadien-2-one	26.70	>2100	+	-	-
Phenyl methyl ketone	28.31	>2100	+	+	+
Glycocyanidine	29.26	>2100	+	+	+
β-Damascenone	32.09	>2100	+	+	-
trans-Geranylacetone	32.62	>2100	+	+	+
3-Phenyl-2-nutanone	33.02	>2100	+	+	+
β-Ionone	34.62	>2100	+	+	+
2,3-Epoxy-β-ionone	35.77	>2100	+	+	+
cis-Cinerolone	36.84	>2100	+	+	+
6,10,14-Trimethyl-2-pentadecanone	37.99	>2100	+	+	+
3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	41.02	>2100	+	+	+
5,6,7,7a-Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	43.03	>2100	+	+	+
Alcohols (15)					
Hexan-1-ol	19.79	1605	+	+	+
cis-3-Hexen-1-ol	20.80	1684	+	+	+
1-Octen-3-ol	22.60	1838	+	-	+
cis-1,5-Octadien-3-ol	23.62	1931	+	+	+
Octan-1-ol	25.46	>2100	+	+	+
2,6-Dimethyl-cyclohexanol	27.01	>2100	+	+	+
1-Nonanol	28.01	>2100	+	+	+
β-Citronellol	30.48	>2100	+	+	-
α-Methyl-benzenemethanol	31.81	>2100	+	+	+
Benzenemethanol	33.24	>2100	+	+	+
Benzeneethanol	34.02	>2100	+	+	+
cis-Farnesol	34.22	>2100	+	+	+
Nerolidol	36.27	>2100	+	+	+
(1S*,6S*,7S*)-Tricyclo[5.3.2.0(1,6)]dodecan-7-ol	38.96	>2100	+	+	+
D11-Dodecene-1-ol	39.31	>2100	+	-	+

Table 1. (Continued)

Phenyls and Phenols (14)					
1,2,3,4-Tetramethylbenzene	17.32	1443	+	+	-
Safranal	22.78	1855	+	+	+
trans-Anethole	32.23	>2100	+	+	+
2-Methoxy-phenol	32.95	>2100	+	+	+
2-Methyl-naphthalene	33.83	>2100	+	+	+
2-Methoxy-4-methyl-phenol	34.96	>2100	+	+	+
Phenol	35.90	>2100	+	+	+
2-Propenyl-benzene	36.71	>2100	+	+	+
2,6-Dimethyl-phenol	37.32	>2100	+	-	-
4-Methyl-phenol	37.40	>2100	+	-	-
m-Cresol	37.55	>2100	+	-	+
2-Methoxy-4-(2-propenyl)-phenol	39.12	>2100	+	-	+
Tymol	39.78	>2100	+	+	+
Biphenylene	40.05	>2100	+	+	+
Esters (11)					
Benzoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	13.43	1250	+	+	+
Heptanoic acid, methyl ester	17.83	1474	+	-	+
Hexanoic acid, methyl ester	21.00	1699	+	-	+
Nonanoic acid, methyl ester	23.85	1952	+	-	+
Tiglic acid, *cis*-3-hexenyl ester	28.39	>2100	+	+	+
Acetic acid, 2-phenylethyl ester	30.03	>2100	+	+	+
Benzoic acid, 2-hydroxy-, methyl ester	31.27	>2100	+	+	+
Acetic acid, 2-phenylethyl ester	31.96	>2100	+	+	+
Benzoic acid, *cis*-3-hexenyl ester	38.35	>2100	+	+	+
Hexadecanoic acid, methyl ester	39.67	>2100	+	+	+
Phthalic acid, ethyl ester	42.94	>2100	+	+	+
Alkenes (8)					
7-Methyl-3,4-octadiene	20.17	1635	+	+	+
3,7-Dimethyl-1-octene	20.29	1644	+	+	+
trans-3,5-Dimethyl-1,6-octadiene	21.45	1738	+	+	-
2,5-Dimethyl-2,4-hexadiene	24.53	2019	+	-	+
4,8-Dimethyl-1,7-nonadiene	25.88	>2100	+	-	+
1,2,4,4-Tetramethyl-cyclopentene	27.63	>2100	+	+	+
cis-2,6-Dimethyl-2,6-octadiene	28.17	>2100	+	+	+
1,4-Diethyl-1,4-dimethyl-2,5-cyclohexadiene	33.34	>2100	+	+	+
Alkanes (7)					
9-Methyl-nonadecane	22.16	1798	+	+	+
5-Methyl-dodecane	25.00	2064	+	-	+
8-Hexyl-pentadecane	25.57	>2100	+	-	+
Hexadecane	26.38	>2100	+	+	+
Heptadecane	28.79	>2100	+	+	+
cis-1,2-Divinylcyclohexane	30.23	>2100	+	+	+
Cyclododecane	34.77	>2100	+	+	+
Furans (3)					
2-(2-Propenyl)-furan	27.38	>2100	+	+	+
β-Agarofuran	29.18	>2100	+	+	+
Dihydro-β-agarofuran	32.45	>2100	+	+	+
Others (3) (Unkown-Alkyne-Acid)					
Unknown	18.94	1546	+	+	+
1,4-Dimethoxy-2-butyne	27.73	>2100	+	+	+
trans-3-Hexenoic acid	34.71	>2100	+	-	+

RI: retention index based on the identified VCs retention time (RT) and calculated from a linear equation between each pair of straight alkanes (C5-C25).; + and -: compounds are identified and unidentified in leaves, respectively.

α-Cubebene identified in 'Halhalı' olive leaf as the major VC is a dibenzocyclooctadiene lignin and it has a potential antioxidant property [12]. It has previously been identified in *Schisandra chinensis* herb as the most plentiful VC [13]. This plant is a well-known medicinal herb that ameliorates cardiovascular symptoms. Benzenethanol, cycloisosativene and α-farnesene was identified in Gemlik, 'Halhalı' and 'Sarı Hasebi' olive tree leaves as the second most abundant VCs, respectively. Benzenethanol (Phenylethyl alcohol) was the main component of Rose oils obtained from rose blossom, which is used in ophthalmic drug products as preservative and in food and also cosmetic industries as a flavoring substance [14].

Table 2. The relative percentages of the major volatile compounds (VCs) identified in 'Gemlik', 'Halhalı' and 'Sarı Hasebi' olive leaves.

Major Volatile Compounds (26)	Gemlik	Halhalı	Sarı Hasebi	Analyses of Variance
α-Cubebene	5.54±0,65^{b}	31.79±1,74^{a}	3.56±0,28^{b}	F(148,5) = 423.4, p< .001
trans-Caryophyllene	11.17±1,19^{b}	3.85±0,12^{c}	23.16±1,08^{a}	F(148,5) = 219.9, p< .001
α-Farnesene	6.69±0,74	5.55±1,22	9.52±1,35	F(9,55) = 6.50, p> .05
trans-2-Hexenal	5.18±0,47	6.86±0,59	7.30±1,33	F(9,55) = 3.15, p> .05
Benzeneethanol	6.93±0,66^{a}	3.09±0,13b	3.98±0,78^{b}	F(9,55) = 22.6, p< .05
Nonanal	5.07±0,20^{a}	3.05±0,54^{b}	4.03±0,12^{ab}	F(9,55) = 17.5, p< .05
trans,trans-2,4-Heptadienal	4.28±0,06	3.60±0,33	3.84±0,24	F(9,55) = 4.05, p> .05
Cycloisosativene	1.20±0,08^{b}	7.69±0,17^{a}	0.88±0,02^{b}	F(148,5) = 2432.6, p< .001
trans-4,8-Dimethyl-1,3,7-nonatriene	2.65±0,22^{ab}	1.79±0,07^{b}	4.65±1,07^{a}	F(9,55) = 10.72, p< .05
2,4-Heptadienal	3.10±0,05	2.07±0,40	2.56±0,18	F(9,55) = 8.22, p> .05
α-Humulene	1.85±0,05^{b}	0.64±0,01^{c}	3.64±0,05^{a}	F(148,5) = 2778.5, p< .001
α-Muurolene	0.43±0,01^{b}	4.05±0,45^{a}	0.31±0,03^{b}	F(30,82) = 135.8, p< .01
Benzaldehyde	2.17±0,06^{a}	1.25±0,04^{b}	1.18±0,14^{b}	F(30,82) = 74.56, p< .01
Benzenemethanol	2.07±0,20^{a}	0.90±0,03^{b}	1.13±0,20^{b}	F(9,55) = 28.03, p< .05
11H- Dibenzo[b,e][1,4] diazepin-11 one, 5,10-dihydro-5-[3-(methyl amino)propyl]-	1.32±0,62	1.39±0,25	1.29±0,45	F(9,55) = 0.023, p> .05
Benzoic acid, 2-hydroxy-, methyl ester	1.40±0,05^{a}	0.68±0,18^{b}	1.91±0,26^{a}	F(9,55) = 22.66, p< .05
6-Methyl-5-hepten-2-one	1.62±0,07	1.10±0,04	1.10±0,31	F(9,55) = 5.05, p> .05
3,5-Octadien-2-one	1.49±0,03^{a}	0.44±0,05^{b}	1.24±0,17^{a}	F(30,82) = 50.59, p< .01
γ-Terpinene	0.94±0,19	0.85±0,33	1.36±0,83	F(9,55) = 0.54, p> .05
Unknown	0.70±0,29^{b}	1.79±0,20^{a}	0.64±0,14^{b}	F(9,55) = 17.48, p< .05
trans-3,5-Dimethyl-1,6-octadiene	1.76±0,23^{a}	1.32±0,15^{a}	nd	F(30,82) = 66.20, p< .01
Styrene	1.40±0,46	0.41±0,05	1.03±0,33	F(9,55) = 4.71, p> .05
3,7-Dimethyl-1-octene	0.76±0,07^{b}	1.38±0,02^{a}	0.44±0,00^{c}	F(148,5) = 263.5, p< .001
2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde,	1.00±0,03^{a}	0.29±0,12^{b}	0.49±0,04^{b}	F(30,82) = 48.39, p< .01
trans-2-Decanal	0.99±0,15^{a}	0.26±0,07^{b}	0.42±0,11^{b}	F(9,55) = 23.52, p< .05
β-Cubebene	nd	1.16±0,12^{a}	0.50±0,02^{b}	F(148,5) = 153.4, p< .001

Mean values (n = 3) followed by different letters in the same row indicate significant differences (p< 0.05) for the olive leaves. nd: not detected.

Cycloisosativene is one of the major compounds found in *Euphorbia macrorrhiza* which is one of medicinal plants used in the treatment for skin diseases, gonorrhea, migraine, intestinal parasites and warts [15]. Cycloisosativene identified in 'Halhalı' leaf as the second most abundant VC was significantly higher than that of the other olive varieties. While *trans*-2-hexenal was the third most abundant VC in 'Sarı Hasebi' and 'Halhalı' olive leaves, α-farnesene was in 'Gemlik' olive leaf. Both *trans*-β- and α-farnesene occur in a wide range of plant and animal species. *Trans*-β-farnesene molecule was identified to be effective kairomone for the predators. The aphids releasing *trans*-β-farnesene alone were found to be attractive for *Adalia bipuncata* L. [16]. Additionally, β-farnesene has a bitter flavor note, which is high in some hop varieties with bitter flavor [17].

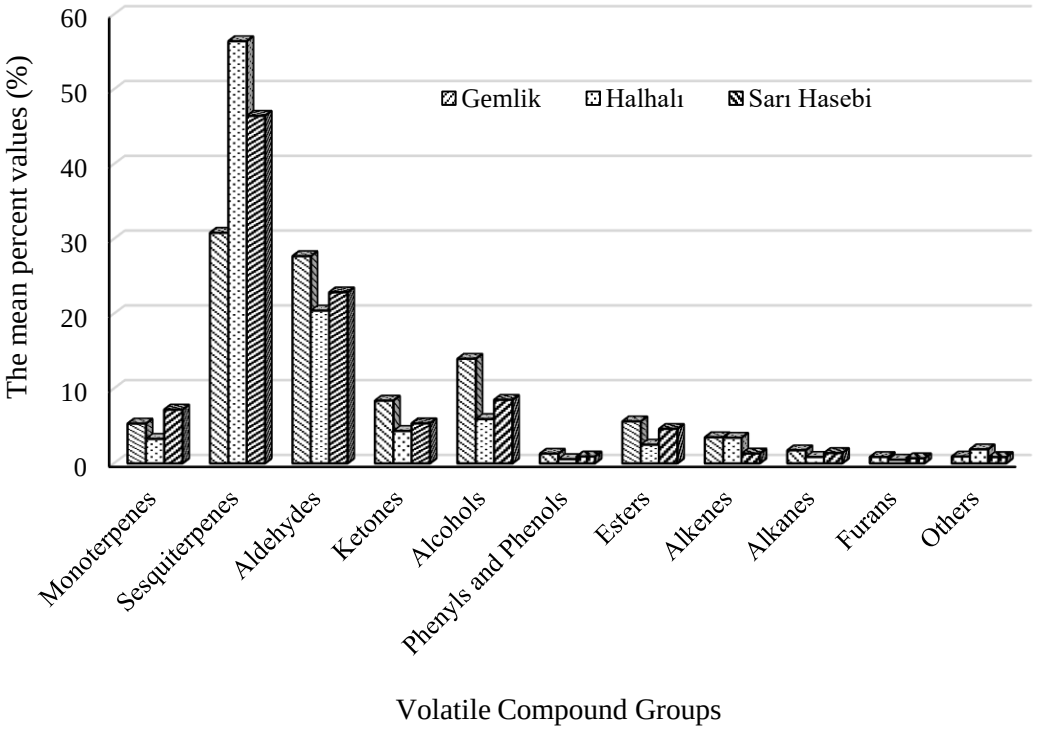

Figure 1. The mean percent values of volatile compound groups of olive leaves according to chemical families.

Another major compound, *trans*-2-hexenal, has an activity against a large number of microorganisms [8]. α-Muurolene is considerably high in 'Halhalı' leaf, compared with the other varieties. It was previously found to be in essential oil of *Calendula officinalis* flowers [18]. On the other hand, 'Halhalı' leaf had the lower percentages of *trans*-4,8-dimethyl-1,3,7-nonatriene and α-humulene than those in 'Gemlik' and 'Sarı Hasebi' leaves. These VCs have been considerably high, especially in 'Sarı Hasebi' leaf. *Trans*-4,8-dimethyl-1,3,7-nonatriene has been previously identified in Bergamot essential oil and maize leaves, which is a defense chemical compound against to herbivores [19]. Nonanal was identified in all the leaves but it was considerably higher in 'Gemlik' leaf than the other varieties. This saturated aldehyde is derived from the oxidation of linoleic acid by 9-lipoxygenase. This finding indicates that 'Gemlik' olive may have had the higher linoleic acid content, followed by 'Sarı Hasebi' and 'Halhalı'. Actually, the fatty acid composition of olive fruit may be estimated by the determining of the profiles of VCs in olive leaves. It is even possible to make a comparison between olive varieties.

VCs including β-bourbonene, caryophyllene, α-cubebene, α-amorphene, γ-muurolene, tridecanal, 1-octen-3-ol, D11-dodecene-1-ol, m-cresol, 2-methoxy-4-(2-propenyl)-phenol,

heptanoic acid methyl ester, hexanoic acid methyl ester, nonanoic acid methyl ester, 2,5-dimethyl-2,4-hexadiene, 4,8-dimethyl-1,7-nonadiene, 5-methyl-dodecane, 8-hexyl-pentadecane, trans-3-hexenoic acid were routinely identified in 'Gemlik' and 'Sarı Hasebi' olive leaves whereas they were not found in 'Halhalı' olive leaf. The remaining 29 VCs were sporadically identified in all the leaves.

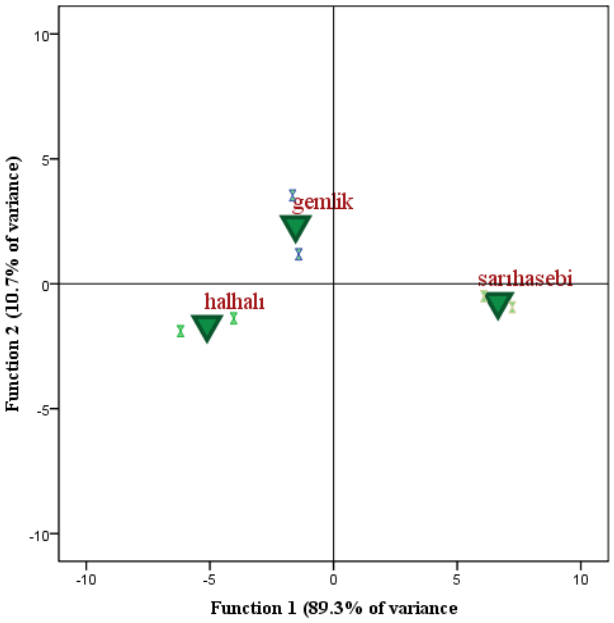

Figure 2. Discriminant analysis of the percentage composition of volatile compounds (VCs) in ''Gemlik'', ''Halhalı'' and ''Sarı Hasebi'' olive leaves.

Discriminant analysis was applied to data on VCs obtained from the leaves of three olive varieties analyzed. According to discriminant analysis based on Eigenvalues, VCs identified in the olive leaves could be used to discriminate and classify the olive tree varieties (Fig. 2). As shown in Fig. 2., the olive varieties were completely different from each other. Table 3 shows that the olives in each discriminant function, in proportion to the magnitude of their variation value (bold numeric), are independent from the olives in the other discriminant function.

Table 3. Results of discriminant function on the volatile compounds of olive varieties.

Factors	**Discriminant function**	
	1	**2**
Gemlik	-1.534	**2.357**
Halhalı	**-5.117**	-1.639
Sarı Hasebi	**6.651**	-0.718
Eigenvalues	48.5109	5.8399
Explained variance%	89.3%	10.7%

Olive varieties in the same discriminant function are related to each other, according to positive and negative variation. According to discriminant function, 'Halhalı' olive leaf showed a negative variation with 'Sarı Hasebi', and also both was related with each other. 'Halhalı' olive leaf had the highest α-cubebene, cycloisosativene and α-muurolene, and also the lowest *trans*-caryophyllene and α-humulene percentages. However, in terms of VCs mentioned, 'Sarı Hasebi' olive leaf has a tendency opposite to that of 'Halhalı' leaf.

The leaf of 'Gemlik' olive variety was clearly different from the other two varieties (Fig. 2. and Table 3.) since it had the highest benzenethanol, benzenealdehyde, nonanal and benzenemethanol percentages. Therefore, the olive variety groups of 'Gemlik', 'Halhalı' and 'Sarı Hasebi' can be grouped based on the volatile compounds identified in their leaves. Additionally, volatile compounds in olive leaves may be a tool for the assessment of olive origin as varietal markers. However, future studies should increase the number of varieties for accurate generalization.

4. CONCLUSION

This study confirmed that the profile and proportions of VCs identified in the leaves collected from olive trees changed depending on the variety. In terms of the proportion of volatile compounds identified in headspace of olive leaves, 'Gemlik' variety was considerably different from 'Sarı Hasebi' and 'Halhalı' varieties. According to discriminant analysis, VCs such as α-cubebene, cycloisosativene, α-muurolene, *trans*-caryophyllene, α-humulene, benzenethanol, benzenealdehyde, nonanal and benzenemethanol can be used to discriminate and classify the olive varieties. In general, the major volatile compounds identified in the leaves of three olive tree varieties were similar to those in medical plants. Thus, olive tree leaves may be used in folk medicine or in food supplements as a raw material. In addition, some VCs identified in olive leaves such as nonanal may give an idea of the fatty acid composition of olive fruit. An important result of this work is that olive tree varieties may be discriminated or classified by volatile compound contents of olive leaves and also a SPME method can be used as a quality assessment system.

Conflict of Interests

Authors declare that there is no conflict of interests.

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The Influence of Licorice Root (*Glycyrrhiza glabra*) on Sex Reversal in Guppy *Poecilia reticulata*

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Abstract: The roots and rhizomes of licorice (Glycyrrhiza) species have long been used worldwide as a herbal medicine and natural sweetener. Licorice is commonly present in menopausal botanical supplements in the United States. The estrogenic activities of different licorice species are variable and likely depend on the type and amounts of bioactive compounds. This study examined the effects of Licorice root extract on sex reversal in guppy, *Poecilia reticulata*. Newly born guppy fry (0.014 ± 0.001 mean weight), were randomly distributed into 30 L aquaria at a density of 80 fish per aquarium and subjected to a sex-reversal treatment by immersion application of licorise root extract for 30 days. The licorise root extract was incorporated into the aquarium water as follows: 0 (control), 0.25 and 0.5 g/ L licorice root extract. Each of the treatment group was randomly assigned to triplicate groups of fish. The treatment was repeated twice (in the beginning and half way through the experiment) during 30 days. At the end of experiment, the highest feminization (88%) was observed at 0.5 g\L licorise root extract group. Morphological and histological examinations of the gonads in all groups revealed no intersex fish. Histological examination of fish treated with licorise root revealed no damage to the testes or ovaries. This study demonstrated successful sex reversal with treatment of licorise root on new-born progenies of *P. reticulate.*

Keywords: Guppy, *Poecilia reticulata*, Licorice Root, *Glycyrrhiza glabra*, Sex reversal

1. INTRODUCTION

In last decade, the total world fishery production decreased slightly and the human consumption for aquatic product increased. The reduction in capture fisheries was partly compensated for the fast growth of aquaculture industry and this sector is the world's fastest growing sector of agricultural [1]. With the increasing intensification and commercialization of aquaculture production, fish culture is globally expanding into new directions, intensifying and diversifying for enhanced disease resistance, feed efficiency, and growth performance of cultured organisms. Therefore, several growth promoters and hormones have been tested for enhancing feed conversion efficiency and for increasing fish culture productivity [2]. However, the recent consumer demand for farmed fish has increasingly stressed quality and safety, and the absence of pollutants, antibiotics and carcinogens. Thus, along with growth performance, the fish rearing strategy needs to focus on food hygiene. This global demand for safe food has prompted the search for natural alternative growth promoters to be used in aquatic feeds. There has been heightened research in developing new dietary supplementation strategies in which

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various health and growth promoting compounds as probiotics, prebiotics, synbiotics, phytobiotics and other functional dietary supplements have been evaluated [3].

Phytooestrogens are estrogenic compounds found in >300 species of plants that can mimic oestrogen activity [4]. Phytooestrogens of current medical interest are the isoflavones, which are widely used as oestrogen replacement in human post-menstrual treatment and as a breast cancer preventive treatment [5]. A conspicuous feature of the chemical structure of phytooestrogen is the presence of a phenolic ring that, with few exceptions, is prerequisite for binding to the oestrogen receptor (ER). It was initially believed that phytooestrogens action would be predominantly hormonal, but as this field of study has expanded over the past decade, it has become apparent from the diversity of properties of isoflavones that no single action can explain many of the in vivo and in vitro effects in the same organism [5, 6]. The oestrogenic potency of these compounds on fish has been documented [7-11].

The roots and rhizomes of licorice (Glycyrrhiza) species have long been used worldwide as a herbal medicine. The active component of licorice, glycyrrhizic acid, is hydrolyzed in vivo to glycyrrhetinic acid, which is responsible for most of its pharmacological properties. In ancient Chinese medicine and during Roman times, licorice was also recommended to cure sterility of women [12, 13]. Theophrastus and Pliny also reported an efficacy of licorice in reducing hunger and thirst [13, 14]. In addition, licorice extract has some estrogen-like, anti-viral, and antioxidant properties [12]. The estrogenic activities of different licorice species are variable and likely depend on the type and amounts of bioactive compounds.

Licorice root (*Glycyrrhiza glabra*) is also able to affect androgen metabolism in human [13]. Various studies have demonstrated that licorice blocks the activity of 3-β-hydroxysteroid dehydrogenase (3HSD), 17- hydroxysteroid dehydrogenase (17HSD) and 17–20 lyase [13, 15] and stimulates the activity of aromatase [16-18]. All these enzymes are involved in the synthesis and/or metabolism of androgens and estrogens. A possible compensatory mechanism is the binding of glycyrrhetinic acid to sex binding globulin, thus increasing free testosterone and estradiol values [19].

Considering these aspects, the present study was focused to evaluate the efficacy of licorise root as potent alternatives for induction of sex reversal in fish. The live-bearing guppy, *Poecilia reticulata* is a popular freshwater species among aquarium hobbyists. In this study, guppies have used as a model fish for investigating the influence of Licorice root (*Glycyrrhiza glabra*) on sex reversal in fish.

2. MATERIAL and METHODS

Newly born guppy fry (0.014 ± 0.001 mean weight), were randomly distributed into 30 L aquaria at a density of 80 fish per aquarium and subjected to a sex-reversal treatment by immersion application of licorise root extract for 30 days.

Licorise root was provided from Hatay province (Turkey). The aqueous extracts of Licorice root was prepared by boiling 7.5 and 15 g Licorice root in 250 ml distilled water for 30 min and then filtering it with a whatman filter paper twice [20,21]. The licorise root extract was incorporated into the aquarium water as follows: 0 (control), 0.25 and 0.5 g/ L licorice root extract. The prepared solutions were poured in the aquariums. The aquariums were aired strongly to spread the solutions in water. Each of the treatment group was randomly assigned to triplicate groups of fish. The treatment was repeated twice (in the beginning and half way through the experiment) during 30 days [10]. The water of the aquaria was changed entirely every 15 days. The aquaria were controlled temperature (26 ± 1 °C) and equipped with aeration. The photoperiod was maintained on a 12-h light: 12-h dark schedule. A control group was also included in this experiment. Hatchlings were fed ad-libitum three times a day until satiation.

The basic food was ornamental fish diet (Aquamaks, Turkey: 28% protein, 12% lipid (on wet basis)). After completion of the treatment, they were transferred to larger aquaria. When individuals were 2 months old, the sex of each was determined by external examination, with gonopodium and other morphological characters [22]. In addition, for histological examination of gonads, guppy were fixed in10% neutral formalin. After fixing the samples, specimens, excluding head and caudal regions were processed for histology using routine de- hydration and parafin-embedding procedures. Cross-sections of thickness 4-6 mm, were stained with Mayer's haematoxylin and eosin phloxine B solution examined, and microphotography [23]. Growth and survival rate were monitored to determine the growth in each treatment groups during the experiment. Each fish was individually anesthetized with 300 mg L^{-1} lidocaine-HCL\1000 mg L^{-1} $NaHCO_3$ [24], weighed and measured (total length) to the nearest 0.0001 g and 0.01 cm, respectively. A Chi-square (χ^2) test was used to determine whether observed sex ratios are different from an expected 1:1. Also, differences in growth were assessed by one-way ANOVA test and Duncan test was used to analyze which dosage groups cause the difference [25].

3. RESULTS and DISCUSSIONS

In the present investigation, both 0.25 and 0.5 g/L groups of licorise root extract– treated fish exhibited more female number than male number comparing to the control group. The sex ratio in the *P. reticulata* in these groups significantly changed ($p<0.001$; Table 1) and the highest feminization (88.35%) was observed at 0.5 g\L licorise root extract group. Also, the sex ratio observed in 0.25 g/L groups of licorise root extract– treated fish was 137:70 (female:male); this difference was statistically significant. The sex ratio observed for the *P. reticulata* was nearly the expected ratio of 1:1 (female:male) in control group. (Table, 1). Morphological and histological examination of the gonads in all treatment groups revealed that no intersex fish were identified in guppy. Histological examination of fish treated with licorise root revealed no damage to the testes or ovaries.

Table 1. Effects of treatment with Licorise root extract on sex reversal of the *Poecilia reticulata.*

	Dose (g/L)	Treatment Duration (day)	Sex Distribution (n) ♀ : ♂	Sex Ratio (%) ♀ : ♂	χ^2
Licorise root extract	0	30	102 : 107	48.80 : 51.20	-
	0.25	30	137 : 70	66.18 : 33.82	12.851^{***}
	0.5	30	182 : 24	88.35 : 11.65	75.105^{***}

χ^2 values are for comparisons of sex ratios with the control (*,P<0.05; **, P<0.01; ***, P<0.001).

Table 2 shows the survival rate and growth rates in total length and body weight of the *P. reticulata*. Total survival rates and growth rate in all treatments and controls were uniformly high ranging from 85.83% to 87.08% and 0.197 to 0.189 g respectively ($p>0.05$). This indicates that Licorise root extract has no negative effect on survival and growth rate of *P. reticulata* at the tested concentrations (Table 2).

Table 2. Effects of treatment with Licorise root extract on survival and growth of the *Poecilia reticulata.*

	Dose (g /L)	Survival Rate (%)	Total length (cm)	Body weight (g)
Licorise root extract	0	87.08 ± 1.10^{a}	1.85 ± 0.06^{a}	0.197 ± 0.01^{a}
	12.5	86.25 ± 0.72^{a}	1.79 ± 0.08^{a}	0.192 ± 0.01^{a}
	25	85.83 ± 0.42^{a}	1.80 ± 0.11^{a}	0.189 ± 0.01^{a}

Values (mean±S.E. of triplicate) with same superscripts in each column indicate insignificant differences (P>0.05) (Water temperature 26±1ºC).

To our knowledge, this is the first report regarding the potential of Licorise root extract as a feminization agent in ornamental fish. The 0.5 g\L licorise root extract group was the most potent dose in this study, having duration of 30days for feminization. Although the treatment of the newly-born progenies using Licorise root extract significantly increased the percentage of females to 88.35%, we cannot conclude that this potency was caused by an increase of estrogen since we did not measure plasma estrogen levels. The Licorise root extract did not produce 100% females; a higher dose may have generated better results. Most literature on Licorise treatments in humans [13,26,27] and rats [28,29] endocrine system because it contains isoflavones (phytoestrogens), which are chemicals found in plants that may mimic the effects of estrogen. Licorice may also reduce testosterone levels, which can contribute to hirsutism in women. Therefore, usage of a higher dose may lead to the production of all-female *P. reticulata* population. Most authors have reported similar observation for other fish species, treated with various phytoestogens [8,9,10,11,30]. However, the use of of Licorice plant in fish studies was rather limited so far [7]. Also, survival rate of the Licorise -treated *P. reticulata* was similar to that of control group of the experiment. Similarly, survival was high in studies on guppy *P. reticulata* and zebra cichlids *C. nigrofasciatum* [10].

In summary, this study demonstrated that sex differentiation of guppies was successfully diverted toward the female gender with treatment of licorise root extract. The findings of this study indicate the probability of using herbal compounds as an alternative method for production of all-female guppy population. However, further studies will be required to determine the optimum treatment regime for induction of 100% sex reversal with these chemicals in guppy.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Effects on Antibacterial and DNA Protection of Organic Dyestuff Extracts Obtained from Hazelnut Nuthusk

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Abstract: Hazelnut is of great important in agricultural export of our country. It has an annual export of one and a half billion dollars. Every new product that will increase the value of the nut is very important. In recent years, natural dye materials used especially in textile materials are important and those of plant origin are preferred. For this purpose, we have made a natural dye extracts from hazelnut nuthusk after collected, dried and grinded of samples. Some biological activities have been carried out showing the suitability for industrial use of the dye extracts obtained from hazelnut nuthusk. Within the scope of our study, the effect of extracts obtained from the hazelnut nuthusk of the Düzce region was evaluated on anti-bacterial and DNA protection. From the extracts obtained, antibacterial activity tests were analyzed by disk diffusion method using 3 different standard bacterial strains as S. aureus, *E. coli* and *P. aeruginosa*. pBR322 plasmid DNA and UV-C method was used for DNA protective activity. Four different extracts of dyestuff has showed antibacterial activities to S. Aureus and two different of them *P. aeruginosa* but none of the samples antibacterial properties to E. coli bacterial strains. It has been determined that the all of dyestuffs extracts obtained from the hazelnut nuthusk have potential for protection against the effects of UV-C and H_2O_2 on DNA.

Keywords: UV-C, reflux, H_2O_2, DNA

1. INTRODUCTION

Before the discovery of the synthetic dyestuff agents in 1876, all the dyestuff processes were made with natural materials [1]. Nowadays, it has become clear that the synthetic dyestuff agents have harmful effects both on the people as well as on the environment since a majority of them are carcinogenic or create carcinogenic intermediates with fragmentation [2]. For this reason, people are turning to the natural dyestuff agents like in the past years which can be divided into two groups including artificial and natural. The main materials used for the synthesis of the artificial dyestuff agents and pigments are coal tar compounds known as aromatic hydrocarbons. The petrochemical industry is gaining importance in providing these materials, while the natural dyestuff agents are obtained from natural materials found in the nature. It is known that the herbal dyestuff agents are obtained from plants which are found in the nature and have dyestuff features. While the whole of some plants are used for coloring,

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there are also plants whose certain parts (flowers, leaves, roots and nark etc.) are used only. The disadvantage of the natural dyes is that they can be produced in a low amount and that it is difficult the catch the same color tone [3]. The advantage of the natural dyestuff agents is that they do not pose a risk to health and do not create environmental pollution as the synthetic dyestuff agents. Textile industry is a sector that uses excess amount of water therefore it generates excessed amount of wastewater too. The content of these wastewaters consists of fiber impurities, used dyestuff agents and various chemicals. The dyestuff agents that are included within the wastewater give color to this water which destroys the aesthetic appearance of the water and also reduces the amount of the dissolved oxygen which is the life source of the living creatures within the aquatic environment by reducing the light permeability and causes the death of these living creatures. Considering that the share of the textile industry in Turkey is 20 % in the industrial production, the necessity of the treatment of the textile wastewater becomes more important. The direct disposal of such wastes into the water is dangerous and may cause infection [4].

As a result of chemical and biological changes the organic materials such as dyes and detergents which are included within the wastes cause the dissolution of the dissolved oxygen in the water and cause the destruction of the fishes' life environment. However, naturel dyestuff agents are biodegradated quickly in the naturel cycle [5]. This demand will be ensured when the textile materials are colored with natural dyestuff agents. Among the natural dyestuff agents, the plant coloring agents are the most widely used. These can be obtained from plants called dyestuff plants or from plant wastes. Turkey which is one of the countries having the most favorable conditions for planting and growing hazelnuts grows hazelnuts on about 600 thousand hectares and gives 65 % of the hazelnuts produced throughout the world. Approximately 550 thousand tons of hazelnut are grown in Turkey each year and from these approximately 275 thousand tons of hard shell and about 500 tons of hazelnut nut husk waste emerge [6]. Therefore this study intended to examine the effects of the herbal dyestuff agents obtained from the hazelnut nuthusk through appropriate optimization and extraction methods on antimicrobial, antibacterial and DNA protection. Bacteria can grow very quickly on the textile surface when the appropriate temperature, humidity and other conditions are provided. It is possible to encounter two different hazards on the textile surface as a result of bacterial growth. The first of them, as a result of the uncontrolled bacterial growth is the emergence of bad odor, loss of comfort and the potential risk to harm the human health. Secondly, there may occur stains, discoloration and performance losses on the fabric surface [7,8]. Therefore, it is very important to use antibacterial dyestuff agents for the coloring of the textile products. It is known that the various solvents obtained from the pomegranate extracts [9,10], aloe Vera [11], green tea [12], chilly seed oil [13], peppermint oil [14] and rosemary oil have antibacterial activities and that these are applied to the textile surface directly or through microencapsulation. However, the recovery and antibacterial properties of the dyestuff agent obtained from the hazelnut shells have not yet been reported.

Since the textile products are constantly in contact with the human body, the synthetic dyestuff agents used in the production can cause skin diseases moreover these materials may have allergic or carcinogenic effects [15].

The European Commission (EC) date of 2004/21 directive of the European Union limited the use of many azo dyes to 30 ppm. Some of the azo dyes cause the formation of free amino groups as a result of dyeing. The carcinogenic effects of these emerging free amino groups are known [16]. It is also known that the UV (Ultraviolet) rays that reach the Earth due to the destruction of the stratosphere layer have negative effects on the living creatures. The UV rays can cause serious diseases that may result in skin cancer or skin aging. The synthetic dyestuff agents used in textile products can cause irreversible damage to the skin when they got

combined with the effects of the UV rays. Therefore, in this study it tried to be detected that the obtained herbal dyestuff extracts had effects on DNA protection against the UV rays.

2. MATERIAL and METHODS

2.1. Plant Material

Hazelnut nuthusk samples from Düzce (district of Akcakoca) in Turkey were collected (August and September 2016). This samples were dried without the sun. The collected samples were ground to powder approximately 70-80 mesh and it was eliminated so as to be smaller than 80 mesh. Dried and sieved samples are wrapped with aluminum foil on the outside of the glass bottle (to be dark) and are stored in cold (+4℃).

2.2. Recovery of the Dyestuff Agents

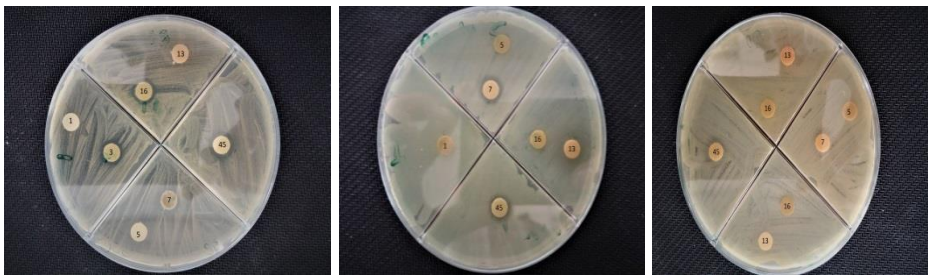
Organic dyestuff (H1, H2, H3, H4, H5, H6, H7) extraction was achieved from the hazelnut nuthusk according to the literature [17]. The obtained dry crude extracts were used for biological activity studies [18].

2.3. Determination of the Antibacterial Activity

In order to determine the antibacterial activity, the tests of the nuthusk dyestuff agents were carried out by considering the CLSI criteria of the disc diffusion test. 500 µl solvent was used for 50 mg DMSO (CH_3) 2SO of the obtained extracts. After the solutions were homogenized in the vortex device, then 20 µl was impregnated to sterile discs of 6 mm diameter. The extract was dried at room temperature for one day in order to remove the solvent from the impregnated discs. By taking an amount of the bacterial strains (S. aureus ATCC 6538, *E. coli* ATCC 25322, *P. aeruginosa* ATCC 27853) as shown that table 1. The extract was adjusted in a densitometer to 0.5 mcFarland standard (Barium Sulfate Turbidity Standard) in separate physiological saline. After vortexing the suspensions, they were spread with a sterile syringe bar by inoculating 100µl to MHA medium. They were left to dry for 10 minutes. The dried discs which were prepared one day before were placed on the medium with the help of a sterile forceps. The petri dishes were placed into an incubator and were left there for 24 hours at 35^0C. At the end of this period the diameters of the inhibition zones formed around the discs was measured according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) with a ruler as shown in Figure 1.

Table 1. Result of the antibacterial from nuthusk dysetuff

Indicator bacteries	H1	H2	H3	H4	H5	H6	H7
S. aurus	+	+	-	-	+	-	+
P. aeruginosa	+	-	-	-	+	-	-
E. coli	-	-	-	-	-	-	-



S. aureus *P. aeruginosa* *E. coli*

Figure 1. Inhibition zone diameters

As a result of these evaluations, as it was shown in Table 1, the 1st, 2nd, 5th and 7th samples of the nuthusk's dyestuff agents showed antibacterial activity against the strains of *S. aureus*. Antibacterial activities were not found in the other samples against these strains.

While antibacterial effects were found in the 1st and 7th samples against *P. aeruginosa*, none of the samples showed antibacterial effects against the *E. coli* strains

2.4. Determining the DNA Protector Activity of the NutHusk's Dyestuff Agent

The plasmid DNA of pBR322 (vivantis) was used to determine the DNA protection effect of the extracts obtained from the nuthusk raw materials against the UV and oxidative damages [19] Imaging was performed on 1.25% agarose gel by applying the method determined by Russo et. al (2000). Seven nuthusk extracts were used for the DNA protection activity test. 50 mg of the extracts were weighed and it was dissolved by adding 1000 µl of methanol in order to prepare a 5% stock concentration [20].

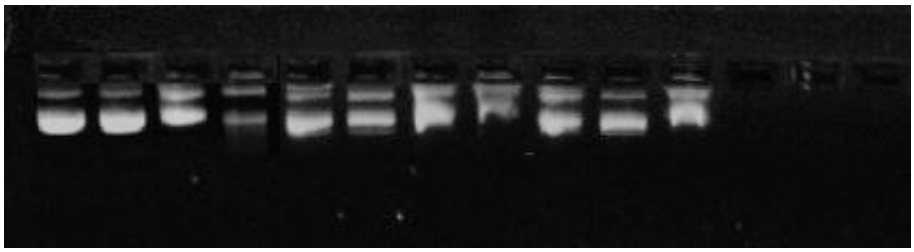


Figure 2. Dysetuff samples DNA protector activities band

Preparation Conditions of Control and Nuthusk samples (H):
C1: Control 1: Plasmid DNA (3µl) + dH_2O (6 µl)
C2: Control 2: Plasmid DNA (3µl) + dH_2O (6 µl) + UV
C3: Control 3: Plasmid DNA (3µl) + dH_2O (6 µl) + H_2O_2 (1 µl)
C4: Control 4: Plasmid DNA (3µl) + dH_2O (6 µl) + H_2O_2 (1 µl) + UV
H1: Plasmid DNA (3µl) +Nuthusk Extract (5 µl) + UV + H_2O_2 (1 µl)
H2: Plasmid DNA (3µl) + Nuthusk Extract (5 µl) + UV + H_2O_2 (1 µl)
H3: Plasmid DNA (3µl) + Nuthusk Extract (5 µl) + UV + H_2O_2 (1 µl)
H4: Plasmid DNA (3µl) + Nuthusk Extract (5 µl) + UV + H_2O_2 (1 µl)
H5: Plasmid DNA (3µl) + Nuthusk Extract (5 µl) + UV + H2O2 (1 µl)
H6: Plasmid DNA (3µl) + Nuthusk Extract (5 µl) + UV + H_2O_2 (1 µl)
H7: Plasmid DNA (3µl) + Nuthusk Extract (5 µl) + UV + H_2O_2 (1 µl)

As a result of examining the DNA protection activity of the nuthusk's dyestuff agents, it was determined that all the samples showed DNA protective activity as shown in Figure 2 but the H4 and H7 samples had lower activity than the other samples. H1, H2, H3, H5 samples had good protector activities than control groups, H4 and H7.

3. DISCUSSIONS

Medical and aromatic plants are plants that are used as medicines to prevent diseases, maintain health or heal diseases. While the medical plants are used in fields such as nutrition, medicine, cosmetics, body care, incense or religious ceremonies, aromatic plants are used in the fields of fine food, aromatherapy and perfumery [16]. The secondary metabolites of the plants are in fact very important chemical compounds with complex mechanisms produced by the plants which have many functions used in various fields recently such as protection, defense, compliance to the environment, survival and the continuation of the generations. Secondary metabolites used as raw materials in many sectors nowadays, are chemical substances that are not directly related to the essential vital function of the plants instead they are as important as

the primary metabolites (protein, fat and carbohydrates) that are directly associated with the vital function of the plant [21]. Although the duties of the secondary products are different in the plants, those with cytotoxic effects against the microbial pathogens can be used as antibacterial agents in medicine. Also some secondary metabolites can be evaluated medically as anti-depressant, sedative and muscle relaxant or can be used as anesthetic drugs. There are various literatures on various chemical components in different mechanism about the use of the plant's secondary metabolites both in industrial as well as in the medical fields [22]. For this purpose, the antibacterial and DNA protective activity of the extracts of the husk was evaluated. The extracts of the husk which is the secondary metabolite of hazelnut were obtained through high temperature and suitable optimization in order to evaluate its usability as coloring material in the textile industry. Many of the dyestuff agents obtained from plants have antimicrobial activities besides of the fact that they do not have damaging effect on health. Many of the plants used for paint extraction are used medically and it have been discovered recently that some of them have significant antimicrobial activity [23].

Punica granatum (Pomegranate) and many commonly used natural dyestuff agents have been reported as antimicrobial agents due to the abundant presence of tannin (commonly referred to as tannic acid) Some other plant sources are rich in naphthoquinone. For example, the anti-bacterial and anti-fungal activity of lawsone in henna, juglone in walnuts and the lapachole in radix alkannae were reported [24]. In this study, in the solvent extracts of the dyestuff agent obtained from the hazelnut nuthusk wase valuated on antibacterial and DNA protection. The natural antimicrobial agents are used to inhibit the growth of mold and bacteria on fabric. A large class of the antimicrobial agents used in textile includes oregano-metallic, phenol, quaternary ammonium salts and oregano silicones [25]. The microbial agents should be safe and biodegradable and should not be toxic and it should be noted if the active agent used in the antimicrobial dyes is effective and can be used safely. Due to the fact that the natural dyestuff agents generally inhibit the growth of microorganisms without any toxic effects, it has become important to study and apply these dyestuff agents [26]. Recently, detailed studies have been carried out on the extracts obtained from medical and aromatic plants in order to investigate new compounds that can control the oxidative DNA damage causing cancer [27].The exposure to oxidative stress and the Ultraviolet rays (UV) can induce the skin damage and can trigger diseases such as aging, inflammation and cancer [28].. It is known that the UV rays reaching the Earth due to the destruction of the stratosphere layer have negative effects on the living creatures. UV rays may cause serious illnesses that can result in skin cancer of aging. In fact, the human skin has a number of mechanism that reduce the harmful effects of VIS (visible rays) and UV rays. However, the high exposure to UV rays can lead to the decrease in the amount of the cellular antioxidants and to UV-induced oxidative DNA damage caused by the reactive oxygen species [20]. There were some research about some additives as colorent of food in the literature. One of them red sorghum bran is known as a rich source for anthocyanins that used of food additives. To study the health benefits of anthocyanin from red sorghum bran, the total antioxidant activity was evaluated by biochemical and molecular methods as use of DNA nicking test. The antioxidant activity of the red sorghum bran was directly related to the total anthocyanin found in red sorghum bran [29].

The another report about a green food additive of chlorophyllin inhibitory effected on the genotoxicities of various carcinogens in Drosophila. The similar reported about purpurin, a component of a red food additive produced from mader root (*Rubia tinctorium*), inhibited the bacterial mutagenicity of hetero cyclicamines [30]. This reports were about food additives on genotoxicity and biochemical properties there were limited report about textile dyestuffs.

For this reason, in this study the DNA protective effect mechanism of the obtained dyestuff agents was evaluated against the UV rays. It was detected that all of the extract samples obtained in order to be used as dyestuff agents in the textile industry had DNA protective effects

against the UV rays. This result is significantly important since it was the first study in the literature that intended to evaluate the dyestuff agents in terms of their DNA protective activity. It is expected to increase the demand to apply this study to the extract of the dyestuff agents obtained from different plants.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Propolis Extract-PVA Nanocomposites of Textile Design: Antimicrobial Effect on Gram Positive and Negative Bacterias

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Abstract: Potential antimicrobial efficiency of propolis extract (bee glue) was experimentally studied on gram positive and negative bacterias by manufacturing propolis extract-based textiles. Pre-samples were prepared by varying percentange concentration of propolis extract in PVA polymer solution and homojenic solutions were electrospun onto polypropylene nonwoven fabric. In-vitro experiments showed that antimicrobial efficiency of extract-containing nanocamposite samples were better than those of not including. According to investigations nanocomposite fabrics with propolis extract sol. were provided antimicrobial effect against to gram positive bacteria (S. aureus) but not to gram negative bacteria (A baumannii and P. aeruginosa). The results indicated that the electrospun PVA/propolis extract nanocomposites provided a good means for healing of wounds or decreasing infection proliferation caused by gram positive bacteria.

Keywords: nanocomposite, PVA polymer, propolis, hospital infections

1. INTRODUCTION

Medical textiles contribute to increase on quality of health service. Improving functionality of textiles used in hospitals can limit some problems due to infection spreading. Bed coverings, bedspreadings and some products for patients' personel care are commonly used for supplying sublevel hygenic demands. In order to prevent from hospital infections, these products can be manufactured with pharmasotical materials by considering controversal effects of bacterias causing these problems. By means of bacteria types, it is seen that commonly-known hospital infections are results of gram positive (Staphylococcus aureus) and gram negative (Klebsiella pneumoniae, Enterobacter spp. and E. Coli from Enterobacteriaceae; Pseudomonas aeruginosa, A. Baumannii and Stenotrophomonas maltophilia from nonpermentative gram-negatives and less frequently Burkholderia cepacia) bacterias. Due to increased drug resistance of these bacterias, preventing of hospital infections take great attention for both patience and personnel health [1].

As considering natural-based pharmasotics, propolis has become a popular material due to its abundant phenolic compounds, mainly with potential antioxidant namely "flavonoids" and aromatic acids. It is a strongly adhesive resinous substance used in traditional medicine and

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been reported to have a broad spectrum of biological activities, such as anticancer, antioxidant, antiinflammatory, antibiotic, and antifungal activities [2-7].

It has recently become popular as a health drink and it has been claimed to prevent diseases such as dermatological wounds, inflammation, heart disease, diabetes, cancer, etc. These facts encourage researchers to draw interest in the extraction of flavonoids from propolis esters and adapt these flavonoids to care products [2, 8-10]

By means of adapting pharmasotics or drug delivery systems onto textile materials, electrospinning is the most preferable method due to producing compatible nanofibers from a large variety of bulk starting materials. Electrospun bicomponent nanofibrous fabrics with high specific surface area, aspect ratio and porosity as a result of random deposition of fibers, could have a great potential in biomedical applications such as tissue engineering scaffolds, drug delivery carriers, wound dressings etc. [11, 12]. By handling of electrospun pharmasotic material, healing period of wounds can be limited and their high surface area of electrospun surfaces can provide optimum healing dosage by using low pharmasotic material extense.

In this study, aqueous poly (vinyl alcohol) (PVA) and propolis extract solutions were blended in proper volume ratio and were electrospun under constant conditions. Electrospun PVA/Propolis extract nanofibrous structure were manufacturedand their wound healing performances and bacterial proliferation rates were macroscopically compared by counting colony occured on both subject and control agars.

2. MATERIAL and METHODS

2.1. Electrospinnability of Materials

PVA polymer (Mw:80.000 g/mol) was dissolved in distilled water at 60 ^{o}C during 8 hours. Propolis extract was diluted with ethanol by concentration of 3% in weight. 3% propolis/ethanol solutions were added into 5%, 7%, 9% and 11%PVA/water solutions drop by drop and mixtures were stirred during 15 hours at room temperature.

(a) (b)

Figure 1. Prepared solutions (a) 7% PVA polymeric solution, (b) mixture of 5% PVA polymeric solution and 3% propolis extract in weight of 5% ratio of PVA solution.

Homogenized solutions were electropun onto PP nonwoven surface (17 g/m^2). Potential differences between the tip and the counter electrode (collector) used to electrospin the polymer /propolis solutions were 37 kV. Stationary collector covered with aluminum foil, placed 10 cm above the capillary tip, was used to collect the electrospun fiber material.Feeding rate was undercontrolled during manufacturing and it was adjusted to 2.5 ml/h. This study was carried out by following two stages: (a) electrospinnability of propolis by keeping constant propolis amount in polymer solutions to observe fiber formation; (b) efficiency of propolis/PVA nanostructures electrospun from solutions containing different amounts of propolis extract. Samples were characterised by SEM method and their antimicrobial efficiency were tested

against to gram positive bacteria (*S. aureus)* and gram negative bacteria (*A baumannii and P. aeruginosa*) either in serum physiologic and human blood.

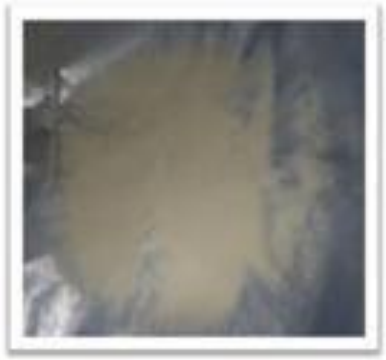


Figure 2. Appearance of sample electrospun from PVA/propolisextract mixture

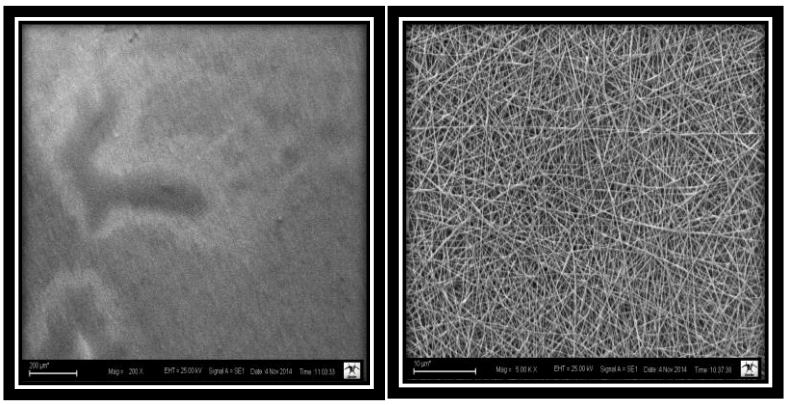
2.2. Efficiency of Samples on Gram-Negative/-Positive Bacterias in Different Medias

0.5 McFarland (0.33 ml) bacterial solutions of gram-positive (S. Aureus) and gram-negative (A. Baumannii and P. Aeruginosa) are prepared by 2 ml serum physiologic. Samples, which are electrospun from *various amounts of propolis extract* in PVA polymer solution, are penetrated into bacterias and serum physiologic containing tubes. Electrospun samples containing tubes are observed before subculturation process and it is seen that samples are completely dissolved. These solutions are subcultured on blood agars and it is repeated 3 times by every other day for each sample. Efficiency of propolis containing nanostructures on human blood is also experienced. 0.5 McFarland (0.33 ml) bacterial solutions of gram-positive (S. Aureus) are prepared by 0.1 ml centrifuged human blood and 2 ml serum physiologic.

3. RESULTS and DISCUSSIONS

3.1. SEM Micrographs of Electrospun Samples

Figure 3 (a) shows that there is no fiber formation on electrospinning of 3% propolis extract solution and electrospun surface of propolis extract is composed of nearly particular substances. Electrospinning of PVA polymer solution succesfully provides nanoweb formation in various concentration rates, meanly in Figure 3 (b). According to SEM micrographs, it is seen that propolis extract can be electrospun by adding into PVA polymer solution.

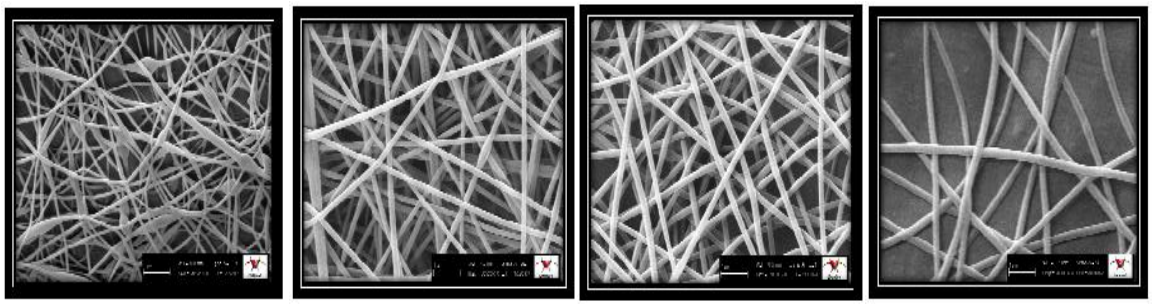


(a) (b)

Figure 3. SEM images of electrospun reference samples (5000x ve 200x)a) 3% propolis extract, b) 7% PVA

Easily electrospinnability of PVA polymer solution contribute to adaptable of propolis by getting further away from electrospraying. Electrospinnability of propolis by help of polymer solutions has been investigated with other types of polymers in recent studies [13, 14].

By means of fiber fineness, it is manufactured finer nanofibers from 100% PVA polymer solution than those from propolis-containing polymer solutions. Addition of propolis into PVA polymer solution cause nano/micro-sized fiber manufacturing with thicker diameter and homogeneous structural properties. Figure 4 shows the compatibility of propolis extract on PVA polymer. At decreasing concentration of PVA polymer in polymer/propolis mixture, beading formation and clustering are seen on electrospun structure. There is no seen any beading formation on higher concentrations of PVA polymer. From Figure 4, it is observed that network clusters are occured in propolis containing nanofibers. But electrospun fibers from 100% PVA polymer solution or high PVA-containing mixtures exhibits more regular, uniform fiber formation and less network clustering.



(a) (b) (c) (d)

Figure 4. SEM micrograps of nanostructures electrospun from PVA/propolis extract solutions, a) 5% PVA-3% propolis b) 7% PVA-3% propolis, c) 9% PVA-3%propolis, d) 11% PVA-3% propolis

3.2. Efficiency of Samples on Gram-Negative/-Positive Bacterias in Serum Physiologic Solutions

Subculturation of samples of 0.5 McFarland bacteria, serum physiologic solution and with/without propolis nanostructure is illustrated in Figure 5 and Figure 6. Figure 5 shows subculturation of bacteria and serum physiologic mixture on blood agar and colony proliferation are available for each bacteria types. 100 colonies are proliferated in sample shown in Figure 5 (a). However, there are4 colonies in Figure 6 (a). It is seen that propolis nanostructure containing solutions prevents proliferation of Staphylococcus aureus colonies effectively but not of Acinetobacter baumannii and Pseudomonas aeruginosa [15]. This proves that propolis extract containing nanostructure is effective on gram positive bacteria, Staphylococcus aureus [16-19].

(a) (b) (c)

Figure 5. Bacterial subculturation of control samples from tubes containing serum physiologic and bacteria a) *S. Aureus*, b*) A. baumannii,* c) *P. Aeruginosa*

(a) (b) (c)

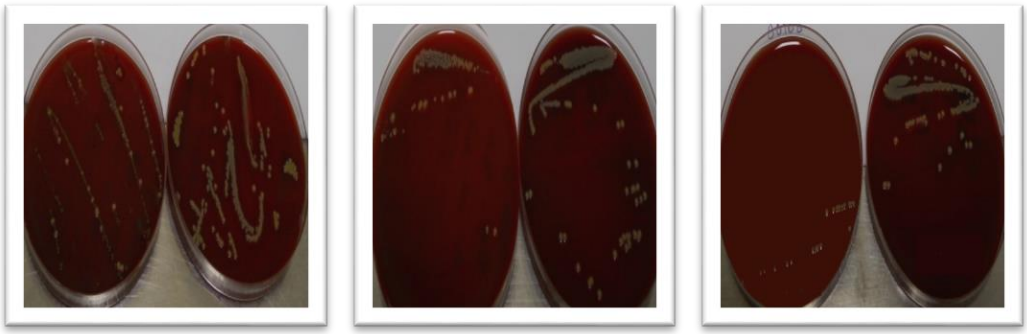
Figure 6. Bacterial subculturation of experimental samples from tubes containing serum physiologic, bacteria and electrospun surface (7% PVA solution / 9% propolis extract) a) *S. Aureus*, b*) A. baumannii*, c) *P. Aeruginosa*

(a) (b) (c)

Figure 7. Colony proliferation of *S. Aureus bacteria* for solutions containing electrospun samples and control samples without electrospun surface*in serum physiologic (a)electrospun 7%PVA / 5% propolis extract (4 colonies) (b) 7%PVA / 7% propolis extract (no colony proliferation) (c) 7%PVA / 9% propolis extract (no colony proliferation)*

3.3. Efficiency of Samples on Gram-Negative/-Positive Bacterias in Human Blood

Subculturation of controls (solution in which propolis containing electrospun is not penetrated) result to occurence of 100 colonies profileration. The least proliferation among control solution subculturation is observed in Figure 8c as 60 colonies. But all subculturations from propolis containing solutions, namely illustrated as“sample”, lead to decrease on proliferation of S. Aureus bacterias although different concentration amount in polymer solutions. This case declares the effectiveness of propolis extract on preventing proliferation of S. Aureus bacterias in human blood [20].



(a: sample/control) (b: sample/control) (c: sample/control)

Figure 8. Colony proliferation of *S. Aureus bacteria* for solutions containing electrospun samples and control samples without electrospun surface *in human blood (a)electrospun 11%PVA / 7% propolis extract (50-60 colonies) (b) 11%PVA / 9% propolis extract (30-40 colonies) (c) 11%PVA / 11% propolis extract (20 colonies).*

4. CONCLUSION

Electrospinnability of propolis is possible by addition of this material into easily electrospun biocompatible polimeric solution. Micro/nanocomposite structure is obtained by mixing propolis extract into PVA polymer solution. According to experiments electropun fabrics with propolis sol., especially 11%, were provided antimicrobial effect against to gram positive bacteria (*S. aureus)* and not provided antimicrobial effect against to gram negative bacteria (*A baumannii and P. aeruginosa*). According to studies, S.aureus bacteria is the most initiative and encountered bacteria type among hospital bacterias. By adapting propolis onto hospital textiles or patients'personal care products, hospital infections caused by S.aureus bacteria can be limited by these resistive natural pharmasotic, propolis.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Macro, trace and toxic elements of 4 different edible wild plants from Karadeniz Region

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Abstract: The study was conducted to assess the content (mg kg^{-1} fresh wt.) of macro, trace and toxic elements in the 4 different edible wild plants. The percentage of dry matter and ash ranged from 6.77 to 20.56 and 0.79 to 2.26, respectively. The contents of Ca (1074), Fe (160.5), Mn (18.21), Ni (6.11), B (2.61), Cr (0.93), Co (0.50), Mo (1.80), Be (0.009) and Se (0.076) of *U. diocia* were richer than in other plants. Other hand, K (7742), Mg (954), Al (10.79), V (9.77) and Ag (0.109) in *T. orientalis*, Zn (12.47), Cu (9.98) and Ba (130.3) in *O. umbellatum* were taken the highest value. Also As, Hg, Tl, Cd and Pb were determined mg kg^{-1} level in fresh plants. Conversely, antimony (Sb) in wild plants could not be detected by ICP-MS. The results of statistical analysis of forty plants showed that moisture, total dry matter, ash, K, Ca, Mg, Fe, Zn, Cu, Mn, Ba, Ni, Al, V, B, Cr, Co, Mo, Be, Se, Ag, As, Tl, Cd and Pb contents were varied significantly compared to plant species except for Na and Hg ($p<0.05$). Consequently, wild plants may be used as popular vegetables in many people diet as a source of minerals (Fe, Cu, Mn, Cr, Mo, K, Zn and Mg). Excessive plant consumption may be adversely affected human health with Cd, As, Hg, Tl and Pb.

Keywords: Edible wild plants, Macro-Microelements, Toxic elements, ICP, Traditional foods

1. INTRODUCTION

Although the number of edible plants on earth is known to be about 70000, today people use about 7000 plant species for food, medicine and other needs. However, the number of consumed plants as culture vegetables only is 150 [1]. Moreover, distribution of edible wild plants on the earth depending on the climate is quite varied. Turkey is one of the richest natural resource for wild plants and has nearly 9000 plant species, of which 3000 are endemic [2, 3].

Nowadays, the demand for wild plants is increasing day by day due to the idea of positive impact on human health. Moreover, wild plants all over the world have an important place in agricultural production also many industrial sectors (agricultural struggle, pharmaceutical, beekeeping, textiles etc.). The protection of wild genetic resources for agricultural production is another important issue [2, 3]. Therefore, harvesters and manufacturers should be responsible for the conservation of plant species for the continuation of biodiversity.

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Many wild species as *Urtica diocia* (regional name is Isırgan), *Trachystemon orientalis* (regional names are Galdırik, Kaldırik), *Similax excelsa* (known names in the region are Melocan, Melvocan, Silcan, Diken otu, Mamula, Melevcen, Sıraca, Kırçan and Çıtırgı) and *Ornithogalum umbellatum* (regional names are Sakarca and Çökülce) are common used for human consumption in Karadeniz region [4, 5]. These species are annual wild herb and their vegetation is concentrated in hazelnuts orchards. These wild plants rarely found in valleys, pastures and forested areas in the region are collected by local people and are preferably cooked like green leafy vegetables. Wild vegetables are used widely as traditional foods such as soups, pickles, meals, pastries, salads and fried products. Their formulations in Turkey varies according to local consumption habits (fried or cooked with wheat or corn meal, egg, cheese, onion, garlic, meat, rice, spices, oil etc.).

Wild vegetables also are popular foods for vegetarians. Moreover, these plants are widely considered a potential rich source of minerals (Ca, Fe, Mg, P, K, Na, Zn etc.), phytochemicals (phenolics, carotenoids, strerols), vitamins (vitamin A, B and C) and dietary fibres for human consumption [1, 6-8].

The main constraint for the nutritional use of these species is the presence of certain anti-nutritional and toxic substances such as nitrates [9, 10], pesticides [10], oxalate [6, 7], saponins [12, 13], tannins [7], glicosinolate [14], alcoloids [13] and heavy metals [15].

On the other hand, it is known that the natural components of vegetables such as minerals, vitamins, phytochemicals and dietary fibers contribute significantly to the protection of human health and the increase of body resistance. For this reason, studies conducted in recent years are focused on relationships health and nutrient resources. Indeed, it has been determined that complaints related to serious health problems such as cardiovascular diseases, osteoporosis, breast, prostate and bowel cancer are less common in societies fed on rich diet with basic minerals [16].

Despite the known macro and some micro mineral composition (K, P, Mn, Ca, Na, Mg, etc.) of wild vegetables, there is scarce literature about its micro (trace) and toxic element contents such as Co, Cr, Mo, As, Tl, Pb, Hg etc. The aim of the present work is to assess the concentration of macro, micro and toxic elements in wild vegetable samples from grown in Karadeniz region (Ordu-Giresun) of the Turkey.

2. MATERIAL and METHODS

2.1. Sample Preparations

Four plant species were collected from the ten locations of the natural vegetation (hazelnut orchard) in the Karadeniz region of Turkey (Ordu-Giresun province) during the harvest period between March and May 2008. Before analysis, the leaves were washed, first with tap water and then with distilled water, and residual moisture was removed by Whatman No:1 filter paper and evaporated at room temperature. Only the edible parts of young plant leaves were used. One-hundred-gram plant samples for each plant were taken randomly from portions of about 1.0 kg. Samples were ground with a mortar and pestle. Then, the ground samples were packed in plastic bags and stored in deep freeze until analysis.

2.2. Analytical Procedures

2.2.1. Dry Matter and Ash Analysis

Dry matter was determined gravimetrically by drying by convection in an oven at 105 °C for 2 h. Ash analysis were performed by incineration in an oven at 550°C [17].

2.2.2. Digestion Procedure

Approximately 6 g samples per plant were accurately weighed to the nearest milligram (0.001 g) into six Teflon microwave digestion vessel (about 100 ml with standing a pressure of at least 800 psi) in 1 g portions. After adding 10 ml concentrated HNO_3 (65%), the vessel was sealed and placed into the microwave digestor (CEM MARS 5, CEM Corporation, North Carolina, USA) for dissolution. Six weighed samples were digested in each digestion program. The applied digestion program at the first stage time was 600 W power, 10 min ramp time, 600 psi pressure, 180^0C temperature and 5 min hold values. For the second stage time, these values were 600 W, 10 min, 800 psi, 200^0C and 10 min, respectively. Thereafter, the vessel was cooled and 0.5 ml H_2O_2 (30%) was added in a microwave digestion system for 30 min. Digested samples were transferred to a volumetric flask and diluted to 100 ml with deionized water (18 MV/cm). Nitric acids (Merck 100452) and H_2O_2 (Merck 107209) used were of highest purity grade. A blank digest free from samples was carried out in the same way.

2.2.3. Mineral Analysis

Minerals in samples were determined by ICP-MS (Model Agilent 7500a) using argon as the inert gas (99.99%). The instrumental operating conditions for the determination of the elements are summarized in Table 1. In order to detect the polyatomic interferences for V, Zn, As, Cd and Pb, the different isotopes of V (51-52), Zn (66-69), As (75-77-82-83), Cd (106-108-111) and Pb (206-207-208) were measured for standard mode. The proposed method was standardized utilizing standard solutions of different concentrations from micro and macro elements.

Table 1. Instrumental operating conditions for ICP-MS

Spectrometer	Mass
RF power (W)	1350
Plasma gas flow rate (L/ min) (Argon)	15
Auxiliary gas flow rate (L/ min)	0,90
Carrier gas flow rate (L/ min)	1,02
Sampling depth (mm)	7,0
Acquisition Mode	Spectrum
Number Replicates	3
Spray chamber temp.(^{0}C)	2
Nebulizer	Babington type
Sampler Cone	Nickel cone
Analytical masses	^{9}Be,^{11}B, ^{23}Na, ^{24}Mg, ^{27}Al,^{39}K,^{43}Ca,^{51}V,^{53}Cr, ^{55}Mn,^{57}Fe, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{78}Se, ^{95}Mo, ^{107}Ag, ^{111}Cd, ^{121}Sb, ^{137}Ba, ^{202}Hg, ^{205}Tl,^{208}Pb

The standard solutions for calibration were 0, 5, 10, 25, 50, 100 and 200 µg l^{-1} for minor elements (Fe, Zn, Cu, Mn, Ba, Ni, Al, V, B, Cr, Co, Mo, Be, Se, Ag As, Cd, Tl and Pb) and 0, 25, 50, 100, 200 and 400 mg l^{-1} for major elements such as K, Ca, Mg and Na. Furthermore, standard reference materials (SRM) of NIST-SRM 1515 apple leaves were used as controls. The recovery values of this procedure were nearly quantitative for SRM (>93%) and standards (>95%). Table 2 shows the detection limits (LOD) and the quantification limits (LOQ) for the elements studied. In order to prevent mineral element contamination, no glassware was used (particularly for B contamination). Dilutions were made with deionized water (Millipore Water Purification System, Billerica, MA, USA) in volumetric flasks manufactured from High-density polyethylene (HDPE) and kept until used.

Table 2. LOD and LOQ values for the elements

Minerals	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Minerals	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
K	0,025	0,075	Cr	0,005	0,015
Ca	0,021	0,063	Co	0,004	0,012
Mg	0,020	0,060	Mo	0,007	0,021
Na	0,030	0,090	Be	0,008	0,024
Fe	0,010	0,030	Se	0,004	0,012
Zn	0,005	0,015	Ag	0,006	0,018
Cu	0,004	0,012	As	0,002	0,006
Mn	0,005	0,015	Hg	0,001	0,003
Ba	0,010	0,030	Tl	0,005	0,015
Ni	0,006	0,018	Cd	0,002	0,006
Al	0,004	0,012	Pb	0,002	0,006
V	0,008	0,024	Sb	0,004	0,012
B	0,015	0,045			

Note: LOD: limits of detection, LOQ: limits of quantification

All the plastic equipment was cleaned by NaOH solution (130 g NaOH+130 ml distilled water+880 ml ethanol) and then by HNO3 solution (500 ml HNO3 (65%)+4,500 ml distilled water) and rinsed four or five times with deionized water.

2.2.4. Statiscal Analysis

All results of mineral elements were obtained from ten repetition and the data were expressed as the arithmetic mean, min, max, standard deviation, standard error and coefficient of variation. The data were also analyzed by one-way analysis of variance using the MINITAB statistical program, and Duncan's multiple range test was used to detect significant differences ($p<0.01$) among the variety means [18].

3. RESULTS and DISCUSSIONS

Dry matter, ash and the mineral matter contents of the investigated samples are shown in Table 3. The amount of moisture in wild plant species as consumed vegetables were changed between in *U. diocia* 79.44-86.35%, in *T. orientalis* 89.23-93.23%, in *S. excelsa* 85.28-89.76% and in *O. umbellatum* 80.44-85.53%. On the other hand, the ash content of these plants ranged from 0.79 to 2.26%. These results are in agreement with previously reported values [19, 20].

All trace element concentrations were expressed as mg kg^{-1} in fresh weight. The concentrations of macro, micro and toxic elements in edible wild plants varieties to be min, max, mean, SD, SE, CV (%) and results of Duncan's Multiple Comparison tests are given in Table 3, 4 and 5. According to variance analysis (One-way ANOVA), the effect of species on moisture, ash and minerals were found to be significant except for Na and Hg ($p <0.05$). In addition, amount of Sb (antimony) from the toxic elements in majority of samples was below detection limit. A large variation in minerals among plant species were observed from the coefficients of variation (14.7-138.6%).

In the result of this study, K concentration in wild plants species was determined highest level than other elements, followed by Mg, Ca, Fe, Na, Mn, Ba, Zn, Cu, V, Al, Ni, B etc. In our samples, potassium from the macro elements was determined a very wide range of oscillation as 213-7742 mg kg^{-1}. The highest mean of K was determined in *T. orientalis* (4850 mg kg^{-1}) and followed by *U. diocia* (4342 mg kg^{-1}), *O. umbellatum* (2189 mg kg^{-1}) and *S. excelsa* (349 mg kg^{-1}) respectively. The amount of Ca in plants was similar to statistically for *T. orientalis* and *S. excelsa*. The highest mean of Ca was found in *U. diocia* with 623 mg kg^{-1} that were

followed by *O. umbellatum* (334 mg kg^{-1}). According to the Duncan Multiple Comparison Test, the highest and lowest Mg values were found in *T. orientalis* (535 mg kg^{-1}) and *S. excelsa* (94 mg kg^{-1}), respectively. The Na from macro-elements in plants was determined in *U. diocia* between 26 and 66 mg kg^{-1}, in *T. orientalis* between 11 and 56 mg kg^{-1}, in *S. excelsa* between 29 and 61 mg kg^{-1}, and in *O. umbellatum* between 17 and 56 mg kg^{-1} (Table 3).

Table 3. The moisture of dry matter (DM), ash and macro-elements composition belonging to wild plant species and Duncan Multiple Comparison Test results [*]

Plant Species	Variation	Moisture (%)	DM (%)	Ash (%)	Mineral Matters (mg kg^{-1} fresh weight)			
					K	Ca	Mg	Na
U. diocia (Isırgan) n=10	Min	79.44	13.66	1.54	2487.67	283.88	268.48	26.63
	Max.	86.35	20.56	2.26	6327.68	1074.52	484.85	65.71
	Mean	82.17c	17.83a	1.92a	4342.5a	623.44a	386.45b	42.83ns
	SD	2.4	2.4	0.22	1184.81	256.71	73.57	11.26
	SE	0.76	0.76	0.07	374.67	81.18	23.26	3.56
	CV (%)	2.92	13.48	11.2	27.28	41.18	19.04	26.28
T. orientalis (Kaldırık) n=10	Min	89.23	6.77	0.93	1703.61	41.84	194.7	11.61
	Max.	93.23	10.77	1.52	7741.89	142.49	953.73	56.32
	Mean	90.98a	9.02c	1.17b	4850.37a	90.36c	534.74a	34.43ns
	SD	1.23	1.23	0.2	1674.07	26.15	218.54	16.21
	SE	0.39	0.39	0.06	529.39	8.27	69.11	5.13
	CV (%)	1.35	13.58	17.17	34.51	28.94	40.87	47.09
S. excelsa (Melocan) n=10	Min	85.28	10.24	0.87	213.34	14.96	66.79	29.67
	Max.	89.76	14.72	1	590.35	24.4	123.52	60.88
	Mean	86.94b	13.06b	0.93c	349.44c	19.52c	94.09c	43.02ns
	SD	1.43	1.43	0.04	123.17	2.87	16.82	10.34
	SE	0.45	0.45	0.01	38.95	0.91	5.32	3.27
	CV (%)	1.64	10.93	4.77	35.25	14.71	17.88	24.04
O. umbellatum (Sakarca) n=10	Min	80.44	14.47	0.79	1147.01	168.19	69.06	17.33
	Max.	85.53	19.56	0.95	3593.16	657.78	179.64	56.14
	Mean	82.64c	17.36a	0.86c	2188.7b	334.06b	112.22c	33.49ns
	SD	1.67	1.67	0.06	821.89	141.91	34.5	12.37
	SE	0.53	0.53	0.02	259.9	44.88	10.91	3.91
	CV (%)	2.02	9.6	6.5	37.55	42.48	30.74	36.95

Notes: SD- Standard Deviation SE- Standard Error CV- Coefficient of Variation ns: not significant [*] : Same letter marked as statistical averages are not different from each other ($P<0.05$).

While the highest and the lowest amount of Fe were in *U. diocia* (108 mg kg^{-1}) and in *S. excelsa* (1.79 mg kg^{-1}) respectively, the amounts of Fe in *O. umbellatum* and *T. orientalis* were between these two values. The Zn contents of *U. diocia, T. orientalis, S. excelsa* and *O. umbellatum* plants localized under the hazelnut were varied between 3.80-8.39 mg kg^{-1}. The traditional leafy vegetables with the highest values of Cu concentration were *O. umbellatum* (5.66 mg kg^{-1}). While the amount of Mn in *U. diocia* (10.55 mg kg^{-1}) was more than other 3 wild plants, this plant was followed by *T. orientalis* (7.49 mg kg^{-1}) plants. The amount of Mn of *S. excelsa* (1.31 mg kg^{-1}) and *O. umbellatum* (2.26 mg kg^{-1}) were statistically similar. K, Ca, Mg, Fe, Zn, Cu, and Mn values of the studied wild plants are similar to that previously reported by other authors [7, 16, 21, 22, 23, 24, 25, 26]. One other hand, our Na, Mn and Cu results are

higher than those reported earlier [18]. Our Cu and Zn values are lower than literature reports [27-29].

The highest mean of Ba concentration, on a fresh mass basis, was found in *O. umbellatum* (80.01 mg kg^{-1}), and followed by *U. diocia* (17.99 mg kg^{-1}), *S. excelsa* (8.48 mg kg^{-1}) and *T. orientalis* (4.61 mg kg^{-1}). Barium values are similar to values found in India [30]. The wild vegetable species with the lowest concentration of Ni were found in *T. orientalis* (0.87 mg kg^{-1}), followed by *O. umbellatum* (1.48 mg kg^{-1}), *U. diocia* (3.45 mg kg^{-1}) and *S. excelsa* (3.60 mg kg^{-1}) respectively. Our Ni values are similar to values found in Saudi Arabian [24] and higher than values found in Ghana [28] and in Niger [27]. Duncan Multiple Comparison test showed that Al contents of *O. umbellatum* (6.15 mg kg^{-1}) and *T. orientalis* (5.54 mg kg^{-1}) were statistically higher than those of *U. diocia* (2.75 mg kg^{-1}) and *S. excelsa* (0.196 mg kg^{-1}). Three wild plants, *U. diocia*, *T. orientalis*, *S. excelsa* and *O. umbellatum*, had V levels above 1 mg kg^{-1} and the highest value was found in *T. orientalis* (9.77 mg kg^{-1}). Tables 1, 2 and 3 show that, the amount of B in *U. diocia, T. orientalis, S. excelsa* and *O. umbellatum* varied among 0.29-2.61 mg kg^{-1} and the average B value of plant species taken value of 1.01 mg kg^{-1}. Cr content oscillated from 0.046 mg kg^{-1} (*S. excelsa*) to 0.93 mg kg^{-1} (*U. diocia*) in fresh weight of edible portion. The Cr levels of our samples are comparable to those present in common green leafly vegetables consumed in India [7]. Among edible wild plants, the highest Co level was found in *U. diocia* (0.21-0.50 mg kg^{-1}) and were followed by *O. umbellatum* (0.08-0.30 mg kg^{-1}), *T. orientalis* (0.02-0.10 mg kg^{-1}) and *S. excelsa* (0.01-0.05 mg kg^{-1}). Our Co values are similar to those reported earlier [30]. The median Mo content was ranged from 0.040 to 0.887 mg kg^{-1} in our samples. Beryllium identified only in *U. diocia* (0.0047 mg kg^{-1}). On the other hand, Be contents of other wild species were not detectable levels. The nutritionally significant element Se was determined as the highest value in *U. diocia* (0.0498 mg kg^{-1}), followed by *O. umbellatum* (0.0102 mg kg^{-1}) and *T. orientalis* (0.0032 mg kg^{-1}) but the Se level *of S. excelsa* was not at the level of detection. The Ag content ranged between 0.01 and 0.16 as mg kg^{-1} fresh weight (Table 4).

Table 4. The micro-elements composition belonging to wild plant species and Duncan Multiple Comparison Test results [*].

Plant Species	Variation	Mineral Matters (mg kg^{-1} fresh weight)														
		Fe	Zn	Cu	Mn	Ba	Ni	Al	V	B	Cr	Co	Mo	Be	Se	Ag
U. diocia (Isırgan) n=10	Min	61.49	5.17	2.89	7.59	6.23	1.12	1.07	2.52	1.123	0.219	0.216	0.382	0.001	0.022	0.045
	Max.	160.53	10.61	6.61	18.21	30.42	6.11	3.87	8.52	2.611	0.928	0.502	1.798	0.009	0.076	0.121
	Mean	108.4a	7.59a	4.26a	10.55a	17.99b	3.45a	2.75b	4.78a	1.743a	0.538a	0.393a	0.887a	0.005a	0.05a	0.089b
	SD	31.91	1.59	1.15	3.14	6.77	1.64	1.01	2.25	0.481	0.268	0.086	0.517	0.003	0.018	0.021
	SE	10.09	0.50	0.36	0.99	2.14	0.52	0.32	0.71	0.152	0.085	0.027	0.164	0.001	0.006	0.007
	CV (%)	29.44	20.94	26.85	29.75	37.62	47.5	36.53	47.11	27.59	49.78	21.93	58.3	53.27	37.08	23.88
T. orientalis (Kaldırık) n=10	Min	4.89	2.58	1.4	5.05	1.9	0.54	2.98	3.97	0.297	0.112	0.021	0.021	0.000	0.000	0.011
	Max.	18.23	5.59	7.57	11.65	7.93	1.36	10.79	9.77	0.78	0.412	0.097	0.102	0.000	0.012	0.109
	Mean	11.62bc	3.8b	4.59a	7.5b	4.61b	0.87b	5.54a	6.21a	0.488c	0.275b	0.064c	0.046b	0.000b	0.003bc	0.062a
	SD	4.32	0.92	1.99	2.41	1.99	0.23	2.88	1.88	0.156	0.103	0.022	0.024	0.000	0.004	0.028
	SE	1.37	0.29	0.63	0.76	0.63	0.07	0.91	0.59	0.049	0.033	0.007	0.008	0.000	0.001	0.009
	CV (%)	37.14	24.32	43.45	32.19	43.21	26.77	52.01	30.2	32.04	37.64	34.52	53.08	0.00	138.61	46.11
S. excelsa (Melocan) n=10	Min	0.63	4.15	1.05	0.98	5.11	2.65	0.11	0.03	0.708	0.046	0.01	0.024	0.000	0.000	0.065
	Max.	3.36	8.96	3.25	1.66	14.51	4.74	0.3	0.15	1.791	0.126	0.045	0.11	0.000	0.000	0.129
	Mean	1.79c	6.08ab	2.2b	1.31c	8.48b	3.6a	0.2c	0.1b	1.145b	0.086c	0.023c	0.066b	0.000b	0.000c	0.095b
	SD	0.93	1.71	0.72	0.28	3.02	0.65	0.06	0.04	0.307	0.027	0.012	0.029	0.000	0.000	0.019
	SE	0.29	0.54	0.23	0.09	0.95	0.2	0.02	0.01	0.097	0.008	0.004	0.009	0.000	0.000	0.006
	CV (%)	52.12	28.06	32.8	21.67	35.59	17.93	31.62	35.75	26.77	30.7	52.72	43.4	0.00	0.00	19.46
O. umbellatum (Sakarca) n=10	Min	20.03	3.88	3.41	1.3	56.21	0.84	3.28	1.07	0.314	0.163	0.085	0.017	0.000	0.002	0.034
	Max.	46.73	12.47	9.98	3.49	130.33	3.01	8.03	7.94	1.064	0.483	0.302	0.071	0.000	0.029	0.161
	Mean	29.51b	8.39a	5.66a	2.26c	80.01a	1.48b	6.15a	4.51a	0.647c	0.329b	0.181b	0.040b	0.000b	0.01b	0.085b
	SD	8.82	2.99	2.04	0.67	21.13	0.67	1.48	2.33	0.213	0.099	0.079	0.015	0.000	0.008	0.041
	SE	2.79	0.94	0.65	0.21	6.68	0.21	0.47	0.74	0.067	0.031	0.025	0.005	0.000	0.003	0.013
	CV (%)	29.88	35.59	36.05	29.63	26.41	45.08	24.11	51.75	32.95	30.14	43.48	37.1	0.00	82.97	48.3

Notes: SD- Standard Deviation SE- Standard Error CV- Coefficient of Variation.[*] : Same letter marked as statistical averages are not different from each other ($P<0.05$).

The contents of Ca, Fe, Mn, Ni, B, Cr, Co, Mo, Be and Se of *U. diocia* were richer than in other plants. On other hand, K, Mg, Al, V and Ag in *T. orientalis*, Zn, Cu and Ba in *O. umbellatum* were taken the highest value. There was no significant difference between the Na contents of wild plants.

Table 5. The toxic-elements (heavy metals) composition belonging to wild plant species and Duncan Multiple Comparison Test results [*].

Plant Species	Variation	Mineral Matters (mg kg^{-1} fresh weight)				
		As	Hg	Tl	Cd	Pb
U. diocia (Isırgan) n=10	Min	0.169	0.167	0.271	0.011	0.378
	Max.	0.586	0.496	0.635	0.04	1.728
	Mean	0.366a	0.376ns	0.391a	0.026b	0.997a
	SD	0.146	0.104	0.11	0.009	0.416
	SE	0.046	0.033	0.035	0.003	0.132
	CV (%)	39.85	27.78	28.21	34	41.71
T. orientalis (Kaldırık) n=10	Min	0.058	0.143	0.087	0.002	0.225
	Max.	0.157	0.448	0.302	0.02	1.394
	Mean	0.116bc	0.313ns	0.21b	0.01b	0.539b
	SD	0.029	0.11	0.075	0.005	0.338
	SE	0.009	0.035	0.024	0.002	0.107
	CV (%)	25.07	35.05	35.72	49.72	62.79
S. excelsa (Melocan) n=10	Min	0.014	0.127	0.253	0.016	0.239
	Max.	0.054	0.425	0.419	0.053	1.233
	Mean	0.043c	0.282ns	0.327ab	0.032b	0.628ab
	SD	0.012	0.109	0.062	0.012	0.335
	SE	0.004	0.034	0.02	0.004	0.106
	CV (%)	29.38	38.64	18.97	38.07	53.31
O. umbellatum (Sakarca) n=10	Min	0.112	0.084	0.149	0.206	0.463
	Max.	0.204	0.605	0.594	0.771	2.673
	Mean	0.151b	0.267ns	0.321ab	0.418a	0.963a
	SD	0.033	0.144	0.149	0.193	0.637
	SE	0.011	0.045	0.047	0.061	0.201
	CV (%)	21.95	53.76	46.45	46.13	66.13

Notes: SD- Standard Deviation SE- Standard Error CV- Coefficient of Variation ns : not significant [*] : Same letter marked as statistical averages are not different from each other ($P<0.05$).

The levels of As, Cd, Hg and Pb in the wild vegetable samples are presented in Table 5. The level of As that has toxic effects with trace amounts was observed between 0.014 and 0.586 mg kg^{-1} in 4 different edible wild plants. These values are in agreement with reported values [30]. Another element with toxic effects, Hg, was determined in *U. diocia*, *T. orientalis*, *S. excelsa* and *O. umbellatum* to be 0.376±0.104 mg kg^{-1}, 0.313±0.110 mg kg^{-1}, 0.282±0.109 mg kg^{-1} ve 0.267±0.144 mg kg^{-1}, respectively. Hg results are in agreement with those reports for leafly fresh vegetables [15]. Thallium (Tl) is caused by fossil fuels and cement production also it take part in the formulation of pesticides as the active ingredient of the poison [31]. The Tl contents of *U. diocia*, *T. orientalis*, *S. excelsa* and *O. umbellatum* ranged from 0.087 to 0.635 mg kg^{-1} according to plant species. The average Cd contents of *U. diocia*, *T. orientalis*, *S. excelsa* and *O. umbellatum* were 0.026, 0.010, 0.032 and 0.418 mg kg^{-1}, respectively. The

amounts of Pb known as heavy or toxic elements, in *U. diocia*, *T. orientalis*, *S. excelsa* and *O. umbellatum* plants consumed as vegetables by local community were 0.997±0.416, 0.539±0.338, 0.628±0.335 and 0.963±637 mg kg^{-1}, respectively (Table 5). The Cd and Pb results are similar to those reported values [15, 24]. Our Pb results are lower than those reported in the literature [27]. The distribution of toxic elements in plants showed that the amount of As and Tl in *U. diocia,* the content of Cd in *O. umbellatum* roots, the amount of Pb in *U. diocia* and *O. umbellate* were higher than the other plants. Hg in all plants also varied within the same limits.

The fact that macro, micro and heavy metals varies according to plant species, this may have been occured from different plant parts (root, leaf, and stem) which are used for food. Indeed, local people are generally used the leaves of *U. diocia* plants, the stems of *T. orientalis*, the shoots of *S. excelsa* and the roots and leaves of *O. umbellatum* as food. The result of mineral differences can be expected because of plant edible parts were analyzed in this study. Mineral contents of our examples changed within broad limits according to both plant species and literature data on same plant species. This may be due to plant type, species, environmental conditions (soil mineral composition, soil type, contamination level, the industrial zone, use of pesticides and fertilizers, climate, irrigation, lighting, temperature, aeration, pH, nutrient type and concentration of these nutrients, the mutual effects with each other, etc.) [25, 30, 32], and the differences of minerals absorption capability of plants (root structure, young-old plants, etc.), differences of mineral deposition in plants parts (leaf, stem and roots) [24, 25], as well as sample preparation and the sensitivity of the apparatus used in the analysis [22].

In this study, most of plants were obtained from vegetation of hazelnut orchard. Therefore, fertilizers and agricultural laundering drugs (insecure, or more than the amount used in pesticides and fungicides) applied to hazelnut may have increased heavy metals in plants to be directly or indirectly. At the same time, the hazelnut orchard in the city center may be affected by heavy metal pollution causing sources. These sources are very diverse and are mainly small-scale industrial wastes (metal processing, metal-wire melting, coating, etc.), flue and exhaust gases (brick furnaces, diesel generators, and vehicle emissions, poor quality coal), the dust created during road construction. Moreover, other important factors of increase of heavy metals in growing plants in urban and rural areas are city garbage, sewage, industrial wastes, wastewater treatment facility sludge and contaminated water [32].

It has been reported that wild plants are richer in terms of macro and micro elements than cultured vegetables [8, 33, 34]. Indeed, in this study, the mineral content of 4 different wild plant compared with the mineral content of some culture vegetables such as *Spinacia oleracea* L., *Portulaca sativa* Haworth, *Lactuca sativa* L., *Allium porrum* L., *Petroselinum sativa* Hoffmann, *Brassica oleracea* L. var. Italica P., *Brassica oleracea* L. var. botrytis [23], the amounts of K, Ca, Mg, Zn, Na were dispersed in similar limits, but, some micro-elements in wild plants were found to be very high, such as Fe 4 folds, Cu 7 folds and Mn 2.5 folds. Finally, 100 g *U. diocia* can supply the majority of daily minerals needs as Cr (% 216) Mo (% 265), Fe (% 133), Mn (61%), Cu (60%), Ni (34%), V (27%), Mg (15%), Zn (11%) and Se (11%). Other hand, 100 g fresh plants of *T. orientalis* and *O. umbellatum* are rich resources to supply need of daily Cr (120-132%), Cu (66-81%), Fe (14-36%), V (34-25%), Mn (42-13%) and Mo (15-12%). *S. excelsa* are found to be adequate resources of Cr (36%), Ni (36%), Cu (31%) and Mo (21%) (Table 6).

Table 6. Evaluation of mineral contents of wild plants according to daily mineral requirements for humans.

Mineral	Daily requirement[a] (mg day^{-1})	Wild vegetables species							
		U. diocia		*T. orientalis*		*S. excelsa*		*O. umbellatum*	
		mg 100g^{-1}	Supply the requirement (%)	mg 100g^{-1}	Supply the requirement (%)	mg 100g^{-1}	Supply the requirement (%)	mg 100g^{-1}	Supply the requirement (%)
K	4700	434	9	485	10	35	0.7	219	5
Ca	1000	62	6	9	0.9	1.95	0.2	33.4	3
Mg	255	39	15	53	21	9.4	4	11.2	4
Na	2300	4.2	0.2	3.4	0.2	4.3	0.2	3.35	0.2
Fe	8.1	10.8	133	1.16	14	0.18	2	2.95	36
Zn	6.8	0.76	11	0.38	6	0.61	9	0.84	12
Cu	0.7	0.42	60	0.46	66	0.22	31	0.57	81
Mn	1.8-2.3	1.1	61	0.75	42	0.13	7	0.23	13
Ni	1.0	0.34	34	0.09	9	0.36	36	0.15	15
V	1.8	0.48	27	0.62	34	0.01	0.6	0.45	25
B	20	0.17	0.9	0.05	0.3	0.11	0.6	0.065	0.3
Cr	0.025	0.054	216	0.03	120	0.009	36	0.033	132
Co	25	0.04	0.2	0.006	0.02	0.002	0.01	0.018	0.07
Mo	0.034	0.09	265	0.005	15	0.007	21	0.004	12
Se	0.045	0.005	11	0.0003	0.7	0.000	0.0	0.001	2

Reference: [35].

The Al content received by an adult with daily diet varies in many countries (Australia (1.9-2.4), Finland (6.7), Germany (8-11), Japan (4.5), Netherlands (3.1), Sweden (13), Switzerland (4.4), UK (3.9) and USA (7.1-8.2)). The amounts of Ba can take daily from various food by adult have been reported to be 0.44-1.8 mg day^{-1}. In light of these data, the Al concentration of all plant species is not a health risk. Similarly, the high Ba content of *O. umbellatum* species (8 mg 100 g^{-1}) has no toxic effect. Because, it is emphasized that Ba taken up to 200 mg / body weight per day has no toxic effect on reproduction and development of human [36, 37].

Wild plants are used as vegetables for human nutrition. On the other hand, toxic compounds for human are absorbed with the consumption of these plants. The adverse effects of wild plants containing As, Cd, Hg, Tl and Pb has been reported by many researchers [8, 28, 30]. World Health Organization has determined limits and tolerable values to reduce negative effects on human health of heavy metals. Accordingly, the daily dose of the heavy metals can be tolerated for adults (70 kg) have been identified as 0.150 mg kg^{-1} for As, 0.070 mg kg^{-1} for Cd, 0.250 mg kg^{-1} for Pb, and 0016 mg kg^{-1} for Hg [38]. Thallium is more toxic to mammals than Hg, Pb and Cd. Its toxicity is more dependent on environmental source than food-borne, and the lethal dose of thallium and salts for adults is 10-15 mg per kilograms of body weight [31]. Consequently, contents of As, Cd, Tl and Pb of our examples (100 g) does not hazard for human health but the amount of Hg in samples supplied from near the city center reached up to 3.7 fold of daily tolerable limit values.

4. CONCLUSIONS

More demand for edible wild plants result from the idea of benefits on health. Research results show that, these plants are located in the vegetable group, is one of rich mineral resources that are effective on human nutrition and health. Therefore, wild plants can significantly contribute with the minerals composition especially for the diet of consumers with low purchasing power of the economic. Moreover, these plants can use for enrichment of diets with low mineral content. Other hand, excessive and continuous uptake of metals (As, Cd, Hg, Tl and Pb) by four plant species may produce toxicity in human nutrition. In order to ensure the sustainable use of wild plant resources as a food, the plant must not be damaged during the harvesting process. Wild plants should not be collected from areas affected by environmental pollution, and wild plants must be cleaned and washed very well before being used. If there is

any doubt about the collected area, consumers should prefer the consumption of cooked rather than raw. In addition, if wild plants that are directly or indirectly affected by pesticides are used for consumption, care must be taken that a certain period of time passes between the last drug application time and the harvesting period before consumption. Most important of all, these plants must be cultured, the use of wild plants for human nutrition should be increased, and more detailed studies should be done about the composition of these plants.

Conflict of Interests

Authors declare that there is no conflict of interests.

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The Colours and Fastness Values obtained from Basil (*Ocimum basilicum*) and Lemon Balm (*Melissa officinalis*) Plants of the *Lamiciacae* Family

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Abstract:Until the invention of synthetic dye substances, natural dyes were used for the purpose of dyeing in textiles, food, medicine and cosmetics fields. With the introduction of synthetic dyes, their use increased and the demand for natural dyes decreased. However, as a consequence of studies made, the carcinogenic properties of synthetic dyes were revealed and that they could create serious problems for human health. Therefore, plant dyes have come to the fore again. In this study, an examination was made of the dye properties and colour and friction fastness values of basil (*Ocimum basilicum*) and lemon balm (*Melissa officinalis*) plants. According to the data obtained, basil and lemon balm dyeing were made with different mordants and different colors were obtained. The highest colour fastness values for basil were seen to be obtained with copper II sulphate, iron II sulphate, potassium bi chromate and citric acid mordants. The highest friction fastness values were obtained with copper II sulphate, potassium aluminium sulphate and citric acid mordants. For the lemon balm, the highest colour fastness values were obtained with all the mordants and the highest friction fastness values with acetic acid and citric acid mordants.

Keywords: *Ocimum basilicum*, *Lemon Balm*, Vegetable dye, fatness to light colour, fatness to abrasio

1. INTRODUCTION

Today, around 20,000 plant species are used for medical purposes in the world. In this context, medical and aromatic plants, which are an important part of the Earth's flora, are widely distributed in different floristic regions [1]. Because Turkey is located at the intersection of three floristic regions, there are ten thousand plant species in the natural flora, provided that three of them are endemic. It is known that about 1000 of these plants are used for medical purposes [2]. Turkey is an important gene center for the family of ballibagiller (Labiateae = Lamiaceae) which is among aromatic plants. The family is represented in Turkey with a total of 731 taxa and 546 species, 45 genus [3].

Basil belongs to the *Lamiaceae* family and possesses 65 denier species in the world [4]. Ocimum species are single-year, herbaceous or small-scale herbaceous plants. Leaves are oval and hairless [5]. This plant is widely used in spices, medicines, food and perfumery because of its essential oils. bacillus essential oils, antifungal, antioxidant and insecticide properties as well

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as antiseptics for midwives, expectorants and urinary tracts [6], [7]). Linalool (46%), α-Terpinen (16%), Borneol (4.4%), Linalyl acetate (4.4%), β-Myrecene (2.1%), Thymol (1.7%) and Camphor) [8].

Lemon Balm belongs to Lamiaceae family, a perennial and herbaceous plant [5]. Lemon Balm naturally grows in southern Europe, the Balkans, North Africa and Turkey [9]. The leaves of Lemon Balm are deeply knotty, hairy or oval in shape [5]. The essential oils of the herb are enriched in terms of aldehydes and terpenes with therapeutic effect [10]. The main essential oil components are 39% citronellal, 33% citral (citronellol, linalool) and geraniol. It possesses antioxidant properties due to these phenolic acid components [11]. It has also been determined that it is effective in the treatment of Alzheimer's disease and in the prevention of tumor formation, as well as being used as a sedative, spasm remover, immune system enhancer due to the properties of essential oil. It is also used in perfumery and cosmetics industry due to hydrosol in volatile oil [12].

Today, as a result of the increase in demand for natural dyes, the determination of the colors obtained by different plants and mordants and their light and friction fastness values have become important. In this study; basil and lemon balm, which have important medicinal and aromatic properties, have been evaluated in this context.

2. MATERIAL and METHODS

Rye and son grass plants were used as experimental material. Natural leaves were obtained with the leaves of these plants in two different ways: mordant (8 different mordants, each at 2% and 4% concentration) and without mordant. As a mordant; aluminium sulphate (KAI (SO4) 2), copper sulphate (CuSO4.5H2O), ferric sulphate (FeSO4.7H2O), tartaric acid (C2H2 (OH) 2 (COOH) 2-C4H6O6), acetic acid, zinc chloride, sodium hydrosulphite, copper II sulphate, potassium bi chromate (K2Cr2O7) mordants were used.

2.1. Preparation of Paint Extract

The leaves of the plants containing the stain were made into small pieces so that the contained stain could pass through the water. Later, 100% of the weight of the wool yarn will be dyed, and the plants will be boiled for 1 hour in water at a rate of 1/20 compared to the dye to be dyed.

2.2. Unmordant Dyeing of Wool Yarns

Hot extracts were obtained using 100% of the plants. The wools, which have been soaked in water for 1 hour, are put into extracts prepared at 1/20 ratio. After reaching the boiling point, it was boiled continuously for one hour. Minor water was added during boiling. It is then rinsed with plenty of cold water and dried in a low light and airy place.

2.3. Mordanting of Wool Yarns

Wool yarns are moored separately with each of the mordants specified in the material section. For this, 2% and 4% mordants were used according to the weight of the wool yarn to be painted, and each wool yarn was treated separately with each mordant. The amount of mordant calculated according to yaw is melted in 1/20 of warm water, pre-moistened wool yarn is pressed into this mordant water. After one hour of boiling, the wools were cooled in boiling water, the resulting wool was then squeezed and dried and ready for coloring.

2.4. Mordant Dyeing of Wool Yarns

Previously mordanted wools were kept in water for at least one hour before starting the dyeing process, and then boiled for 1 hour in a hot extract prepared at 1/20 after being soaked

and allowed to cool. At the end, it was rinsed with plenty of cold water and dried in a low-light airy place.

2.5. Determination of Light Fastness

The determination of light fastness in dyed wool yarns was based on TS 867 (Color Sensitivity Testing Method for Daylight) [13] and DIN 5033 (Farbmessung Begriffe der Farbmetrik) Anonymous 1970) prepared by Turkish Institute of Satellarians. Wool yarn samples painted with a blue wool scale (wool fabric strips painted using various blue dyes graded from 1 to 8) were used to determine the light fastness [14], [15]. 1 indicates the lowest light fastness degree, and 8 indicates the highest fastness degree.

2.6. Determination of Friction Fastness

According to TS 717 [16], which is prepared by Turkish Standards Institute [16] and TS 423 (Textile Products Color Difficulty Determination of stains (dye flow) and solmanin (color). The method of using grey scales for evaluation) was done according to [17]. Uncolored cotton gauze color flow is evaluated according to TS 423 with grey scale [17]. Grey gives 1 the worst friction fastness value according to the (flow-staining) scale and 5 gives the best friction fastness degree.

2.7. Identification and naming of obtained colors

The colors obtained in consequence of this painting are named by a commission created in the Sivas Vocational High School/ Carpet & Decoration Program.

3. RESULTS and DISCUSSIONS

According to the obtained data; as a result of painting with different colors and different mordant applications (acetic acid, copper II sulphate, zinc chloride, iron II sulphate, potassium aluminium sulphate, potassium birochromate, citric acid, tartaric acid) lemon mold 1, coffee bean 1, coffee bean 2, almond green 1, almond green 2, coffee bean 3, coffee bean 4, almond green 1, coffee bean 1, coffee bean 2, potato crust 1, potato crust 2, almond green 3, almond green 4, almond green 3, almond green 4, 16 different colors were obtained in total.

The different mordants used provide different colors to be obtained from the plant [18]. For example, a copper sulphate mordant applied in a study on walnut fruit husks ensured that the color obtained was greenish-brown [19]. The mordant application in this study was carried out at two different concentrations of 2% and 4%. According to the obtained data; the highest light fastness values in both mordants (both 2% and 4%) in both concentrations were determined in copper II sulphate (7), iron II sulphate (7), potassium bi chromate (7) and citric acid (7) mordants. The mordants used in vegetable dyestuffs provide better adhesion of the dyes to the material and strengthen the durability ratings. As mordant materials, more water-soluble metal salts are used [20]. The highest light fastness value among the mordants that had been applied to the thyme plant was determined in the ferric sulphate mordant. If the obtained data will be evaluated in terms of friction fastness values; the highest rubbing fastness values were obtained from the zinc chloride mordant (2-3) at the concentration of 2%, while the highest value was reached at the mordant of tartaric acid (2-3) at the concentration of 4% (Figure 1, Figure 2). However, Kayabaşı and Ölmez [22] stated that they provided the highest friction fastness values in mordant-free applications.

As a result of different mordants (acetic acid, copper II sulphate, zinc chloride, iron II sulphate, potassium aluminium sulphate, potassium birochromate, citric acid, tartaric acid) applied to the psyllium plant; eight different colors were obtained, including boiled chickpeas 1, boiled chickpeas 2, pimento 1, pimento 2, coffee beans 1, coffee beans 2, olive oil green 1, olive oil green 2. Barber [18] reported that in copper mordant applications, the colors obtained

from the plants are more greenish in color. When potassium bi chromate mordant is used, more burgundy color is obtained [23]. When copper sulphate mordant is applied on the walnut fruit coat, it is providing brown close to khaki and greenish brown color [19]. The highest light fastness value at all concentrations (2% and 4%) of all mordants in the study was 7. The highest friction fastness values were obtained from the citric acid (3-4) mordant at a concentration of 2%, while at the concentration of 4% copper II sulphate (3), zinc chloride (3) and potassium birochromate (3) mordants (Table 1, Table 2). Iron mordant applications show increased light fastness values in plants [24]. Akan [21] was obtained the highest friction fastness value as the result of dyeing the apple leaf with mordants of vine stone and citric acid.

Table 1. Colors Obtained at 2% Concentration of Mordant Application Result

Plants	Mordants	Colors	Light Fastness Value	Friction Fastness Value
Basil (*Ocimum basilicum*)	Acetic acid	Lemon mold 1	5	2
	Copper II sulphate	Coffee Beans 1	7	2
	Zinc chloride	Almond green1	4	2-3
	Iron II sulphate	Coffee bean 3	7	1-2
	Potassium aluminium sulphate	Almond green1	4	1-2
	Potassium bi chromate	Coffee beans 1	7	2
	Citric acid	Potato crust 1	7	2-3
	Tartaric acid	almond green 3	4	2-3
Lemon Balm (*Melissa officinalis*)	Acetic acid	Boiled chickpeas 1	7	2
	Copper II sulphate	Pimento 1	7	3
	Zinc chloride	Boiled chickpeas 1	7	3
	Iron II sulphate	Coffee beans 1	7	2
	Potassium aluminium sulphate	Boiled chickpeas1	7	2
	Potassium bi chromate	Olive oil green1	7	3
	Citric acid	Boiled Chickpeas 1	7	3-4
	Tartaric acid	Boiled chickpeas	7	2-3

Table 2. Colors Obtained at Mordant Application Result at 4% Concentration

Plants	Mordants	Colors	Light Fastness Value	Friction Fastness Value
Basil (*Ocimum basilicum*)	Acetic acid	Lemon mold 1	5	2
	Copper II sulphate	Coffee Beans 1	7	1-2
	Zinc chloride	almond green1	4	1-2
	Iron II sulphate	Coffee bean 3	7	1-2
	Potassium aluminium sulphate	almond green1	5	2
	Potassium bi chromate	Coffee beans 1	7	1-2
	Citric acid	Potato crust 1	7	2-3
	Tartaric acid	almond green 3	4	2-3
Lemon Balm (*Melissa officinalis*)	Acetic acid	Boiled chickpeas 1	7	2-3
	Copper II sulphate	Pimento 1	7	3
	Zinc chloride	Boiled chickpeas 1	7	3
	Iron II sulphate	Coffee beans 1	7	1-2
	Potassium aluminium sulphate	Boiled chickpeas1	7	2
	Potassium bi chromate	Olive oil green1	7	3
	Citric acid	Boiled Chickpeas 1	7	2
	Tartaric acid	Boiled chickpeas	7	2

Conflict of Interests

Authors declare that there is no conflict of interests.

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Some Aromatic and Medicinal Plants from Bingöl (Turkey)

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Abstract: The use of aromatic and medicinal plants in developing countries has been widely observed. The increasing human population and demands in the late decades has led to over exploitation of land in many areas thus reducing the biodiversity of medicinal plants. Aromatic and medicinal plants possess odorous volatile substances and the characteristic aroma is due to a variety of complex chemical compounds. This study was carried out in order to contribute aromatic and medicinal plants knowledge of Eastern Anatolia Region of Turkey. This investigation included twenty medicinal or aromatic plant specimens collected and photographed from Bingöl provinces during the vegetation seasons 2016. With this study some medicinal and aromatic plants recorded and photographed; that might be useful for health-care programme, aromatic and medicinal plants knowledge, aromateraphy, phytoteraphy, economic agricultural policy development, alternative food programme, ethnobotany and development of drug sector.

Keywords: Bingöl, Medicinal and Aromatic plants

1. INTRODUCTION

Our country has a rich plant diversity due to the fact that it contains many different habitat types, is located at the intersection of three plant phytogeographical regions, is home to many different species, connects the Asian and European continents, has ecologic differences and rich water resources in addition to change in elevation ranging between 0-5000 meters and this plant diversity of our country has resulted in an increase in the medicinal and aromatic plant taxa [1]. Medicinal and aromatic plants are used as ground cover in agriculture, as decorative plants in parks and gardens, in erosion control, borders, parterre and rock gardens in addition to being used for food, drug and treatment purposes. In addition, medicinal plants are also used in areas such as nutrition, cosmetic, body care, incense or religious ceremonies, whereas aromatic plants are widely used in food, cosmetic and parfume sectors due to their nice scents and tastes [2].

It's different habitats and ecological properties in addition to new plant types that have recently been discovered put forth the richness of Bingol with regard to number and diversity

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of plants [3-6]. Even though our country is richer than Europe with regard to the number of plant species as well as with regard to medicinal and aromatic plants, more than 70% of the herbal substances used for producing drugs are still being imported which prevents our money from remaining inside our country. There are also various plants which are naturally found in the flora of our country among the exported herbal substances. Random, excessive and insensible collection of the economically valuable medicinal aromatic plants place their future generations at risk resulting in economic losses. Medicinal aromatic plants should be well known in order to benefit from them completely for our country's economy, ways to adopt them into our culture should be researched while their sectoral use should be determined based on their effective substances and the academic, technical knowledge and skills of people who carry out studies on the isolation of these effective substances should be increased [2]. Majority of the medicinal and aromatic plants are also rich in essential oils and have significant use in various sectors such as perfume, nutritional additives, cleaning products, cosmetics [7]. However, our medicinal aromatic plant flora is being damaged due to unplanned urbanization, illegal lumbering, forest fires, pollution due to industrial and domestic wastes, radiation emission, insensible use of pesticides, excessive grazing, collecting from nature and erosion and studies should be carried out for preventing this damage, reducing our dependence on foreign countries and for increasing the contributions to the country's economy in this field while also trying to ensure the sustainability of studies in this area.

In this study, twenty plants in Bingol with medicinal-aromatic were determined and photographed after which a brief information was provided regarding their intended use and properties and it was aimed to attract attention to the medicinal aromatic plant potential in Bingol. In addition, it is also stated that awareness and protection of this potential in our country and city along with decrease of our foreign dependency in herbal products despite this rich potential will make significant contributions to the economy of the country.

2. MATERIAL and METHOD

Twenty medicinal-aromatic plants [*Achillea biebersteinii* Afan., *Achillea millefolium* L. subsp. *pannonica* (Scheele) Hayek., *Hypericum scabrum* L., *Lamium garganicum* L. subsp. *reniforme* (Montbret & Aucher) R.R. Mill., *Nepeta nuda* L. subsp. *nuda*, *Origanum acutidens* (Hand.-Mazz.) Letswaart, *Salvia multicaulis* Vahl., *Salvia palaestina* Benth., *Salvia verticillata* L. subsp. *verticillata*, *Salvia sclerae* L., *Salvia syriaca* L., *Stachys lavandulifolia* Vahl var. *brachydon* Boiss., *Scutellaria orientalis* L. subsp. *orientalis*, *Tanacetum densum* (Lab.) subsp. *amani* Heywood, *Tanacetum zahlbruckneri* (Nàb.) Grierson, *Tanacetum parthenium* (L.) Sch. Bip., *Teucrium parviflorum* Schreb, *Teucrium multicaule* Montbret et Aucher ex Benth., *Mentha longifolia* (L.) Huds. subsp. *typhoides* (Briq.) Harley var. *typhoides*, *Thymus kotschyanus* Boiss. & Hohen. var. *kotschyanus*] were determined in the Bingöl city during the 2016 vegetation period and these plants were used material in this study. In addition, literatures were also used as sources of data. Plants were pressed in accordance with the herbarium technique following the keeping of locality and field records and "Flora of Turkey and the East Aegean Islands" [8] was used as the main reference for identification of these samples. After the identification procedures were completed, studied samples were deposited in Bingol University Park and Garden Plant Department. The plants locality information are: *Achillea biebersteinii*: Around Şaban village, steppe-rocky areas, 1750-1850 m. *Achillea millefolium* subsp. *pannonica*: West of Dikme village, steppes-rocky areas, 1800-1850 m. *Hypericum scabrum*: around Haserek mountain, steppes and inclined areas, 1850-1950 m. *Lamium garganicum* subsp. *reniforme*: south of Yelesen village, rocky areas, 1600-1700 m. *Origanum acutidens*: around Aşağıköy exit and steppes, 1500-1600 m. *Nepeta nuda* subsp. *nuda*: south of Şaban village, steppes, 1300-1400 m. *Salvia multicaulis*: 3 km after Şaban village, side of the road and slopes, 1550-1600 m. *Salvia palaestina*: 3 km to Aşağıköy from Bingöl to the right of

the road, slopes and around *Quercus*, 1500-1600 m. *Thymus kotschyanus* var. *kotschyanus*: Towards Yelesen from Şaban village, slopes, 1600-1650 m. *Salvia syriaca*: North of Dikme plains, slopes , 1750-1800 m. *Scutellaria orientalis* subsp. *orientalis*: North of Yelesen village, slopes-rockies, 1650-1700 m. *Tanacetum zahlbruckneri*: upper areas of the Dikme plain, slopes, 1750-1850 m. *Teucrium parviflorum*: exit of Aşağıköy, towards Yelesen, left of the road, steppe, 1700-1750 m. *Tanacetum parthenium*: 5 km to Aşağıköy from Bingöl left of the road, along the river, 1500-1550 m. *Salvia verticillata* subsp. *verticillata*: North of Dikme village, forest clearance, 1700-1800 m. *Mentha longifolia* subsp. *typhoides* var. *typhoides*, West of Şaban village, towards Yelesen, right of the road, moist area, 1400-1500 m. *Teucrium multicaule*: 2nd km of the Çiriş village road, right of the road, slopes, 1600-1650 m. *Salvia sclerae*: West of Direkli village, Elazığ-Bingöl main road side of the forest, 1500-1600 m. *Stachys lavandulifolia* var. *brachydon*: West of Dikme village, steppes and rocky areas, 1750-1800 m. *Tanacetum densum* subsp. *amani*: North of Şaban village, rocky areas, 1700-1750 m.

3. RESULT and DISCUSSION

Many medicinal and aromatic plants can naturally be grown in our country due to its suitable ecological properties, majority of these can be collected directly from nature as is the case in many regions of the world whereas some are cultured. It is a known fact that the importance and industrial use of medicinal and aromatic plants are increasing every day. These plants are provided by way of collecting from nature in our country as well as partially from cultured plants. However, insensible collection of plants with medicinal, aromatic or economic value result in the disruption of the natural vegetation, extinction of rare, endemic and medicinal aromatic plants as well as the increase of erosion which is an important problem in our country [9]. Our country has a rich flora due to its geographical location, geomorphological structure, many different soil types and climate diversity and is among the top countries in terms of plant variety and diversity with studies indicating that our country is richer than Europe in terms of plant variety [10]. However, plants in the flora of our country and especially the medicinal aromatic ones are under various pressures thus many species face difficulties in continuing their existence. These are; industrialization and urbanization, land clearing and excessive grazing, tourism, export and domestic use, reclamation of arid, halophilous lands, agricultural pest control and pollution, afforestation and fires which cause damages in medicinal and aromatic plants as well. There is a small number of studies in literature on Bingol flora and especially on medicinal aromatic plants and recent studies carried out by the researchers of our university on the detection and analyses of various flora and medicinal aromatic plants along with the new plant species identified in Bingol [6] are indications of the number of plants in Bingol, its diversity and richness and as the number of such floristic studies as well as studies on medicinal aromatic plants increases, the richness of Bingol with regard to vegetation and especially medicinal aromatic plants will become clearer which will also contribute to the rural development of the region. In addition to a rich vegetation, intensive apiculture and animal rearing activities, natural beauties, various types of nature tourism (Plain-Flora-Winter-River-Hunting-Cave tourism areas, wildlife observation and trekking) are all indications of the special place of Bingol for our country. It was determined as a result of the study entitled the Flora of Dikme (Kür) plain (Bingol-center) and Its Environs that the Lamiaceae family members were among the most abundant plants in the region [6]. It is noteworthy with regard to studies on medicinal and aromatic plants that the Lamiaceae members are ranked high with regard to the number of plants in floristic studies carried out in Bingol. The Lamiaceae family which includes plants that are most frequently visited by bees is a family with plants that are mostly fine scented, annual or perennial, herbaceous and rarely bushy. Majority of the family members include medicinal, aromatic, scented plants and since these plants contain high amounts of essential and aromatic oils, they can be used in many areas such as raw materials for drugs, in

the cosmetic industry, they can be consumed as tea and they can also be used as decorative plants due to their fine scent and appearance. In addition to the Lamiaceae family, there are also other plant families with medicinal, aromatic and fragrant plants with many economically valuable plants which are found in the natural environment but which are yet to be cultured. Natural vegetation and especially medicinal aromatic plants should be better known in order to ensure the continuity of studies on plants with high economic value in our country and Bingol, necessary precautions should be taken to protect them from harm and the required sensitivity should be shown so that our dependence to foreign countries decreases and the income level of both our country and Bingol increases. These precautions can be listed as follows: continuity of incentives and support for increasing plant production via micro-cultivation in laboratory conditions in order to decrease the collection of medicinal aromatic plants from nature. Significant contributions will be made to our country's economy as studies are continued within the scope of the project for the development of perfumery and medicinal plants and dye plants which are currently in application at Bingol as well as in many different cities, our dependence on foreign countries will decrease, collection from nature will decrease and hence especially medicinal, fine scented plants will be able to spread further in nature thereby leading us to understand the importance of this and similar studies. We can all show the required sensitivity by placing greater emphasis on our national, conscientious and humane responsibility by increasing studies on the cultivation of medicinal aromatic plants in culture areas, benefiting from the knowledge of experts in the field, continuing organizations such as workshops, conferences and scientific studies. Cultivation studies should be carried out by taking into consideration the secondary substance content of species as well as research results on their genetics and heredity and they should be carried out firstly at ecologic conditions where they are naturally grown. Hence; higher yield will be obtained from unit area, thereby obtaining pure, clean drugs that are in accordance with standards. The market volumes of medicinal and aromatic plants continue to increase parallel to their use in different areas and fields of industry. The number of plants that are currently collected from nature and produced is still very low despite the fact that our country has a rich source of medicinal aromatic plants. The increase of medicinal and aromatic plant cultivation in our country can be attained in addition to the development of many relevant branches of industry in a short period of time by taking the necessary precautions.

4. CONCLUSION

Many medicinal and aromatic plants are collected from nature in our country and some of them are cultivated to some extent. However, there are no regular statistical data on these and cultivation is not carried out based on the relationship between supply-demand. Data banks should be formed from where information related with these plants can be accessed. In addition, local consumption and foreign trade data should be taken into consideration for medicinal and aromatic plants for determining how much should be collected from which plant in nature and how much should be cultivated. It will be beneficial to establish an interdisciplinary committee that will provide information on which plants should be cultivated in addition to the supply-demand situation in the global market as well as prices. Annual imports worth millions of dollars will thus be decreased to some extent and significant income will be provided both to the local public and our country's economy by cultivating and exporting the medicinal and aromatic plants determined in the natural environment of our country and our city.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Determination of Phenolic Composition of *Tilia Tomentosa* Flowers Using UPLC-ESI-MS/MS

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Abstract: Phenolic compounds, which are secondary metabolites of plants, are one of the main groups of compounds that provide antiallergic, antiinflammatory, antimicrobial, antioxidant, cardioprotective properties of medicinal and aromatic plants. These broad physiological effects that they possess lead researchers to examine the phenolic contents of plants. *Tilia tomentosa* Moench is one of 45 species belonging to *Tiliaceae* family, and the use of flowers in traditional treatment methods is quite common. Although it is well known that *T. tomentosa* flowers are rich in phenolics with various biological functions, there is no recent study on determination of phenolic compounds of *T. tomentosa* flowers using UPLC-ESI-MS/MS. In this study, firstly, *T. tomentosa* flowers were extracted using hexane and volatile oil fractions were separated from the plant. Distilled water:methanol (50:50) mixture was added to the remaining flower part at 40 °C and that subjected to extraction for 15 min. The obtained extract was filtered and dried in a lyophilizer at -70 °C. The residue was redissolved in a mixture of distilled water:methanol (80:20). The sample was analyzed by UPLC-MS/MS (Waters Acquity Ultra Performance LC, Xevo TQ-S MS/MS) by passing through Macherey-Nagel Chromafil Xtra PTFE-20/25 0.20 µm filters. According to the analysis results, 3,4-dihydroxybenzoic acid (66.820 mg/kg), myricetin (29.395 mg/kg), rutin (21.421 mg/kg), ferulic acid (12.334 mg/kg) and 3,4-dihydroxybenzaldehyde (10.383 mg/kg) were detected. *T. tomentosa* flowers have great potential to usage in industries such as food, medicine and cosmetic due to its rich content of phenolics.

Keywords: Phenolics, 3,4-dihydroxybenzoic acid, *Tilia tomentosa*, UPLC-ESI-MS/MS

1. INTRODUCTION

Phenolics or polyphenols are secondary plant metabolites generally found in edible plants and have always been of interest because of their biological functions [1]. Phenolic compounds are important determinants in sensory and nutritional specifications of plants [2, 3]. These compounds presence an aromatic ring with one or more hydroxyl groups and their structures may vary from a simple phenolic molecule to a complex polymer with high-molecular mass [4]. Phenolics have been used for centuries for various medicinal purposes. Phenolic compounds, especially phenolic acids and flavonoids, which are of great interest mostly due to

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their biological functions in human health-related issues, are accepted as main source of pharmacological properties with their presence [5]

Ones of the most widely occurring phytochemicals in plants are phenolic compounds. Phenolic compounds are known to be extremely beneficial in terms of human nutrition, cosmetic and pharmacological [6-9]. As a large group of biologically active chemicals, they have large number of biological functions [10]. As previously reported in literature, phenolic compounds have cardio-protective effect, anti-cancer effect, anti-diabetic effect, anti-aging effect, neuro-protective effect, anti-viral effect, analgesic and anti-inflammatory effects, anti-bacterial effect, anti-parasitic effect and anti-oxidant effects [11, 12]. At the basis of the antioxidant capacities of the phenolic compounds are redox properties which allow them to function as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelating agents [4].

As a result of bioactive compounds found in different parts of plants such as flowers, peel, leaf and increased interest in natural products, medicinal and aromatic plants have found application fields such as pharmaceutical, cosmetic and dye industries. Therefore, they have been subjected to numerous researches [13-15]. Throughout the history of humanity, many diseases (diabetes, jaundice, shortness of breath, etc.) have been studied and tried to be treated using plants. The World Health Organization (WHO) reports that approximately 4 billion people around the world are trying to get rid of health problems with herbal drugs in the first place (80 % of the world population). Furthermore, in developed countries, about 25 % of prescription drugs constitute plant-based active ingredients [16].

Tilia tomentosa Moench is one of 45 species belonging to *Tiliaceae* family [17] and the use of flowers in traditional treatment methods is quite common [18]. Researches on different parts of *T. tomentosa* showed that the plant possesses spasmolytic, diuretic and sedative effects due to its flavonoids, essential oil and mucilage components and has been used to treat disorders such as nervous tension, cough, flu, migraine [19, 20].

T. tomentosa have been studied as novel phenolic compound source and qualitative and quantitative analyzes of phenolic compounds have been carried out using various techniques until today and reported that *T. tomentosa* contains flavonoids, mainly quercetin glycosides (rutin, quercitrin, and isoquercitrin), kaempferol glycosides, tyliroside and phenolic acids (caffeic, *p*-coumaric, and chlorogenic acids) [21, 22]. Additionally, polysaccharides, tannins and terpenoids were identified [22, 23].

Although it is a well-known and frequently used plant, it is seen that the number of studies on *T. tomentosa* is not much. To the best of our knowledge, there is no previous study using UPLC-ESI-MS/MS for the determination of the phenolic composition of *T. tomentosa* flowers.

In this study, the phenolic composition of *T. tomentosa* flowers was determined using UPLS-ESI-MS/MS and the importance of the plant as a medicinal and aromatic plant was evaluated according to the results.

2. MATERIAL and METHODS

2.1. Chemicals and Standards

Phenolic reference standards (pyrogallol, homogentisic acid, 3,4-dihydroxybenzoic acid, gentisicacid, pyrocatechol, galantamine, 4-hydroxy benzoic acid, 3,4-dihydroxybenzaldehyde, catechin hydrate, vanillic acid, caffeic acid, syringic acid, vanillin, epicatechin, catechin gallate, p-coumaric acid, ferulic acid, rutin, trans-2-hydroxy cinnamic acid, myricetin, resveratrol, trans-cinnamic acid, luteolin, quercetin, naringenin, genistein, apigenin, kaempferol, hesperetin, chlorogenic acid, and chrysin) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

All chemicals were analytical grade and purchased from local suppliers. High-Performance Liquid Chromatography (HPLC) grade water (18.2 MΩ) was purified by Millipore Milli-Q system (Molsheim, France) that contains reverse osmosis, ion exchange, and filtration steps.

2.2. Plant Material

T. tomentosa flowers were collected in the time of flowering season (June, 2016) in Muğla, Turkey. The plant was identified from Muğla Sıtkı Koçman University, Faculty of Science, Department of Molecular Biology and Genetics. *T. tomentosa* flowers were collected carefully and studied in fresh form without exposure to extreme temperatures.

2.3. Sample Preparation for the Determination of Phenolic Compounds

Phenolic compounds of *T. tomentosa* flowers were extracted according to the previously reported method [24, 25] with slight modifications. Briefly, flowers of *T. tomentosa* were extracted with hexane at first, and essential oil was removed from the plant. After this extraction, portion of flower was extracted using distilled water:methanol (50:50) at 40 °C for 15 min. This extract was filtered and dried at -70 °C with freeze dryer. Then, the residue was redissolved in water:metanol (80:20) mixture. The mixture was filtered from Macherey-Nagel Chromafil Xtra PTFE-20/25 0.20 µm, and analyzed using UPLC-MS/MS (Waters Acquity Ultra Performance LC, Xevo TQ-S MS-MS) instrument.

2.4. Determination of Phenolic Compounds Using UPLC-ESI-MS/MS

The UPLC-ESI-MS/MS instrument includes Waters (Milford, MA, USA) Acquity Ultra Performance LC with a Waters binary system manager and sample manager coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer with ESI probe. The separation was done with Waters analytical C18 column, Acquity UPLC BEH C18 (1.7 µm 2.1 × 100 mm) at 40°C column oven temperature and 2 µL injection volume with the two mobile phases (mobile phase A, 0.5 % (v/v) acetic acid in ultrapure water and mobile phase B, 0.5 % (v/v) acetic acid in acetonitrile) with a linear gradient mode, 0–1 min 99 % A, 1–10 min 70 % A, 10–12 min 5 % A, 12–13 min 99 % A at 0.650 mL min^{-1} flow rate. The multiple reaction monitoring (MRM) mode executes the transitions of parent ion to daughter ions of m/z, and the optimal instrument parameters of the mass spectrometer were as described in previous methods [25, 26]. MassLynx mass spectrometry software and TargetLynx data processing software (Waters) were used for the identification and evaluation of phenolic compounds by comparing retention time and m/z transitions of commercial standards using established calibration curves.

3. RESULTS and DISCUSSIONS

UPLC analyses of phenolic compounds in flowers of *T. tomentosa* revealed that flowers were highly rich in phenolics. In total, 24 of phenolic compounds were detected among 32 phenolic compounds were scanned.

Genistein, galanthamine, quercetin, pyrocatechol, gentisic acid, trans-2-hydroxy cinnamic acid, homogentisic acid and chlorogenic acid in analyzed sample were not determined.

3,4-dihydroxybenzaldehyde (10.383 mg/kg), 3,4-dihydroxybenzoic acid (66.820 mg/kg), ferulic acid (12.334 mg/kg), myricetin (29.395 mg/kg) and rutin (21.421 mg/kg) were found to be major phenolic compounds while 4-hydroxy benzoic acid (3.542 mg/kg), vanillic acid (5.275 mg/kg), kaempferol (3.683 mg/kg), and catechin hydrate (6.685 mg/kg) were determined as minor compounds. Phenolic compounds which were identified in flowers of *T. tomentosa* are given as ppm (mg/kg) with their method parameters in Table 1.

3,4-dihydroxybenzoic acid was determined as the highest rate of phenolics with the amount of 66.820 mg/kg (39.57% of phenolics), while the apigenin was minimum with the amount of 0.012 mg/kg.

Total phenolic content of *T. tomentosa* flowers was found to be 168.850 mg/kg in this study. Phenolic profile of flowers contained phenolic acids and their derivatives, flavonols, flavanols, hydroxybenzaldehydes and other compounds.

Table 1. Phenolic concentrations (mg/kg ± standard deviation) and method parameters for the analysis of compounds using UPLC-ESI-MS/MS

No	Compounds	Quantification > confirmatory transition (*m*/*z*)	Cone voltage (V)	Collision Energies (V)	Concentration (mg/kg) ± SD
1	Pyrogallol	125.01 > 69.10, 79.04, 81.02	20	17, 17, 14	0.701 ± 0.003
2	Gallic acid	168.95 > 125.02, 107.02, 97.02	20	25, 20, 14	0.232 ± 0.007
3	Homogentisic acid	167.03 > 123.03, 122.08, 108.00	10	20, 20, 10	ND
4	3,4-Dihydroxybenzoic acid	153.06 > 108.00, 81.01, 91.01	10	20, 25, 20	66.820 ± 0.007
5	Gentisic acid	153.05 > 109.04, 108.03, 81.00	10	20, 20, 12	ND
6	Pyrocatechol	153.06 > 81.01, 108.00, 109.04	8	20, 25, 20	ND
7	Galantamin	288.10 > 198.00, 213.09, 230.95	20	32, 23, 17	ND
8	4-Hydroxy benzoic acid	136.98 > 93.03, 65.10	10	25, 14	3.542 ± 0.005
9	3,4-Dihydroxybenzaldehyde	137.00 > 91.93, 107.94, 136.00	8	21, 20, 18	10.383 ± 0.008
10	Catechin hydrate	288.88 > 109.15, 124.99, 245.26	30	25, 20, 15	6.685 ± 0.005
11	Vanillic acid	166.98 > 151.97, 108.03, 123.03	20	18, 12, 14	5.275 ± 0.005
12	Caffeic acid	179.10 > 135.14, 107.10, 133.9	32	23, 23, 24	1.824 ± 0.004
13	Syringic acid	197.20 > 123.00, 167.00, 182.00	15	22, 18, 14	0.320 ± 0.005
14	Vanillin	150.95 > 135.94, 91.90, 107.97	30	20, 20, 14	0.051 ± 0.002
15	*p*-Coumaric acid	163.01 > 119.04, 93.00, 117.01	5	27, 27, 15	0.730 ± 0.005
16	Ferulic acid	193.03 > 134.06, 178.00, 149.02	20	16, 12, 13	12.334 ± 0.011
17	Epicatechin	189.18 > 151.00, 203.00, 205.00	20	20, 20, 20	4.002 ± 0.005
18	Chlorogenic acid	353.02 > 191.01, 179.09, 161.02	30	30, 28, 24	ND
19	Catechin gallate	441.00 > 168.98, 288.97	30	20, 20	0.140 ± 0.005
20	Rutin	609.00 > 254.99, 270.93, 299.90	17	55, 55, 40	21.421 ± 0.005
21	*trans*-2-hydroxycinnamic acid	163.04 > 119.04, 117.01, 93.07	10	25, 22, 13	ND
22	Myricetin	316.90 > 107.07, 137.01, 150.97	30	30, 25, 25	29.395 ± 0.010
23	Resveratrol	227.01 > 143.01, 159.05, 185.03	30	25, 18, 18	0.020 ± 0.004
24	*trans*-Cinnamic acid	146.98 > 103.03, 62.18	30	10, 10	0.922 ± 0.003
25	Luteolin	284.91 > 107.01, 133.05, 151.02	20	30, 33, 30	0.314 ± 0.004
26	Quercetin	303.00 > 137.00, 153.00, 229.00	20	30, 32, 30	ND
27	Naringenin	270.98 > 107.00, 119.04, 150.97	20	25, 25, 20	0.060 ± 0.005
28	Genistein	271.00 > 153.00, 215.00, 243.00	20	27, 25, 24	ND
29	Apigenin	269.10 > 107.00, 117.00, 149.00	20	30, 30, 25	0.012 ± 0.002
30	Kaempferol	284.90 > 158.97, 117.10, 227.14	10	34, 40, 30	3.683 ± 0.006
31	Hesperetin	301.02 > 108.01, 136.00, 163.99	20	36, 30, 24	0.015 ± 0.002
32	Chrysin	252.99 > 63.05, 107.05, 142.99	20	30, 25, 25	0.024 ± 0.005

ND : Not detected

Total ion chromatograms of major phenolic compounds determined in *T. tomentosa* flowers using ultra-performance liquid chromatography with electrospray ionization coupled to tandem mass spectrometry (UPLC-ESI-MS/MS) were given in Figure 1.

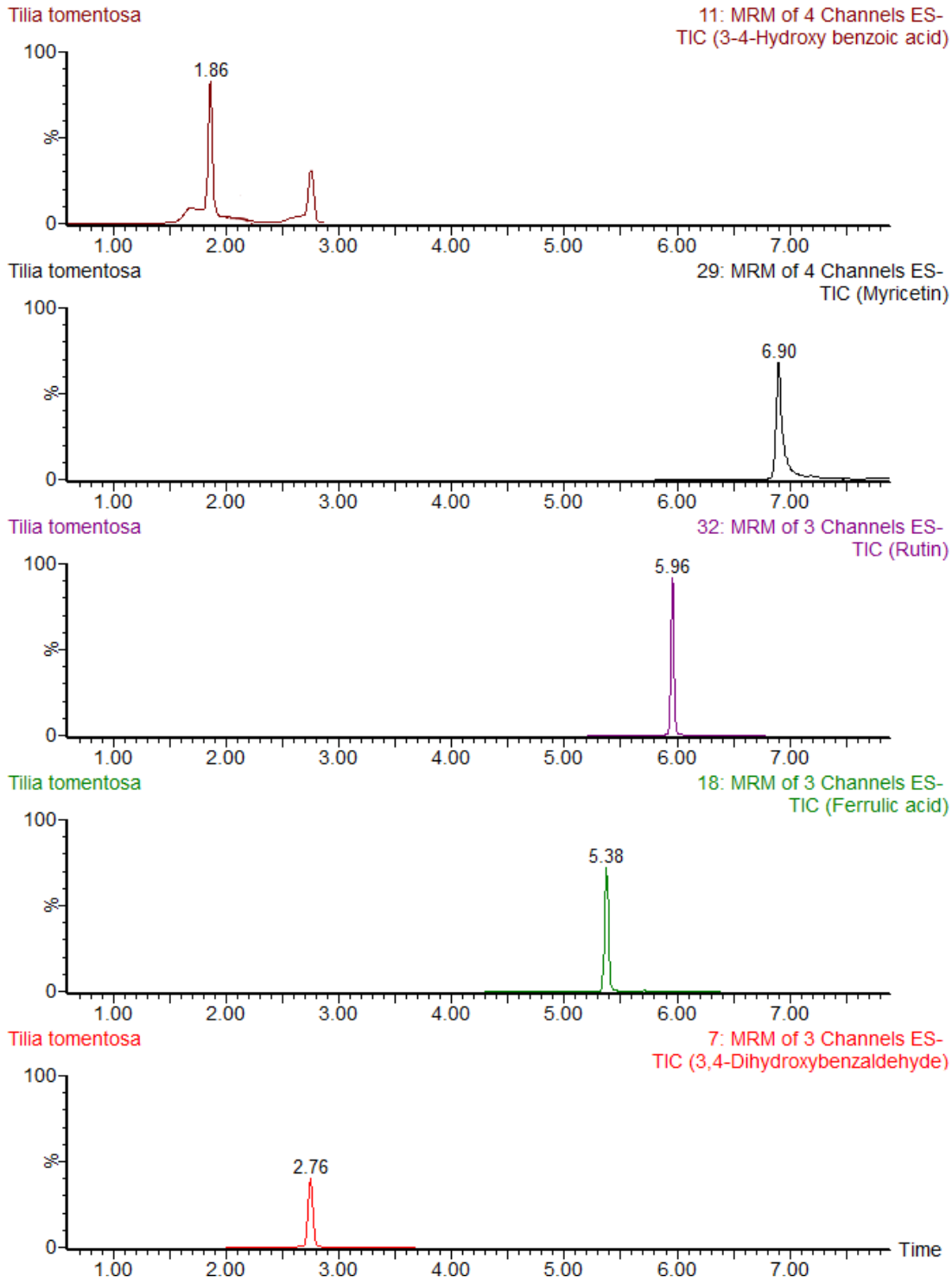

Figure 1. Total ion chromatograms (TIC) of major phenolic compounds analyzed using UPLC-ESI-MS/MS.

Although there is no previous study for the determination of the phenolic composition of *T. tomentosa* flowers, there few literatures related to leaves of *T. tomentosa* (syn. *T. argentea*) Toker et al. [27] reported that kaempferol 3,7-O-α-L-dirhamnoside (I) and quercetin 3,7-O-α-

L-dirhamnoside (II) were isolated from the leaves of *Tilia argentea* (Tiliaceae) in the leaves of *Tilia argentea*. Also Demiray et al. [28] indicated that protocatechuic acid is the major free phenolic compound in acetone and methanol extracts of *T. argentea*. Distilled water showed the highest extraction capacity for catechin, chlorogenic, caffeic and gallic acids.

Aromatic plants are broadly used by food industries but their properties also justify their application by other industries like food packaging, cosmetics, perfumery and pharmaceutical. According to the results of phenolic composition of *T. tomentosa* flowers we can conclude that flowers of *T. tomentosa* are potent natural ingredients for scientists, manufactures and producers to replace their syntetic materials with natural ones.

4. CONCLUSION

Nowadays, it is clear that the escape from artificial substances will further increase the importance of natural phenolic substances. In addition to the possibilities for use food, pharmaceutical and cosmetic industries. It is necessary to understand the mechanisms of action of phenolic substances, which have important effects on human health, and to investigate ways to quantify and use them technologically. For this purpose, phenolic compositions of medicinal and aromatic plants or their different extracts need to be investigated with accuracy and precision using modern instruments.

In this study, the phenolic composition of *T. tomentosa* flowers were identified first time using UPLC-ESI-MS/MS instrument. The lack of information about phenolic composition of *T. tomentosa* flowers using UPLC-ESI-MS/MS makes this study unique and important. The UPLC-ESI-MS/MS demonstrated to be reliable for the unambiguous detection of a large number of compounds, by enabling the determination of phenolic profiles. According to the results, it is understood that *T. tomentosa* flowers are very rich in phenolic acids and flavonols which are the two most important phenolic substance groups. The most abundant ingredients in the samples were 3,4-dihydroxybenzoic acid, myricetin, rutin, ferulic acid and 3,4-dihydroxybenzaldehyde.

The study is a guide for those who want to study the biological activities of *T. tomentosa* flowers include but not limited for antioxidant activity, anticancer activity or antitumor activity.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Antibacterial activities of *Calendula officinalis* callus extract

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Abstract: The purpose of this study was to determine the antibacterial activity of *C. officinalis* callus derived from cotyledon explants. Cotyledons excised from *in vitro* germinated seedlings were used as explants. Explants were transferred on MS medium supplemented with benzil amino purine (BAP; 2 mg l^{-1}), α-naphthalene-acetic acid (NAA; 2 mg l^{-1}) for callus studies. The cultures were maintained on the same media compositions and were sub-cultured at an interval of 4 weeks. Callus cultures were harvested at the end of the 16th week. Calli were dried at 40° C in the dark for antimicrobial studies. *Calendula officinalis* callus extracts were tested for their antibacterial activities by using agar well diffusion method. Ethanol and chloroform extracts from these plants were assayed against nine bacteria species (*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium* CCM 5445, *Proteus vulgaris* ATCC 6896, *Enterococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, and *Kocuria rhizophila* ATCC 9341). The test antibiotics penicillin G, novobiocin, amphicillin, chloramphenicol and erythromycin were used for comparison. Callus formation was observed at the end of the 5th week on cotyledon explants. *C. officinalis* callus extracts showed 38 mm inhibition zone against *S. aureus*, and chloroform extracts showed 32 mm inhibition zone against *B. cereus*. These results are very close to the test antibiotics used and *C. officinalis* is found more effective on gram positive bacteria.

Keywords: *Calendula officinalis*, callus, antibacterial activity, BAP, NAA.

1. INTRODUCTION

Plants are able to synthesize substances called secondary metabolites in addition to the production of carbonhydrates, proteins and fats, the primary building blocks of life, that is, primary metabolites. The discovery of the bioactive properties of secondary metabolites and their widespread use in many areas of industry such as medicine, cosmetics, paint making, and fragrance progressively increase the significance of medical and aromatic plants in the world markets [1, 2]. Biotechnological methods, especially callus culture of plant cell tissue cultures, appear as alternative methods in the production of secondary metabolites in order to solve encountered problems by wild environments [3]. In many studies callus cultures represent an alternative source for producing natural antimicrobial compounds and plant tissue cultures produce a variety of secondary metabolites, sometimes in higher percentage than the original

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plant, particularly in the polyphenolic class in which high yields have sometimes been obtained [4].

Calendula officinalis L., known for its ornamental plant characteristics, is a medicinal plant belonging to Asteracea family. It is annual or perennial taproot plant with 20-40 cm height and has 20 varieties [5]. The chemical compounds identified in methanol extracts of *C. officinalis* include polar compounds phenolic acids and flavonoid glycosides [6]. As a result of the many studies performed with *C. officinalis*, the anti-cancer [7], anti-microbial [8], anti-leishmanial [9], anti-HIV [10], antioxidant [11], cytotoxic, anti-tumor [12], anti-viral [13], anti-inflammatory [14], edema amplifier [15], hypoglycemic [16], uterotonic [17], effects and its utilization in the treatment of venous ulcers [18], as well as the biligenic [19] pharmacologic effects of this plant have been reported.

In this study, the effect of BAP and NAA plant growth regulators applied on *C. officinalis* cotyledon explants and the antibacterial effect of the obtained calluses have been investigated.

2. MATERIAL and METHODS

2.1. Plant material

Calendula officinalis seeds used in the study were obtained from Hekim Sinan Medicinal Plant Research Center of in the municipality of Kutahya, Turkey.

2.2. Medium & culture conditions

Murashige and Skoog medium was used as nutrient medium. For germination studies without any plant growth regulators and for callus studies MS medium supplement with 2 mg l^{-1} BAP and 2 mg l^{-1} NAA were respectively used [20]. They were supplemented with 100 mg l^{-1} myo-inositol and 30 mg l^{-1} sucrose. The pH of the medium was adjusted between 5.7-5.8 using 0.1 N NaOH or 0.1 N HCl before the addition of 0.7 % (w/v) agar and then the medium was autoclaved at 121°C for 15 min at 105 kPa. Cultures were maintained at 24 ± 2°C, under 16:8 photoperiod at a 4000 lux white fluorescent light, in a growth room.

2.3. Establishment of the aseptic culture

Initially, *C. officinalis* seed pods were unshelled, separated, and washed using distilled water. Later surface sterilization was made in 70% ethyl alcohol for 3 min and 0.5% NaOCl for 5 min, the seeds were rinsed with sterilized distilled water to remove the traces of sterilant. They were transferred on MS medium for germination.

2.4. Callus culture & induction

Calendula officinalis L. cotyledon explants were used as explants for the induction of callus. Cotyledons were excised from aseptic seedling and were and cultured on MS medium suplemented with; 2 mg l^{-1} BAP and 2 mg l^{-1} NAA for callus studies. The cultures were maintained on same medium compositions and sub-cultured in the same medium every four weeks. The calli were harvested at the end of 16th week and were dried at 40° C under the dark for antimicrobial studies.

2.5. Test microorganisms and growth conditions

Test microorganisms included following bacteria: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium* CCM 5445, *Proteus vulgaris* ATCC 6896, *Enterococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, and *Kocuria rhizophila* ATCC 9341. Cultures of these bacteria were grown in Mueller Hinton broth (Oxoid) at 37ºC for 24h [21].

Test microorganisms were obtained from the culture collection of Ege University, Faculty of Science, Basic and Industrial Microbiology Department.

2.6. Antibacterial activity assay

The dried and powdered plants were reduced to coarse powder. Two g of powder was extracted with 20 ml of ethanol and chloroform at room temperature with stirring for 3 days (125 cycles/minute). The solvents were evaporated to dryness after extraction progress. Sample solutions were prepared by dissolving the extracts in same solvents (1 ml). *In vitro* antibacterial studies were carried out by the agar well diffusion method against test microorganisms. Bacterial strains grown on nutrient agar at 37ºC for 24 h were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 MacFarland standards [10^6 Colony Forming Units (CFU)/ml]. Briefly, 50 microlitres (µl) inoculum (containing approximately 10^5 bacteria per milliliter) was added to 25 mL melted Mueller Hinton Agar (MHA) medium cooled at 45ºC. This was then poured into 90 mm diameter Petri dishes and maintained for 1h at room temperature. Small wells (6 mm diameter) were cut in the agar plate using a cork borer; 60 µl of extract concentration with a negative control (ethanol and chloroform, 60 µl) were loaded in the wells. The dishes were preincubated at 4ºC for 2 h to allow uniform diffusion into the agar. After preincubation, the plates were incubated at 37ºC for 24h. The antibacterial activity was evaluated by measuring the inhibition zone diameter observed. In addition, commercial antibiotics (penicillin G, novobiocin, amphicillin, chloramphenicol and erythromycin) were used as positive control to determine the sensitivity of the strains [22]. All experiments were performed in triplicate.

3. RESULTS and DISCUSSIONS

Plant tissue culture techniques enable the production of plant tissue or cells in sterile environments under controlled conditions, allowing the growth and development of the cells or tissues to be manipulated for a variety of applications. One of these methods callus cultures, could be produced pharmacologically active molecules at the desired amount and constant quality at any time in laboratory conditions [23, 24]. In this study, the antimicrobial effect of the calli obtained from the cotyledon explants of the *C. officinalis* plant was examined.

It is more feasible to take explants that will be used in tissue culture studies from plants grown in *in vitro* conditions. The risk of contamination for plants which grow under these conditions is almost nonexistent, and they regenerate much easier and can be used directly as explants [25]. Thus, the explants to be used for this study were obtained from the plantlets produced from seeds that were germinated on MS media. At the end of the surface sterilization procedure, which was performed by incubating the seeds in 70% ethyl alcohol for 3 min, in 0.5% bleach for 5 min, and rinsing 3 times with incubation for 3 min in distilled water, sterile plantlets were successfully obtained. The cotyledones to be used as explants were isolated from these plants.

Result of some studies revealed that auxins played an important role in the callus induction. Furthermore, they showed that cytokinins facilitated the effect of auxins in callus induction [26]. It has been reported that callus formation is high in many tissue culture studies in which plant growth regulators, namely BAP from among cytokines and NAA from among auxins are applied [27-29]. In the present study, 2 mg l^{-1} BAP ve 2 mg l^{-1} IBA cytokinins that were applied caused a swelling of the explants after 5–7 d of culture, and callus induction was observed on the cotyledon explants within 5th wk of culture (Figure 1).

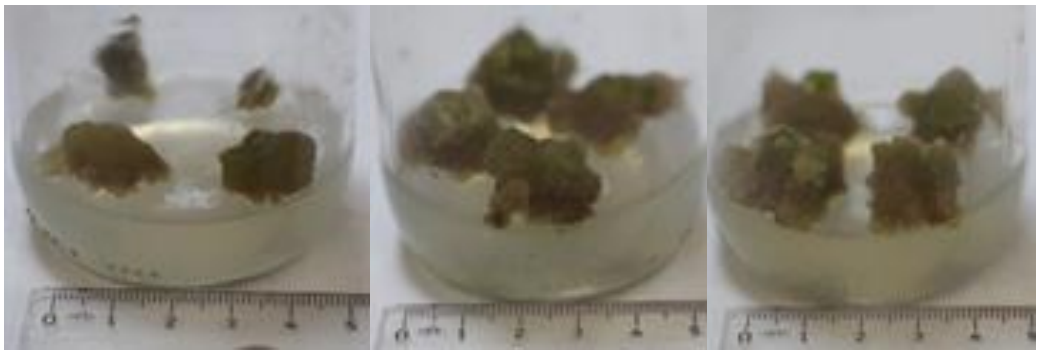

Figure 1. Calli obtained from *C. officinalis* cotyledon explants

Table 1. Screening for antibacterial activity of callus from cotyledons of *C. officinalis* extracts against standart microorganisms.

Bacteria	Zone of inhibition (mm)						
	Extracts		Antibiotics				
	Eth.	Chl.	Pe. G	Nov.	Amp.	Chlor.	Eryt.
S. aureus	38	26	24	32	20	18	12
E. coli	12	8	6	6	26	9	28
B. cereus	28	32	10	25	28	12	15
B. subtilis	24	20	8	13	32	11	30
S. typhimurium	14	10	6	40	6	13	28
P. vulgaris	18	12	10	26	12	9	22
E. faecalis	20	18	24	28	30	18	16
E. cloacae	10	10	12	22	12	11	30
K. rhizophila	16	13	20	28	10	12	15

Eth: Ethanol; Chl: Chloroform; Pe: Penicillin; Nov: Novobiocin; Amp: Amphicillin; Chlor: Chloramphenicol; Eryt: Erythromycin

When literature is reviewed, it is evident that many studies have been carried out in recent years on the secondary metabolite production [30-32] and on the antimicrobial effects of these metabolites in addition to studies such as cell culture, microbial growth, organogenesis, embryogenesis by callus culture method [33-37]. However, no literature has been found on the antimicrobial effect of *C. officinalis* calli.

In the present study, we observed *in vitro* antibacterial activities of ethanol and chloroform extracts of the anti-bacterial activity of cotyledon derived calli of *Calendula officinalis* and standard antibiotics (Table 1.).

The extracts of *C. officinalis* showed various antibacterial activities against the test bacteria. All extracts studied in this work showed antibacterial activity against at least one of the test microorganisms with inhibition zones ranging from 8 to 38 mm (Table 1). Ethanol extract of *C. officinalis* callus showed 38 mm inhibition zone against *Staphylococcus aureus* and chloroform extract showed 32 mm zone against *Bacillus cereus*. These results are very close to the test antibiotics used and *C. officinalis* found more effective on gram positive bacteria.

The results of the current investigation clearly indicate that the antibacterial activity vary with the *C. officinalis*. Further, the active phytocompounds of this plant against some bacteria should be characterized and their toxicity should be evaluated *in vivo*.

Conflict of Interests

Authors declare that there is no conflict of interests.

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The Investigation on Drug Yield and Some Quality Characteristics of Mountain Tea (*Sideritis congesta*) Cultivated in Turkey

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Abstract: This research has been conducted under Konya ecological conditions to determine the effect on yıeld and quality some characters of nitrogen fertilizers applied at the different doses of *Sideritis congesta* in Medicinal Plants laboratory and Medicinal Plants Experimental Farm of Agriculture Faculty, Selcuk Universty. Experiment was designed and applied in randomized complete plotdesign with three replications in the year of 2012-2013. According to results of this researh; the plant height of *Sideritis congesta* was varied between 58.66-64.33 cm, the number of flowering branches 49.00-55.00, fresh flowering yield 446.66 - 623.33 kg/da, essential oil yield 0.24-0.33 % and major essential oil component (β –pinene) 43.24 and 48,45. The highest drug flower yield and essential oil yield for mountain tea in Konya and similar ecology 10 kg/da nitrogen fertizer application is reasonabled.

Keywords: Mountain tea, *Sideritis congesta*, Essential oil, β –pinene, Fertilizer

1. INTRODUCTION

Mountain tea (*Sideritis congesta* et Huber-Morath) is a perennial plant which belongs to the Labiatae / Lamiaceae family. Lamiaceae family plants are grown in almost every habitat types and altitudes spreading out in a wide area from the North Poleto the Himalayas, South East Asma, Hawaii and Australia, Africa and America, but actually grown in the Mediterranean region [1]. Showing a wide distribution in our country and the world, the *Sideritis* species which are an important species of Labiatea family, are represented by approximately 150 taxon species of annual and perennial plants especially grown in the Mediterranean basin and the world [2] as well as by 54 species and sub-species, 41 of which are endemic (78%) especially grown in Mediterranean region of Turkey [3]. *Sideritis congesta* is endemic to Turkey and located in natural flora of our country. Most types of the endemic mountain tea species in our natural flora, especially the *Sideritis congesta* to the point of extinction as a result of uncontrolled, intensive, continuous and unconscious collected from the flora where they are naturally grown. The mountain tea species naturally grown in our country are the species in the hairly or rarely glabrous structure with or without secretory glands, simple leafed, fulled gedcrenata-dentate and stalked or stemless perennial herbs or small shrubs, consisting of erected and four-cornered stems [4-5]. The mountain tea species (*Sideritis* spp.) are not cultivated in our country. The mountain tea species (*Sideritis* spp.) whose consumption has been rapidly and increasingly are collected from natural flora in our country. The mountain tea species continuously collected

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from nature do not have good yield and quality, so are not sustainable. Drug plant at high quality and economic values cannot be produced from the mountain tea species collected from the natural flora because of the fact that these species are not collected, dried and stored in a suitable time. Expected effect cannot be achieved by consumption of the mountain tea species collected from the nature as they are not standardized in terms of physical, chemical and biological values. A great number of chemical studies have been conducted on the mountain tea species, mainly focus on essential oils, diterpenes and flavonoids [6-7-8]. In addition to such studies, there are also pharmacological studies conducted on the *Sideritis* species [9-10]. In addition to studies on their biological activity, some recent studies have especially been conducted on their anti-inflammatory, analgesic, diuretic, antiulcer, anti-depressants, antimicrobial and insect-repellent effects [11-12]. Also, antispasmodic effects of *Sideritis congesta* and *Sideritis arguta* have been reported [13]. The mountain tea species are used as tea form in the treatment of certain diseases due to their anti-inflammatory, antirheumatic, digestive and antimicrobial activity properties [14]. *Sideritis* species are widely consumed as herbal tea due to their aroma profiles. *Sideritis* species are publicly known to have sedative, antitussive, digestive system regulatory and anti-inflammatory effects [15]. When the yield of the mountain tea flowers consumed by the public as a tea is considered, the yield of fresh flower per decare was observed to be higher than 1 tone irrespective of applied plant density, but more than 1.5 tone in many densities. Average drug flower yield was reported to range between 348-419 kg/da [16]. In this study the yields were determined as 1567-2404 kg per decare for fresh herba and 530-629 kg drug herba but varying depending on the plant density [16]. This study aim was determined to drug yield and some quality parameters of cultivated *Sideritis congesta* which is one of the important endemic species of Turkey.

2. MATERIAL and METHOD

The research has been done at Selcuk University Agriculture Faculty Medical Plants Research and Application Farm in Konya which is in the Central Anatolia region in Turkey, between 2012-2013 years. In this study, seeds that has been used were obtained from the collection that belongs to Department of Medical Plants. Properties of the soil of research farm are shown in the Table 1.

Table 1. Chemical and physical properties of the soil

Soil Proporties	Quantity
Clay (%)	18.3
Silt (%)	14.3
Sand (%)	67.4
Texture	Sandy loamy
pH (1:2.5)	8.1
EC (1:5) (µS/cm)	12.5
$CaCO_3$ (%)	31.3
Organicsubstance(%)	2.9
Suitable Cu (ppm)	0.2
Suitable Fe (ppm)	0.9
Suitable Mn (ppm)	2.4
Suitable Zn (ppm)	0.1
B (ppm)	0.2
P (ppm)	17.7
Field capacity(%)	22.5
Total N (%)	0.2

Note: *Soil analyses performed at S.Ü Agriculture Faculty, Laboratory of Department

When Table 1 is evaluated, it can be seen that the ground has loamy and sandy soil, is rich in lime and phosphorus. Also, it's organic ingredients at intermediate level, it has an alchali character and does not have saltiness problem. In addition, it is intermediate level in point of mangan and it is poor in terms of other trace elements. Climate datas that are belongs to the years that experiments done, 2012 and 2013 are given in the Table 2 with the datas from other years. In this study, trials have carried out with three repeats as the "Random Blocks Trial Design" in the fields of Selçuk University, Agriculture Faculty, Department of Medical Plants Research Farms. In this trial which planned as the "Randomized Blocks Trial Design", three different nitrogen doses (0, 5, 10 kg/da) were used. Mountain tea seedlings were planted as 60 cm squence intervals and 30 cm on top on 25th April 2012 in the field. Nitrogen fertilization was applied when the height of plants is 10-15 cm in mountain tea before the blooming period (%33 Ammonium Nitrate). During the growing period, study plots been irrigated 3 - 5 times according to climate conditions and the water need of mountain tea. It was cleaned to the weeds. The flower has been harvested from 50 cm on the top 10th September.

The height of the plant (cm) was determined as the height from ground surface to the highest point of the plant using 5 plants that has been chosen randomly when their growing reached its highest level. The number of branches with flower (number/branch) has been determined when the plant was in its blooming period and the plants that has been chosen randomly from every plot. Fresh flower yield (kg/da) was obtained by weighting the plants after side efficacy of the plots were thrown out. Fresh flower yields were determined by the calculations that were made through the plot yield. Drug flower yield (g) were designated by drying the 1000 g wet samples that was harvested from each and every plots, using three different drying methods (shadow, sun, drying oven). Drying rates were determined by weighing the remaining dry weights. Drying methods;

- Drying in shadow 6 days (144 h)
- Drying in oven 40^{o}C 2 days (48 h)
- Drying in The Sun 3 days (72 h)

Essential oil yield (%), dried samples were weighed 100 g. These samples were distilled 3 hours. Essential oil yield were accumulated by the Clevenger apparatus. Essential oil was put in bottles and it was given to the GS-MS device. Herbal essential oil yield (%) was calculated as the volume of the essential oil in the 100 g dry matter (ml/100 g). essential oil components (%) For determining the volatile oil components GC-MS device has been used. Essential oil components that belongs to every plant has been determined by using the parameters below. Identification of the essential oil components were studied based on Wiley and Nist Mass Spektral library datas. Statistical analyses using randomized blocks trial design, variance analyses have been done and according to these analyses average values that are important were grouped as "Least Significant Difference" (LSD). Statistical evaluations have been done by using Jump program.

Gas Cromatography mass spectrometry (GC-MS)

Machine: Agilent 6890N Network GC system combined with Agilent 5973 Network 30

Mass Selective Detector (GC-MS Agilent)

Colon: Agilent 19091N-136 (HP Innowax Capillary;60.0mx0.25mmx0.25 m)

Carrier Gas: Helium

Flow rate: 1.2 ml/min

Injection Volume: 1 µl

Split ratio: 50:1

Injection Tempature: 250°C

Tempature programme: 60-220-240

Scanning range (m/z): 35-450 atomic mass units (AMU)
Ionization: Electron bombardment (EI - 70 eV).

Table 2. Some climatic values for the long growing term (1980–2012) and for the term (2012–2013)

Months	Average monthly temperature level			Average monthly total rain fall (mm)			Average monthly relative humidity (%)		
	1980-	2012	2013	1980-	2012	2013	1980-	2012	2013
January	2,1	-0,3	1,6	38,6	0,0	33,7	78,0	78,4	80,6
February	3,6	-0,9	4,7	35,5	0,2	31,9	66,8	69,2	70,6
March	7,3	5,1	7,7	24,5	10,0	16,6	57,8	55,5	55,4
April	11,3	14,4	11,9	44,9	4,6	41,6	58,1	43,7	58,1
May	16,4	16,3	18,4	41,8	51,0	54,8	52,1	55,2	45,9
June	20,5	23,0	21,6	41,0	11,0	8,8	48,7	39,3	36,3
July	25,4	26,2	23,2	6,4	0,2	0,9	36,4	33,1	34,0
August	25,0	23,2	23,5	3,1	0,0	0,0	33,7	38,3	32,3
September	19,5	20,9	18,6	6,6	1,0	4,0	35,6	34,0	37,8
October	12,5	15,2		48,5	31,5		61,1	57,9	
November	6,7	7,8		17,1	39,1		65,6	78,0	
December	3,5	3,8		48,8	60,8		74,7	82,1	
Average	12,8	12,9	14,6				55,7	55,4	50,1
Total				356,8	209,4	192,3			

3. RESULTS and DISCUSSIONS

The variance analysis of the yield and some quality characteristics examined in the experiment are given in Tables 3. and 4.

3.1. Plant Height (cm)

In this study, the doses of fertilizer applied on *S. congesta* plant height were not obtained statistically significant. The highest plant height was obtained from 10 kg/da nitrogen application (64.33 cm). In this was obtained from control (60.66 cm) and plots (58.6 cm) applied with 5 kg/da nitrogen, respectively. By the reason that it is the first study related to the cultivation of mountain teas which are endemic plants in Turkey, the references has not been found within the scope of this subject.

3.2. Number of Flowering Brunch (number / plant)

In this study, the doses of fertilizer applied on the number of flowering branches per *S. congesta* plant were not obtained statistically significant. The number of flowering branches per plant was obtained from 55.00 pieces with nitrogen application 10 kg/da, this was followed by nitrogen application (50.30 pieces) with 5 kg/da and control plots (49.00 pieces), respectively. An significant increase in the number of flowering brunch for per plant was determined in with the doses nitrogen applied in this research.

3.3. The Yield of Fresh Flowers (kg/da)

In this study, the doses of fertilizer applied on the yield of wet flowers belonging to *S. congesta* plant were obtained statistically significant as 0.01 %. The highest yield of fresh flower was determined from nitrogen application (10kg/da). This was followed by nitrogen application (546.66 kg/da) with 5kg/da and control plot (446.66 kg/da) respectively. According to these results, the yield of fresh flower were significantly increased when the amount of applied nitrogen increases. It has been recorded 499-818 kg/da in the experiment with mountain tea [16].

3.4. Essential Oil Yield (%)

In this study, the doses of fertilizer applied on the essential oil of yield of mountain tea were obtained statistically significant. The highest essential oil yield was determined from shade dried application of control plots (0,33 %). This was followed by sun dried application of control plots (0,26 %). The lowest essential oil yield was determined from 10 kg/da nitrogen application of control plots and oven dried application (0,23 %). As a result, while the highest essential oil yield of samples was obtained by the shade-dried, the lowest essential oil yield of samples was obtained by the oven-dried method.

3.5. Essential Oil Compositions

The effect of fertilizer doses and drying methods applied on the essential oil components of *S. congesta* plant obtained from this study is statistically significant. The average percentage of β-pinene, the most important component of *S. congesta's* essential oil, varied between 43.2-48.4%. The highest amount of β-pinene was obtained from the control plots and from the drying methods of the sun, the lowest amount of β-pinene was obtained in 10 kg/da nitrogen treated plots and in a shade drying method. In this study; the amount of α-pinene from other important essential oil components varied from 29.2% to 33.9%. The amount of β-pinene, which is the major component of *S. congesta*, was also obtained by control plots and by the drying method of α-pinene. It can be said that nitrogen application in high doses increases the drug flower yield in mountain tea species such as *S. congesta*, but does not increase chemical composition of essential oil.

Table 3. The agronomic characteristics of *S. Congesta* cultivated at the different fertilizier doses

Fertilizier dose	Plant height (cm)	Number of flower branches (number/ flower bud)	Fresh flower yield (kg/da)
S.congesta 0 kg/da	60.6	49.0	446.6 b
S.congesta 5 kg/da	58.6	50.3	546.6 a
S.congesta 10kg/da	64.3	55.0	623.3 a
LSD	10.2	8.2	158.9

Table 4. Essential oil compositions (%) and yield values (%) obtained at different drying methods applied to *S. congesta*

Drying method	Essential Oil Yield	B-pinene	α-pinene	Linalool	Sabinene	GermacreneD
S.congesta (in the sun)	0.26	48.3	33.7	3.72	1.57	0.48
S.congesta incubator	0.24	47.7	32.6	3.45	0.60	1.37
S.congesta shadow	0.28	46.6	29.3	4.53	1.42	1.63
LSD		1.90	3.73			

4. CONCLUSION

According to our results, the plant height was varied between 58,66- 64,33 cm, the number of flowering brunch is 49,00-55 the yield of fresh flower is 446,66- 623,33 kg/da, the essential oil yield 0,23-0,33 ml/100 g and the yield of β–pinene in the main compounds of essential oil 43,24-48,45 % for *S. congesta*.

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Summarized from Master's Thesis Prepared by Emine BİLGİNOĞLU.

Conflict of Interests

Authors declare that there is no conflict of interests.

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The Biological Activities of *Lavandula stoechas* L. against Food Pathogens

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Abstract: Foodborne pathogens are microorganisms as well as a number of parasites, which are capable of infecting humans via contaminated food or water. In recent years, diseases caused by foodborne pathogens have become an important public health problem in the world, producing a significant rate of morbidity and mortality. In traditional medicine, numerous plants and their extracts have used for thousands of years to treat health disorders. Although many studies were made on natural herbs, those involving the antimicrobial, antioxidant and antimutagenic activity of the herb species are rather rare. This study researches the biological activities of ethanol and methanol extracts of *Lavandula stoechas* L., which are prevalent in Turkey. In this study, 8 food pathogens were used for antimicrobial activity studies. Antimicrobial activity studies were done by disk diffusion assay and MIC (minimum inhibitory concentration). DPPH method was used for non-enzymatic antioxidant activity. The *Lavandula* extracts were screened for their antimutagenic activity against sodium azide by Ames test in absence of rat microsomal liver enzyme (-S9). The ethanol and methanol extracts of *Lavandula stoechas* showed antibacterial activity (7 mm) against most of bacteria. The antifungal activity of *L. stoechas* was not determined against *C. albicans* RSKK02029. The lowest MIC value was determined as 3250 µg/mL. The highest radical inhibition was determined as 79 % by *Lavandula stoechas* flower methanol extract. The flower extract of *L. stoechas* (12500 µg/plate) was found to have its highest antimutagenic activity for *Salmonella* Typhimurium TA98. This inhibition value is 42 %. *L. stoechas* leaves extracts (6250 and 3125 µg/plate) showed a moderate positive inhibitory effect for *Salmonella* Typhimurium TA98, and TA100. *L. stoechas* flower extracts (12500 and 6250 µg/plate) showed a moderate positive inhibitory effect (respectively 31 and 30 %) for *Salmonella* Typhimurium TA100. The extracts of *L. stoechas* have antimicrobial, antioxidant and antimutagenic activities.

Keywords: *Lavandula, Antimicrobial Activity, Antioxidant Activity, Antimutagenic Activity*

1. INTRODUCTION

More than 200 known diseases are transmitted through food by a variety of agents that include bacteria, fungi, viruses, and parasites. The Center for Disease Control and Prevention [1,2] estimates that 76 million people get sick, more than 300,000 are hospitalized, and 5,000 Americans die each year from foodborne illness. Food items most likely associated with antibiotic resistant pathogens included dairy products, ground beef, and poultry. Pathogens exhibiting multi-drug resistance to five or more antibiotics were identified in more than half of the outbreaks [3]. Scientists have taken up the issue to solve this problem.

Medicinal plants are natural resources, yielding valuable herbal products which are often used in the treatment of various ailments [4]. The important advantages claimed for therapeutic uses of medicinal plants in various ailments are their safety besides being economical, effective,

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fewer side effects and their easy availability [5-7]. Because of these advantages the medicinal plants have been widely used by the traditional medical practitioners in their day to day practice. Today, it is estimated that about 80% of the world population relies on botanical preparations as medicine to meet their health needs [8].

Genus *Lavandula* belongs to Lamiaceae family and it includes about 39 species however, the most important species are lavender (*Lavandula angustifolia* Mill.), lavandin (*Lavandula intermedia* Emeric.) and spike lavender (*Lavandula spica* L.) [9]. Lavender is native to the Mediterranean and grows in natural sites of lower parts of mountains. Lavender, small shrubby plant, grows 20–60 cm high with irregular, much branched stems. The leaves are opposite, sessile, lanceolate, linear or lance-shaped and hairy. Flowers are produced in the long spikes on long stems. The spikes consist of rings of 6–10 flowers which are bilabiate, small, 0.8 cm long with blue, tubular and ribbed calyx and violet-blue corolla. The majority of the oil, extracted from the flowers, is contained in the glands on the calyx [10]. According to Nartowska [11] lavender flowers contain essential oil and its components: linalyl acetate (40%), linalool (30%), limonene, β-ocymene, 1,8-cineole, camphor, α-terpineol, borneol, but also phenolic acids, ursolic acid, cumarins flavonoids and sterols. Lavender oil (*Lavandulae aetheroleum*) is known for its antibacterial, antifungal, carminative, antiflatulence, antiholic, sedative and antidepressive activities [12]. Lavender oil is used in phytotherapy to relief cough, neuralgia, insomnia but also for bath and compress [13-15]. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy [16].

The aim of the investigation presented in this paper is to evaluate the antibacterial, antioxidant and antimutagenic activities of extracts of *Lavandula stoechas* on several food pathogens, as there is a significant lack of information on such activities in literature.

2. MATERIAL and METHODS

21. Plant Material

Lavandula stoechas flowers and leaves were collected in May 2014 form 0- 700 m height above sea level in Izmir. The identity was confirmed by Dr. Olcay Ceylan, Department of Biology, Mugla Sitki Kocman University. The voucher specimens were deposited at the Herbarium of Department of Biology, Mugla Sitki Kocman University. The identification of these specimens was carried out using the Flora of Turkey [17].

2.2. Plant Extraction

The flowers and leaves were washed thoroughly 2-3 times with running water and once with sterile distilled water. Fresh plant materials were air dried, and then the dried materials were powdered in a blender. All samples were stored at ambient temperature until initial sample preparation, after which they were stored at 4 $^{\circ}$C until required for analysis. The air dried and powdered flowers and leaves of the plant samples (40 g) were extracted with ethanol and methanol (250 mg/mL) using the Soxhlet apparatus. All experiments were continued for 4 hours. All of extracts were evaporated and then the extracts were dissolved in their solvent and then kept in small sterile opac bottles under refrigerated conditions until used. All of extracts concentrations were set to 100- 200 mg/mL.

2.3. Microorganisms and Cultivation

The extracts were individually tested against food pathogenic strains such as *Bacillus subtilis* RSKK245, *Staphylococcus aureus* RSKK2392, *Salmonella* Typhimurium RSKK19, *Enterococcus faecalis* ATCC8093, *Escherichia coli* ATCC11229, *Listeria monocytogenes* ATCC7644, *Yersinia enterocolitica* NCTC11174 and *Candida albicans* RSKK02029. The

bacteria were grown for 24 hours at 37 °C in Mueller- Hinton Broth (Merck). *C. albicans* was grown for 24- 48 hour at 30 °C in Sabouraud Dextrose Broth (Merck). These strains of bacteria and *C. albicans* were obtained from ATCC (American Type Culture Collection, USA), RSKK (Refik Saydam National Type Culture Collection, Turkey) or NCTC (National Collection of Type Cultures).

2.4. *In vitro* Antimicrobial Activity

Kirby-Bauer method applied for antimicrobial activity [18]. The extracts of plant were tested by disc diffusion assay. The concentration and quantity of extracts were used as 45 µL of 100-200 mg/mL. In this study, ethanol and methanol were used as organic solvents. The bacteria were maintained on Mueller-Hinton agar plates (MHA, Merck) at 37 °C and yeast was maintained on Sabouraud Dextrose agar plates (SDA, Merck) [18]. Bacteria and *C. albicans* RSKK02029 cultures were adjusted to 0.5 McFarland. The experiments were performed in triplicate. Bacteria were incubated at 37 °C in 24 hours. *C. albicans* RSKK02029 was incubated at 30 °C for 24 hours. After incubation, the inhibition zones formed and then the values of zone were measured. Ethanol and methanol used as negative control. Chloramphenicol (30µg), and nystatin (100µg) antibiotics used as positive control.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

The MIC was evaluated on plant extracts as antimicrobial activity. The MIC was taken as the lowest concentration that inhibits growth after incubation. The broth dilution assay was performed as described in the CLSI standards [19,20]. This test was performed at final concentrations of each extract (6500; 3250; 1625; 812.5; and 406.25 µg/mL).

2.6. Determination of non-Enzymatic Antioxidant Activity

The non-enzymatic antioxidant activity was determined using DPPH as a free radical. The stable 2,2-dipheny l-1-picrylhydrazyl- hydrate radical (DPPH) was used for determination of free radical scavenging activities of the flower and leaf extracts. Extract (0.1 mL) was added to 3.9 mL of a 0.1 mM methanol DPPH solution. After incubation for 30 minutes, absorbance of extract was measured at 515 nm using spectrophotometer. Methanol was used as a blank, while methanol with DPPH solution was used as a control [21]. Trolox was used for reference antioxidant. The DPPH scavenging capacity expressed in percentage (%) was calculated using the formula.

2.7. Determination of Antimutagenic Activity

Antimutagenic activity was evaluated by the *Salmonella*-microsome assay, using the *Salmonella* Typhimurium tester strains TA98 and TA100, kindly provided by Dr. B.N. Ames (Berkeley, CA, USA), without (−S9) metabolisation by the pre-incubation method [22]. The *Salmonella* histidine point mutation assay of Maron and Ames [22] was used to test the antimutagenic activity of extracts without S9 mix. In this study, two different tester strains were employed to measure the antimutagenicities of *Lavandula stoechas* extracts. These strains included *Salmonella* Typhimurium TA98 and TA100. The calculation percentage of inhibition was done according to the formula given by Ong *et al.*, [23]. Sodium azide was used as positive control. Methanol is negative control. Concurrently, a positive control (where mutagen but no extract was added) and a negative control (where no mutagen was added) were also set. The test sample was dissolved in methanol. But mutagen was dissolved in distilled water. In our study, non-toxic concentrations of the test sample used for investigating were 12500, 6250 and 3125 µg/plate. These concentrations were categorized as non-toxic because they showed a well-developed lawn, almost similar size of colonies and no statistical difference in the number of spontaneous revertants in test and control plates.

3. RESULTS and DISCUSSIONS

The antimicrobial activities of ethanol and methanol extracts of *Lavandula stoechas* were evaluated *in vitro* against 8 microorganisms test species, which are known to cause some diseases in foods. Results of antimicrobial activities of used plant extracts against the test bacteria are shown in Table 1. Besides, the inhibition zone diameters of the reference antibiotics to the test microorganisms are shown in Table 2.

The results of antibacterial activities were recorded as zone of inhibition in mm for all the materials used as follows. Results show that the ethanol extracts of *L. stoechas* inhibited the growth of six bacteria and the inhibition zones were 7 mm. Whereas methanol extracts of this plant inhibited the growth of seven bacteria and similarly the inhibition zones were 7 mm. In addition the ethanolic and methanolic extracts of this plant did not determine any anticandidal effects against used yeast. The extracts showed the same effect on *B. subtilis, S. aureus, L. monocytogenes* and *Y. enterocolitica* (Table 1). Chloramphenicol and nystatin antibiotics used as positive control. Chloramphenicol very strongly inhibited the bacterial growths (Table 2).

Table 1. Antimicrobial activities of *Lavandula stoechas* extracts

Microorganisms	Concentration (mg/mL)	Inhibition zone diameters (mm)				Solvents	
		Lsc		Lsy			
		EE	ME	EE	ME	E	M
B. subtilis RSKK245	100	7	7	-	-	-	-
	200	7	7	-	-	-	-
S. aureus RSKK2392	100	7	7	-	-	-	-
	200	7	7	-	-	-	-
S. Typhimurium RSKK19	100	-	-	-	-	-	-
	200	-	-	-	-	-	-
E. faecalis ATCC8093	100	-	-	-	-	-	-
	200	-	-	-	-	-	-
E. coli ATCC11229	100	-	-	-	-	-	-
	200	-	-	-	-	-	-
L. monocytogens ATCC7644	100	-	-	-	-	-	-
	200	-	7	7	-	-	-
Y. enterocolitica NCTC11174	100	7	7	-	-	-	-
	200	7	7	7	7	-	-
C. albicans RSKK02029	100	-	-	-	-	-	-
	200	-	-	-	-	-	-

Lsc: *Lavandula stoechas* (flower) Lsy: *Lavandula stoechas* (leaf) EE: Ethanol extract ME: Methanol extract.
(-): zone did not occur E: Ethanol M: Methanol

Table 2. Antibiotic profils of food pathogens

Microorganisms	Inhibition zone diameter (mm)	
	Chloramphenicol	Nystatin
B. subtilis RSKK245	12	nt
S. aureus RSKK2392	15	nt
S. Typhimurium RSKK19	22	nt
E. faecalis ATCC8093	22	nt
E. coli ATCC11229	21	nt
L. monocytogenes ATCC7644	22	nt
Y. enterocolitica NCTC11174	20	nt
C. albicans RSKK02029	nt	7

NOTE: nt =not tested

Table 3. shows MICs of *Lavandula stoechas* extracts obtained by the broth dilution method. Three bacteria showed the lowest sensitivity to extracts of *Lavandula stoechas* (3250 µg/mL).

Table 3. Minimum inhibitory concentrations of *Lavandula stoechas* extracts

Microorganisms	Lsc (µg/mL)		Lsy (µg/mL)	
	EE	ME	EE	ME
B. subtilis RSKK245	6500	6500	nt	nt
S. aureus RSKK2392	3250	3250	nt	nt
S. Typhimurium RSKK19	nt	nt	nt	nt
E. faecalis ATCC8093	nt	nt	nt	nt
E. coli ATCC11229	nt	nt	nt	nt
L. monocytogenes ATCC7644	nt	3250	3250	nt
Y. enterocolitica NCTC11174	6500	3250	6500	6500
C.albicans RSKK02029	nt	nt	nt	nt

NOTE: Lsc:*Lavandula stoechas* (flower) Lsy:*Lavandula stoechas* (leaf) nt: Not tested EE: Ethanol extract ME: Methanol extract

The non-enzymatic antioxidant activity of plant extract was evaluated by the DPHH radical scavenging capacity. Table 4 shows the percent of DPPH radical scavenging capacity with trolox as reference. The methanol extract showed 79% inhibition at 200 mg/mL concentration. Trolox equivalent value was 2.2 mM/g (Table 4).

Table 4. DPPH radical scavenging capacity of *Lavandula stoechas*(200mg/mL)

Plants	Ethanol extracts		Methanol extracts	
	DPPH İnhibition (%)	TE	DPPH Inhibition (%)	TE
Lsc	40	1,4	79	2,2
Lsy	26	1,1	67	2,0

NOTE: Lsc: *Lavandula stoechas* (flower) Lsy:*Lavandula stoechas* (leaf) TE: Trolox equivalent (mM/g DW); DW: Dry weight

The antimutagenic activities of the extracts were evaluated by the against sodium azide by Ames test in absence of rat microsomal liver enzyme (-S9). Table 5, 6, and 7 shows the percent of inhibition. The flower extract of *L. stoechas* (12500 µg/plate) was found to have its highest antimutagenic activity for *Salmonella* Typhimurium TA98. This inhibition value is 42%. *L. stoechas* flower extracts (12500 and 6250 µg/plate) showed a moderate positive inhibitory effect (respectively 31 and 30%) for *Salmonella* Typhimurium TA100 (Table 5).

Table 5. Antimutagenic activity of *Lavandula stoechas* extract (12500 µg/plak)

Test substances	*Salmonella* Typhimurium TA98		*Salmonella* Typhimurium TA100	
	Revertant	% Inhibition	Revertant	% Inhibition
Control	22		80	
Negative control	24		88	
Positive control	45		131	
Lsc	26	% 42,2	90	% 31,3

NOTE: Lsc: *Lavandula stoechas* (flower)

Table 6. Antimutagenic activity of *Lavandula stoechas* extract (6250 µg/plak)

Test substances	*Salmonella* Typhimurium TA98		*Salmonella* Typhimurium TA100	
	Revertant	% Inhibition	Revertant	% Inhibition
Control	22		80	
Negative control	24		88	
Positive control	45		131	
Lsc	31	% 31,1	92	% 29,8
Lsy	33	% 26,7	91	% 30,5

NOTE: Lsc:*Lavandula stoechas* (flower) Lsy:*Lavandula stoechas* (leaf)

Table 7. Antimutagenic activity of *Lavandula stoechas* extract (3125 µg/plak)

Test substances	*Salmonella* Typhimurium TA98		*Salmonella* Typhimurium TA100	
	Revertant	% Inhibition	Revertant	% Inhibition
Control	22		80	
Negative control	24		88	
Positive control	45		131	
Lsy	35	% 22,2	93	% 29,0

NOTE: Lsy**:** *Lavandula stoechas* (leaf)

4. DISCUSSION

Medicinal plants have proved to be abundant sources of biologically active compounds, many of which have been used as compounds to develop new pharmaceuticals [24]. *L. stoechas* flowers and leaves were selected based on their relevant ethnomedical use. In the present study, extracts of the plant obtained in solvents were tested against eight microorganisms. The antimicrobial activities were compared with the standard antibiotics. Results show that the ethanol extracts of *L. stoechas* inhibited the growth of six bacteria and the inhibition zones were 7 mm. Whereas methanol extracts of this plant inhibited the growth of seven bacteria and similarly the inhibition zones were 7 mm (Table 1). Gören et al. [25] reported that antimicrobial activities of extracts of *L. stoechas* ssp. *stoechas* were found highly effect on bacteria.

Result of this study showed that tested plant flower extracts were found to same effective against *S. aureus* RSKK2392 and *Bacillus subtilis* RSKK245 (7 mm) (Table 1). Oskay et al. [26] showed that the most susceptible organism was methicillin resistant *S. aureus* (MRSA) which was sensitive to methanol and ethanol extracts. Khosravi and Malecan [27] reported that alcoholic extract of *L. stoechas* has significantly inhibitory effect on the growth of *Staphylococcus aureus*. These results are like with our study.

In this study, the extracts were not inhibited the growths of *S.* Typhimurium and *E. coli* (Table1). Cherrat et al. [28] reported that *Lavandula stoechas* is effective against Gram (+) bacteria and Gram (-) bacteria is weakly effective. Various workers have already shown that Gram positive bacteria are more susceptible towards plants extracts as compared to Gram negative bacteria [29,30]. These differences may be attributed to the fact that the cell wall in Gram positive bacteria is of a single layer, whereas the Gram negative cell wall is multilayered structure [31].

In addition, the ethanolic and methanolic extracts of this plant did not determine any anticandidal effects against used yeast (Table 1). Zuzarte et al. [32] reported that the oil of *Lavandula stoechas* had low antifungal activity in their study. According to Adam et al. [33], *Lavandula angustifolia* showed moderate to low antifungal activities against *Malassezia furfur*,

Trichophyton rubrum, and *Trichosporon beigelii*. Uzun et al. [34] reported that *L. stoechas* extract was not inhibited growth of *Candida albicans*. These results are like with our study.

In this study, three bacteria showed the lowest sensitivity to extracts of *Lavandula stoechas* (3250 µg/mL). Ünsal et al. [35] reported that MIC value of *Lavandula* subsp. *stoechas cariensis* was 19.52 µg/mL. Results of our study are higher than their results. In our results, MIC values are 6500 µg/mL for *B. subtilis* and 3250 µg/mL for *S. aureus* (Table 3). Nunes et al. [36] determined that MIC values are 9830 µg/mL for *B. cereus* and 9830 µg/mL for *S. aureus*. Results of our study are better than their results.

In this study, the extracts of *Lavandula stoechas* have different free radical inhibition. The flower methanol extract showed 79% free radical inhibition at 200 mg/mL concentration (Table 4). Researchers found several compounds to be present in *L. stoechas* essential oils are known to possess antioxidant activities. These include eugenol, carvacrol, thymol, terpinolene, α- terpinene, γ-terpinene [37], and terpinen-4-ol [38]. Matos et al. [39] reported that chemical composition of *L. stoechas* were fenchone (42%), camphor (35%) and oxygen-containing monoterpenes (87%). These chemical composition differences might be caused by geographic origins, climatic and seasonal conditions, the time of collection, the stage of development, the method of extraction and even might be correlated to the existence of new chemotypes [40]. Some authors reported the antioxidant activity of *L. angustifolia* and *L. luisieri* extracts [41,42]. Cherrat et al. [28] reported that *Lavandula stoechas* has high antioxidant properties in their study, which supports our work.

The flower extract of *L. stoechas* (12500 µg/plate) was found to have its highest antimutagenic activity for *Salmonella* Typhimurium TA98. This inhibition value is 42 % (Table 5). In determining the antimutagenic potential of a sample, a value smaller than 25% inhibition of the mutagen activity indicates a weak or non-antimutagenic effect, a moderate effect when the value is between 25 and 40% and strong antimutagenicity when the value is greater than 40% [43].

5. CONCLUSION

It is inferred that extracts of *L. stoechas* is moderately effective against food pathogens and can be utilized as sources of natural antimicrobial agents. Our results support the use of this plant in traditional medicine and suggest that some of the plant extracts possess compounds with good antibacterial properties that can be used as antibacterial agents in the search for new drugs.

The results obtained in this report clearly demonstrate that greater part of tested extracts exhibited strong antioxidant activities, particularly, to scavenge free radicals generated from DPPH reagent, especially *Lavandula* flower extract. The methanolic extract of *L. stoechas* flowers, should be beneficial as an antioxidant protection system for the human body against oxidative damage. This plant with high antioxidant ability can be used on development research and may be a source of natural antioxidants for potential exploit in food, cosmetic and pharmaceutical industries. However, further researches are needed to explore the bioactive compounds in this selected medicinal plant. Undoubtedly, the antimicrobial and antioxidant effects of *L. stoechas* should be investigated further. Fractionation and characterization of the active compounds should be do further works to investigate.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Antimicrobial and Antifungal Activity of Fabrics Dyed with *Viburnum opulus* and Onion Skins

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Abstract: Microorganisms such as bacteria and fungi can cause serious health and hygiene problems for this reason, products with antimicrobial activity gains importance day by day. As is known, textile products can also provide a suitable environment for the development of microorganisms. In this context, we aimed to develop textile products which can provide antimicrobial and antifungal effect. For this purpose, woolen fabrics were dyed with onion (*Allium cepa*) skins and juice of gilaburu (*Viburnum opulus)* plant fruit in our study. The dyeings were carried out without using of any mordanting agent and no prior extraction of the herbal sources was carried out in other words these natural dye sources (onion skins and juice of *Viburnum opulus* plant fruit) were directly added to the dye bath. Samples after dyeing with these natural dye sources were tested in terms of antimicrobial activity using two bacteria (*Escherichia coli*, *Enterobacter aeroginosa)* and a yeast strain (*Candida albicans*). Meanwhile the dyed samples were also investigated in terms of obtained colors. For this purpose, color measurement of the dyed samples has been managed and color efficiencies and color values has been collected. After the evaluation of the results, it was observed that onion (*Allium cepa*) skin and gilaburu (*Viburnum opulus*) fruit juice had an antimicrobial effect. While the dyed fabric with *Viburnum opulus* juice showed higher activity on bacterial strains, the onion skin had a higher effect on the yeast. *Viburnum opulus* provided the highest activity on *E. coli*, whereas onion skins showed the highest activity on *C. albicans*. Meanwhile it was observed that different colors can be obtained with the use of these tested natural dye sources

Keywords: Antimicrobial textiles, bacteria, biotechnology, fungi, natural dye.

1. INTRODUCTION

Natural dyes were used to colour a fiber or to paint. They may be found hidden in such diverse places as the roots of a plant, a parasitic insect and the secretions of a sea snail [1]. Textile materials used to be colored for value addition, look and desire of the customers. Anciently, this purpose of colouring textile was initiated using colors of natural source, until synthetic colors/dyes were invented and commercialized [2]. In this study gilaburu fruit and onion skin has been used for the coloration of wool fabrics and the anti-microbial/fungal activities of the dyed samples have been analyzed.

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Cranberry (*Viburnum opulus* L.), called gilaburu in the Middle-Anatolia region, especially Kayseri city, Turkey and European cranberry bush in English, belonging to the plant family of *Caprifoliaceae*, is widely distributed in Turkey [3]. Allium cepa is commonly called onions and the bulb comprises fleshy layers of modified leaves, surrounded by papery outer layers. Skin of Allium cepa which is a kitchen waste material can be used in natural dyeing of different textile materials [4].

Textile goods, especially those made from natural fibers; provide an excellent environment for microorganisms to grow, because of their large surface area and ability to retain moisture [5]. Growing awareness of health and hygiene has increased the demand for bioactive or antimicrobial textiles [6]. There is increasing interest in adding value to textiles by the use of natural products. Many of the plants from which natural dyes are obtained are, for example, also known to have medicinal properties [7]. It is easy to find different studies on the usability of natural dye sources for ensuring the antimicrobial efficiencies. For example, Davulcu et al. reported the antimicrobial efficiency of cotton fabrics dyed with thyme and pomegranate peel without use of any mordanting agent [8]. In another study, Singh et al. have studied on antimicrobial activity of different natural dye sources such as *Acacia catechu, Kerria lacca, Quercus infectoria, Rubia cordifolia and Rumex maritimus and* tested the antimicrobial efficiency of samples against some common pathogens. Finally they found different antimicrobial efficiencies against the tested pathogens [9].

In this context, the aim of this study was to investigate the antibacterial and antifungal effect of fabrics dyed with the onion skin and juice of gilaburu fruit. By this way it was planned to use a common waste “onion skin” and a regional plant “gilaburu”.

2. MATERIAL and METHODS

In the study woven wool fabrics, which were ready for dyeing processes and in the weight of 160 g/m^2, were used. As a natural dye source gilaburu (Viburnum opulus) plant fruits were provided from the local markets in Kayseri. Onion skins which are vegetable waste were selected as a second natural dye source. The dyeings were carried out without using of any mordanting agent and no prior extraction of the selected herbal sources were carried out. In the dyeing processes the juice of *Viburnum opulus* plant fruits and the milled onion skins were used (Figure 1).

The milled onion skins

Fruits of *Viburnum opulus* plant

Figure 1. Natural dye sources used in coloration of wool

In the dyeing processes of wool fabrics with the *Viburnum opulus* plant fruit, the juice of the fruits were obtained by squeezing the fruits, then for 2 g of textile material 100 ml of this juice was used as a dye bath so a liquor ratio of 1:50 has been obtained. For the dyeings with onion skins, the same amount of grinded onion skins with fabric were used and so for 2 g of textile material 2 g grinded onion skins was added to 100 ml of the bath containing only water to ensure the liquor ratio of 1:50. The dyeing process was carried out in a laboratory-type sample dyeing machine. The dyeing was started at 40°C for 10 minutes then the bath was

heated to 100°C and at 100°C dyeing was conducted for 1 hour. Afterwards, the dyed samples were washed and subsequently dried at room temperature.

In order to evaluate the obtained colors, the color efficiencies (K/S) and the color values (CIE L*a*b*) of the dyed samples were measured with Konica Minolta 3600d spectrophotometer and for visual evaluation the samples were scanned. Moreover the antimicrobial activities of the fabrics were tested too. For this aim the strains were cultured on appropriate media and incubated aerobically at 37°C overnight as detailed in AATCC 100/AATCC 147. *Escherichia coli*, *Enterobacter aeruginosa* and *Candida albicans* are strains used for antimicrobial susceptibility testing. Antimicrobial activity was evaluated by quantitative test methods. The 1 inch^2 fabric (dyed and undyed) was introduced in the 100 mL nutrient broth inoculated with the desired microbe and incubated at 37 °C overnight (16 h). The assay was performed in triplicate and the mean value was reported. The reduction of bacterial growth by dye was expressed as follows:

$$\% \text{ Reduction} = [(A-B)/A] \times 100 \quad \text{(Equation I)}$$

where A and B are the surviving cells (CFU/ml) for the flasks containing the control (blank wool fabric) and test samples (natural dyed wool fabric), respectively, after 18 hrs of contact time.

3. RESULTS and DISCUSSIONS

3.1. Evaluation of obtained colors after dyeing processes

For the evaluation of the obtained colors the CIEL*a*b* values of the samples and the images of the scanned samples were collected in table 1. As seen from the images of the samples onion skins and *Viburnum opulus* plant fruit juice based dyeings have given different colors. In the CIE L*a*b* space, L* indicates the lightness of the color; the colors with a* higher than 0 represent redness and those with a* lower than 0 greenness; b* higher than 0 indicate yellowness and b* lower than 0 indicate blueness [10]. In the light of this it can be easily told that the color after dyeing with onion skins were redder than the samples dyed with Juice of *Viburnum opulus* plant fruit. It was also confirmed from the images of the dyed samples.

Table 1. Colors of the dyed samples

Natural Dye Source	K/S	L*	a*	b*	c*	h*	
Onion (*Allium cepa*) Skins	22.93	40.8	21.16	27.71	34.87	52.63	
Juice of *Viburnum opulus* Plant Fruit	14.74	40.97	7.99	23.68	24.99	71.36	

3.2. Evaluation of antimicrobial activities after dyeing processes

Fabrics treated with natural dyes were screened for their antimicrobial activity against microorganisms. According to results of study, onion skin and *Viburnum opulus* fruit juice had an antimicrobial effect. The *Viburnum* juice inhibited the growth of *E. coli* by 3.19%, E. aeruginosa by 0.77% and *C. albicans* by 2.05%. On the other hand, the Onion has reduced the growth of *E. coli* by 0.52%, *E. aeruginosa* by 0.26% and C. *albicans* by 4.97%. While the dyed fabric with *Viburnum opulus* juice showed higher activity on bacterial strains, the onion skin

had a higher effect on the yeast. *Viburnum opulus* provided the highest activity on *E. coli*, whereas onion skins showed the highest activity on *C. albicans*.

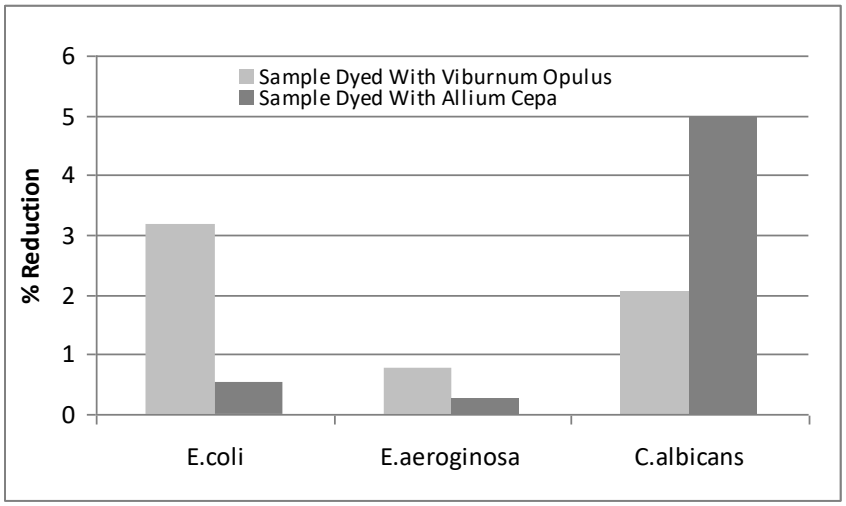

Figure 2. Antimicrobial activity of textile materials dyed with *Viburnum opulus and Allium cepa*.

4. CONCLUSION

Textile materials have an important place in human life and they can be constantly in contact with human body. For this reason the characteristics of textile materials also affect our life comfort. In this context antimicrobial textiles are becoming of interest. In this study wool fabrics were dyed with onion (*Allium cepa*) skins and juice of gilaburu (*Viburnum opulus*) plant fruit and the antimicrobial and antifungal activities of the dyed samples were tested. It was observed that the dyed fabric with *Viburnum opulus* juice showed higher activity on bacterial strains and the onion skin had a higher effect on the yeast. *Viburnum opulus* provided the highest activity on *E. coli*, whereas onion skins showed the highest activity on *C. albicans*. Meanwhile different colors were observed after the dyeings with the tested natural dye sources.

Conflict of Interests

Authors declare that there is no conflict of interests.

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Medicinal and Aromatic Plants of Esenli (Giresun) Forest Planning Unit

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Abstract: Turkey is an extraordinary country in terms of plant species diversity. Besides this diversity, Anatolian lands were hosted by many civilizations. The combination of these two factors has also contributed to a wealth of human-plant use. The plants have been used for different purposes in different civilizations such as food, medical, instrument construction, fuel, paint, feed, incense. The use of plants today, especially medical and aromatic plants, continues to increase. According to the Flora of Turkey, which plant is known to grow in which region. However, there is insufficient information on the status of plant populations. Plant sociology studies can give us satisfactory information about plant populations. In this study, Medicinal and aromatic plants of Esenli (Giresun) Forest Planning Unit, located between Alucra and Yağlıdere districts, were investigated based on plant sociology. During this study; 20 sample plots were taken. The vegetation study was carried out according to Braun-Blanquet's method. Totally, 226 naturally growing plant taxa were identified. Of these plants, 10 taxa (%4,4) belong to Pteridophyta division and 216 taxa belong to Spermatophyta division. 3 taxa (%1,3) belong to Gymnospermae subdivision, while the others 213 (%94,3) are Angiospermae subdivision. As a result of this study, 110 plant taxa which have medicinal and aromatic traits were determined in the area. These plants are 3 taxa Pteridophyta, 3 taxa from Gymnospermae and the rest from Angiospermae (104 taxa). Families, scientific names, Turkish names, usable parts and traditional uses were given in the presentation.

Keywords: Medicinal and Aromatic Plants, Inventory, Plant Sociology, Giresun, Flora

1. INTRODUCTION

Turkey is one of the most important and rich centres of the world in terms of plant resources due to its geographical location, topographic structure, water resources, micro-climate zone diversity, geological structure and plant geography [1]. These factors make our country home to forest, steppe, wetland, sea and coastal, mountain ecosystems [2] and have a very rich biological diversity with a wide variety of habitats. As a matter of fact, this habitat diversity enabled our country to have 11.707 plant taxa and 3.649 endemic taxa [3].

In addition to this diversity, many civilizations hosted from the past to the present Anatolian lands. This hostage brought the civilizations' culture to Anatolia. The combination of these two factors has also contributed to a wealth of human-plant use. The plants have been used in different civilizations for different purposes (food, medical, ware, firewood, paint, feed, incense etc.) [4]. Today, the use of plants, especially medical and aromatic plants, continues to increase.

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Many plant taxa, containing volatile oils, flavonoids, alkaloids, glycosides, various vitamins [5], are accepted as medical and aromatic plants. In addition to the continuity of vital activities in the ecosystem, these plants are used in cosmetics, dye, textile, food, etc., [6]. Furthermore, the increasing use of natural products in place of synthetic products has increased the importance of medicinal and aromatic plants.

The total number of plant species known on the earth is 374.000 [7]. Approximately 80.000 of these plant species are subject to medical purposes [8-10]. The presence of 2.000 plant species traded in Europe, is mentioned [9]. In Turkey, the number of plants used for medical purposes is around 1.024 [4] and about 200 of them have export potentials [11]. In another publication, 337 plant species have been reported to using for commercial purposes [12].

On the other hand, the increasing world population with urbanization and industrialization, have caused the increase of the pressure of human beings on nature. As a consequence of this pressure, it is accepted the fact that natural resources can be exhausted every part of the world [13]. Approximately, 500.000 tons of medicinal and aromatic herb are traded annually in the world [9]. As of 2014, this value has become around 63.500 tons in our country [14]. 90% of the medical and aromatic plants which is traded in Europe [13] are obtained from the nature, while in our country this rate is higher.

A plan to be made in medical and aromatic plants mainly consists of 4 steps. These are: (1) determination of species and populations, (2) selection of sampling method, (3) decision of sample area, and (4) decision of counting method according to product [15]. According to the Flora of Turkey, it is known that which plant species are growing in which vegetation type. However, there is no sufficient information on the status of plant populations (except taxonomic revisions). Plant sociology studies can give us enough information about the populations of medicinal and aromatic plants. In this study, a sample inventory study related with medicinal and aromatic plants is given based on plant sociology.

1.1. General Introduction of Research Area

Esenli Forest Planning Unit is located within the boundaries of Yağlıdere and Alucra (Giresun) provinces. The research area is geomorphologically mountainous and steep, and the average height from the sea level is 1600 m. The area lies within the G41d1, G41d2, G41d3, G41d4 and H41a1 ranges from 1 / 25.000 topographic maps and consists of a total area of 12.573.4 ha. The research area is covered by 4.940 ha of productive forest and 1.410,1 ha of degraded forest areas [16]. There are three settlements (Akpınar, Çakrak and Güllüce villages) within the scope of the planning unit (Figure 1).

The climate assessments of the Esenli Forest Planning Unit were made according to observations between 1964 and 2015 at Şebinkarahisar meteorological station data. The annual average temperature in the region is 7.7 ^{o}C according to Şebinkarahisar Meteorology Station data, while the maximum temperature is measured in July and it is 38.1 ^{o}C. On average annual rainfall measurements, the average annual precipitation in Esenli is 689,1 mm. A majority part of this amount has been recorded in April-May-September.

Looking at the graph drawn for Esenli (Figure 2), it is seen that the highest potential evapotranspiration [PET] values in the area are reached in June, July and August, with the highest rainfall in April. Within the planning unit, water shortage is observed between the end of May and the first weeks of October. The research area is dominated by Oceanic Climate type, which is in the moist, medium temperature (Mesothermal), water deficit in the summer season and medium level.

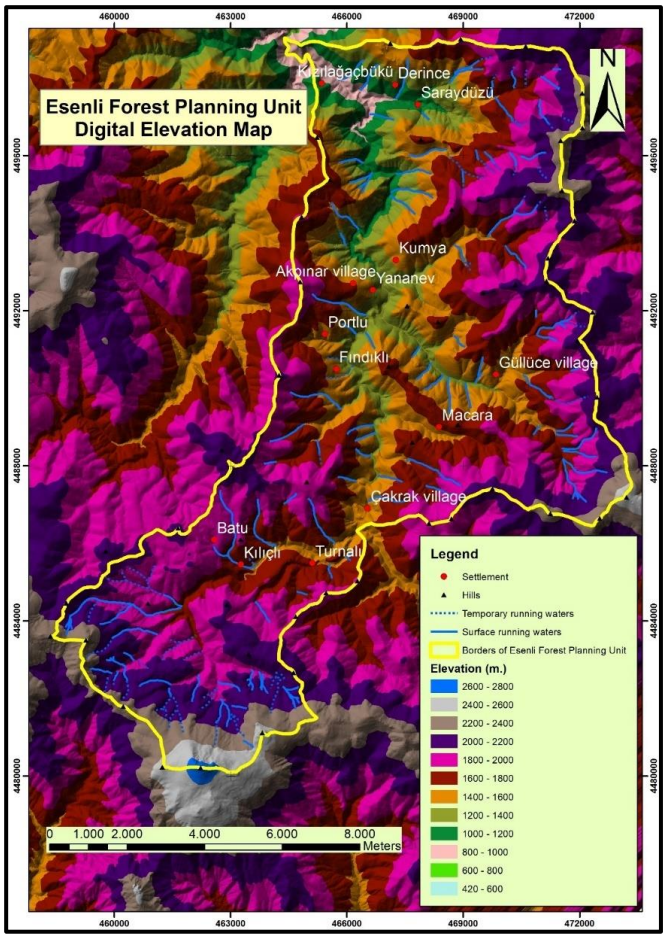

Figure 1. Digital Elevation Map of Research Area

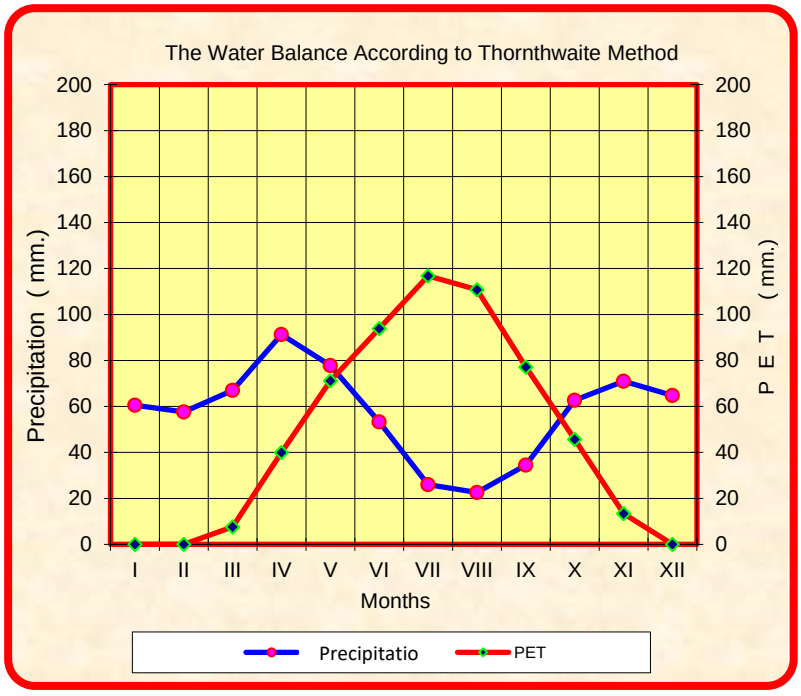

Figure 2. The water balance of research area according to Thornthwaite (interpolated)

1.2. Plant Geography of Research Area

Esenli Forest Planning Unit is located in the Euro-Siberian Floristic Region. The Euro-Siberian flora area encompasses the Black Sea region and is represented by the Euxine (Black Sea) province. Eastern Black Sea region which is also covering the study area is in the Colchic sector which is separated from the west Black Sea region in terms of precipitation from Euxine province [17]. The Colchic sector is characterized by sub-tropical humid forests which are comprise mixed or pure stands of broadleaved deciduous forest, coniferous forest communities. Esenli Forest Planning Unit is located on the A7 of the Eastern Black Sea Region, where Davis [18] divides Turkey's flora in terms of latitude and longitude ratings.

1.3. Vegetation Structure of Research Area

The vegetation types within Esenli Forest Planning Unit are spread between 850-2700 m elevations. At low elevations, there is a mixed forest structure formed by broadleaved deciduous forest. This mixture contains some taxa such as *Carpinus betulus, Fagus orientalis, Castanea sativa, Tilia rubra, Corylus avellana, Cerasus avium, Euonymus europaeus, Quercus petraea* subsp. *iberica*. After this vegetation zone, pure or mixed forest communities formed by *Carpinus betulus* and *Fagus orientalis* together with the elevation are dominant. *Picea orientalis* after 1000 m in the area shows itself and is a dominant species up to 1700 m. From this elevation (1700 m), *Pinus sylvestris* species began to dominate with the influence of the surrounding climate and spreads to about 2000 m. In the higher parts of the study area, subalpine and alpine vegetation are dominant.

2. MATERIAL and METHODS

In order to reveal the floristic structure of the research area, both observation and plant sociology studies were carried out in April-September 2015. For the vegetation studies, 20 samples with an area of 400 m^2 were taken from the areas which were homogeneous in terms of the floristic composition and structure. The cover-abundance values of each plant taxon forming the vegetation in these selected parcels (Table 1) were determined according to Braun-Blanquet [19] method. The identification of the plant species taken from these sample areas was made according to the Turkish Flora [18, 20, 21].

The current status of the plants that have been diagnosed have controlled from the Turkish Plant List [22]. Within the scope of this study, the literature on the utilization of the plant taxa was examined and the plants evaluated within the scope of medical and aromatic plant were determined. Braun-Blanquet cover-abundance values have re-calculated based on the mean values indicated by van der Maarel [23] (Table 1) in order to find the weighted average covers of the medical and aromatic plants determined in the study area.

Table 1. Conversiton cover-abundance values.

Braun-Blanquet Scale	Range of cover [%]	Midpoint of Cover range [%]
5	75-100	87,5
4	50-75	62,5
3	25-50	37,5
2	10-25	15
1	1-10	2,5
+	various individual	0,1
r	Rare	0,05

3. RESULTS and DISCUSSIONS

In this study, 20 sample areas of various vegetation types were taken from the Esenli Forest Planning Unit (Figure 3.). A total of 226 plant taxa were recorded from the 20 sample areas taken within the scope of the study. 10 (4,4%) of these taxa are belonged in division Pteridophyta, 3 (1,3%) Gymnospermae sub-division and 213 (94,3%) Angiospermae sub-division. As a result of the literature research, 110 plant taxa with naturally grow, medicinal and aromatic properties were determined in the field. Three of these taxa belong to the division Pteridophyta, three to the Gymnospermae subdivision and 104 to the Angiospermae subdivision (Table 2.). Rosaceae family is represented 13 taxa and Lamiaceae family is with 9 taxa. These families are followed by Asteraceae (8 taxa), Fabaceae (7 taxa) and Apiaceae and Brassicaceae (5 taxa) respectively.

Among the plants naturally grow in the study area, most of them (111 taxa) can be used for the diseases of the digestive system which used in the literature in terms of the main disease group. This group is followed by treatment methods such as skin diseases, respiratory system diseases, urinary tract diseases (Table 3.).

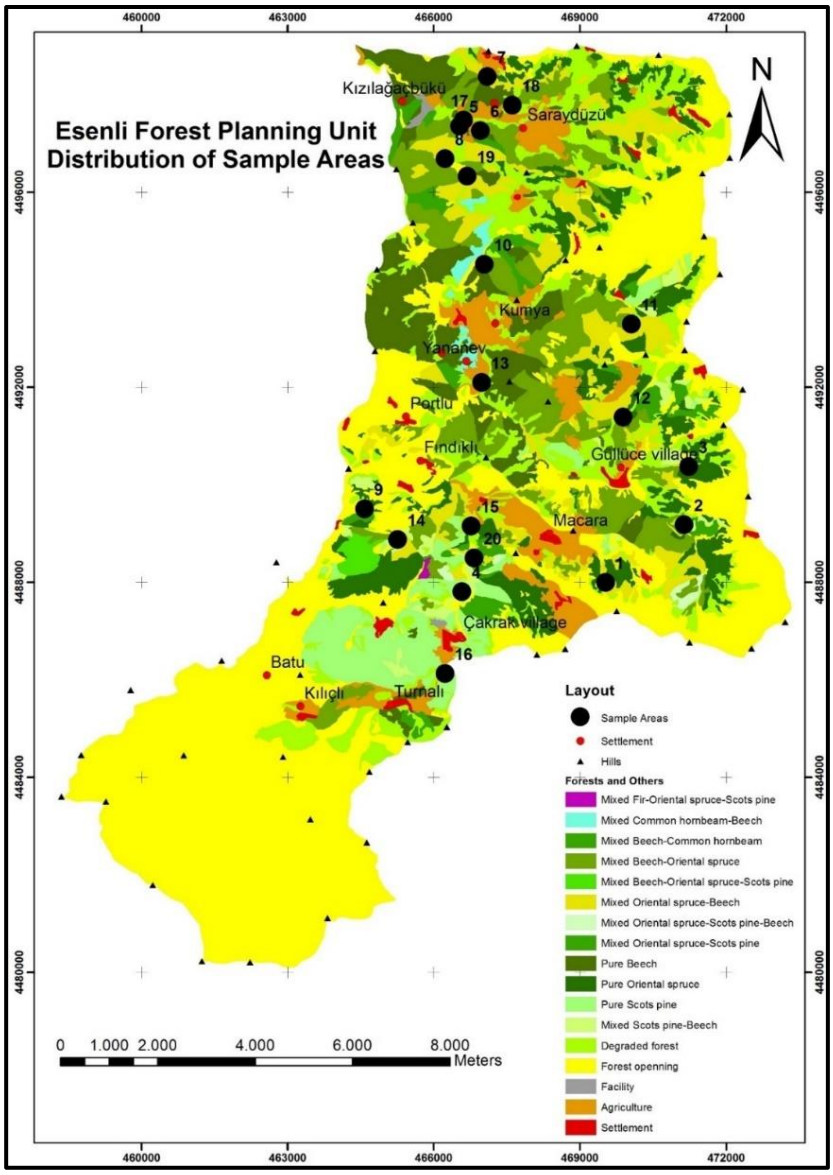

Figure 3. Distribution of sample areas

The result of investigating the literature is that the determined taxa can be used in the treatment of 80 different medicinal purposes (Table 3.). The most important of these forms of treatment are listed following. Diuretic (37), constipation therapy (34 taxa), stomach ache (30 taxa), wound healings (27 taxa), rheumatism (16 taxa), diabetes (15 taxa), haemorrhoids treatment and tonic (14 taxa) and sedative (13 taxa). In addition, 12 of these taxa were found to be poisonous or harmful. When evaluating in terms of protection of biodiversity the plant species distributed in Esenli Forest Planning Unit, it is useful to examine the parts of the plants which are found to be used in the literature. When examined in Table 2, leaves are the first rank in the plant parts used. The leaf organ is followed by flowers (30 taxa), fruit (19 taxa), root (17 taxa), whole plant parts (15 taxa) and finally aerial parts (14 taxa). Plant use in the form of flowers, roots, aerial parts and whole plants is important for biodiversity conservation. Such patterns of use can cause the population of plant species to decrease and species to be destroyed. The disappearance of species will mean the loss of the benefits derived from plant resources. For this reason, sustainable utilization of plants is of importance in terms of conservation biology. In other words, protection-use balance in natural resource use should not be ignored.

In order to determine the average cover of medicinal and aromatic plants within the study area, Braun-Blanquet values were transformed using the median values of mean cover values (Table 4.). With the help of these values, total cover values and weighted average cover values of plant species were calculated. When these values are examined depending on the vegetation structure of the study area, *Picea orientalis* (34,625%), *Fagus orientalis* (11,285%), *Rhododendron ponticum* (6,875%) and *Pinus sylvestris* (6,13%) are in the first ranks in terms of their cover values. These families are followed by *Galium odoratum*, *Oxalis acetosella*, *Sanicula europaea*, *Carpinus betulus*, *Primula veris* subsp. *columnae*, *Vaccinium arctostaphylos*, *Rubus hirtus*, *Fragaria vesca*, *Clinopodium vulgare* subsp. *arundanum*, *Viola sieheana*, *Phedimus stoloniferus*, *Pteridium aquilinum*, *Trifolium repens* var. *repens* respectively.

Within the scope of the study, the existence of 12 more medical and aromatic plants were observed in Esenli Forest Planning Unit as well as observations made on the vegetation studies. These taxa are; *Brassica oleracea*, *Caltha palustris*, *Capsella bursa-pastoris*, *Cerasus avium*, *Hedera helix* f. *Helix*, *Helleborus orientalis*, *Laurocerasus officinalis*, *Mespilus germanica*, *Sambucus nigra*, *Thymus pseudopulegionides*, *Vaccinium myrtillus* and *Zea mays* subsp. *mays*. Since these taxa are not covered by vegetation studies, no information is given in this section on the values of cover.

Today, the importance of medicinal and aromatic plants is increasing day by day. This increase is accompanied by excessive use. In our country, these plants are mostly obtained from the nature by collecting, which endangers natural populations. These attempts, which take place in the form of unconscious gathering (early gathering, excessive gathering, gathering unnecessary parts of plants, etc.), should be avoided. Good quality and standardization should be ensured and products with high added value should be produced instead of raw material sales. Another important issue in our country about medicinal and aromatic plants is that the inventories of these plants are missing. According to the Flora of Turkey, which plant is known to grow in which region. However, there is insufficient information on the status of plant populations. There are many plant sociology studies in almost every region of our country. In this context, plant sociology studies should be used in determining the populations of medicinal and aromatic plants.

As a result of these determinations, it should not be forgotten that all plant species carry chemicals at certain rates in their organs. As the use of medicinal plants may have different effects on each individual person, excessive or some uses other than medical advice can cause poisoning.

Table 2. Medically and aromatic plants grown naturally in the Esenli Forest Planning Unit

No	Family	Plant name	Turkish name	Used Part	Purpose of using
1	Adoxaceae	*Sambucus ebulus*	Mürver otu	Leaves, Fruit, Root	Constipation, joint aches, itch, diuretic, diaphoretic [24], rheumatism [25, 28, 33], hemorrhoids [26].
2	Adoxaceae	*Sambucus nigra*	Ağaç mürver	Flowers, Fruit, Leaves	Diaphoretic, diuretic, constipation [24], hemorrhoids [25], cough [28], stomache ache, cold, flu, dizziness, nausea [30], abscess [32], prostate, hypoglycemia [33].
3	Apiaceae	*Anthriscus nemorosa*	Peçek	Fruit	Carminative [42].
4	Apiaceae	*Anthriscus sylvestris*	Gımı	Fruit	Carminative [42].
5	Apiaceae	*Grammosciadium platycarpum*	Yassı kami	Aerial parts	Stomache ache [31].
6	Apiaceae	*Oenanthe pimpinelloides*	Deli maydanoz	Aerial parts	Burn, hypertension, painkiller [31].
7	Apiaceae	*Sanicula europaea*	Derman otu	Leaves, Root	Constipation, stomache ache, wound healings [24] Shortness of breath, body relaxation, stomache ache [25].
8	Araliaceae	*Hedera helix f. helix*	Orman sarmaşığı	Flowering and Leafy branches	Constipation, intestinal parasites, diaphoretic, callus and abscess, burn [24], stomache ache [25], kidney stone, kidney diseases [28]. Poisonous plant.
9	Asparagaceae	*Muscari neglectum*	Arap sümbülü	Whole plant	Rheumatism [27].
10	Asparagaceae	*Polygonatum multiflorum*	Mührü süleyman	Rizom	Constipation, gout, rheumatism, diabetes, abscess [24].
11	Aspleniaceae	*Asplenium adiantum-nigrum*	Kara saçakotu	Whole plant	Eczema [26], hemorrhoids [28].
12	Aspleniaceae	*Asplenium trichomanes*	Saçakotu	Whole plant	Stomache ache, hemorrhoids [25], eczema [26].
13	Asteraceae	*Achillea millefolium subsp. millefolium var. millefolium*	Civan perçemi	Flowering branches	Diuretic, orexigenic, carminative, women's diseases, wound healing, hemorrhoids [24], shortness of breath [25], nephritis, weight loss [30], kidney stone, blood purifier, liver purifier [37].
14	Asteraceae	*Cota tinctoria*	Boyacı papatyası	Flowers	Wound healing, throat ache, hairpin [24], women's diseases, shortness of breath [25], cold [28] stomache ache, bronchitis [33, 35].
15	Asteraceae	*Bellis perennis*	Koyun gözü papatya	Flowers	Diuretic, diaphoretic, tonic, constipation, pectoral (softener), stomache ache [24], cold [25, 29].
16	Asteraceae	*Centaurea jacea*	Çayır peygamber çiçeği	Aerial parts	Antipyretic, women's diseases, constipation, orexigenic [24].
17	Asteraceae	*Cirsium hypoleucum*	Vişne kangalı		Cardiac disorder, diabetes, rheumatism, eczema [36].
18	Asteraceae	*Leontodon hispidus var. hastilis*	Oklu gulikazer	Leaves	Hematischesis [26].
19	Asteraceae	*Petasites hybridus*	Kabalak	Aerial parts	Carminative, digestive system [29].

20	Asteraceae	*Tanacetum parthenium*	Beyaz papatya	Leaves, Flowering branches	Tonic, antipyretic, headache [24], women's diseases [25].
21	Betulaceae	*Alnus glutinosa subsp. barbata*	Sakallı kızılağaç	Seed, Leaves, Bark	Tonsillitis, intestinal bleeds, diuretic, constipation, tonic, wound healings [24], wound purify [25], hematischesis [26], stomache ache, hemorrhoids [25, 29].
22	Betulaceae	*Carpinus betulus*	Adi gürgen	Leaves	Blood regulator, wound healing, astringent [24], cholesterol [26].
23	Betulaceae	*Corylus avellana var. avellana*	Adi fındık	Leaves, Seed	Diuretic, tonic [24), cough [25, 29], anemia [26], hypertension [29].
24	Boraginaceae	*Cerinthe minor subsp. minor*	Cüce gözü	Aerial parts	Fodder [42].
25	Boraginaceae	*Echium vulgare subsp. vulgare*	Engerek otu	Whole plant, Root	Diuretic, expectorant [24], wound healings [32, 37].
26	Boraginaceae	*Symphytum asperum*	Kaba kafesotu	Root	Cough, pectoral (softener) [25].
27	Boraginaceae	*Trachystemon orientalis*	Kaldirik	Whole plant	Itch, injury, diaphoretic, blood purifier, antipyretic [24], diuretic, Intestinal diseases [25], Inflammatory wounds [26].
28	Brassicaceae	*Brassica oleracea*	Kara lahana	Leaves	Stomach ache, earache, wound healings [26], abscess, stomache ache, rheumatism [33].
29	Brassicaceae	*Capsella bursa-pastoris*	Çoban çantası	Leaves, Flowering branches, Stem	Women's diseases, constipation, diuretic [24], intestinal diseases [26], impotence [29], urinary tract diseases [35], kidney stone [40].
30	Brassicaceae	*Cardamine bulbifera*	Dişli kök	Root	Constipation [24].
31	Brassicaceae	*Nasturtium officinale*	Su teresi	Aerial parts	Blood regulator, kidney diseases, tonic, vitamin supply, diuretic, orexigenic [24], hemorrhoids, prostate [25], stomache ache [25, 29], diabetes [41].
32	Brassicaceae	*Microthlaspi perfoliatum*	Giyle	Leaves	Foodstuff [35].
33	Campanulaceae	*Campanula rapunculus subsp. lambertiana*	Büyük köklü çançiçeği	Leaves	Wound healing, constipation [24].
34	Caprifoliaceae	*Scabiosa columbaria subsp. columbaria var. columbaria*	Küçük uyuz otu	Whole plant	Constipation, diuretic, wound healings [24].
35	Caprifoliaceae	*Valeriana alliariifolia*	Pisot	Rhizome	Sedative, antispasmodic [24].
36	Celastraceae	*Euonymus europaeus*	İğcik ağacı	Fruit	Constipation, diuretic, emetic [24].
37	Cistaceae	*Helianthemum nummularium subsp. nummularium*	Güngülü	Whole plant	Constipation, hematischesis [24].
38	Convolvulaceae	*Calystegia silvatica*	Bürük	Leaves	Wound healings [29].
39	Crassulaceae	*Sedum pallidum*	Koyunörmece	Leaves	Wound healings [43].
40	Crassulaceae	*Phedimus stoloniferus*	Pisikulağı	Leaves	Rennet [41].

41	Dennstaedtiaceae	*Pteridium aquilinum*	Kartal eğreltisi	Leaves	Eczema [28], incense [41].
42	Dioscoreaceae	*Dioscorea communis*	Dolangaç	Root	Painkiller [25], wound healings [28], rheumatism [30].
43	Dryopteridaceae	*Dryopteris filix-mas*	Erkek eğrelti	Rizom, Leaves	Intestinal parasitic [24], stomache ache [25].
44	Ericaceae	*Rhododendron ponticum*	Mor çiçekli orman gülü	Leaves	Painkiller, diuretic, rheumatism [24], eczema [25], itch [26], headache [29].
45	Ericaceae	*Vaccinium arctostaphylos*	Likarpa	Fruit, Leaves	Constipation, shortness of breath [24].
46	Ericaceae	*Vaccinium myrtillus*	Ayı üzümü	Fruit, Leaves	Stomache ache, embolism, antiseptic, tonic [24], bronchitis [25], tooth ache, digestive system [26], diabetes, constipation [24, 29].
47	Euphorbiaceae	*Euphorbia amygdaloides subsp. amygdaloides*	Zerena	Seed, Leafy branches (fresh), latex	Constipation, curing warts. Harmful if used overdose [24], water purifier [33].
48	Euphorbiaceae	*Mercurialis perennis*	Yer fesleğeni	Flowering branches	Diuretic, constipation [24].
49	Fabaceae	*Anthyllis vulneraria subsp. pulchella*	Renkli çoban gülü	Flowering branches	Constipation, wound healings[24].
50	Fabaceae	*Securigera orientalis subsp. orientalis*	Ala körigen	Flowers, Juvenil Leaves	Foodstuff [35], nephritis [42].
51	Fabaceae	*Securigera varia*	Körigen	Whole plant	Fodder [42].
52	Fabaceae	*Lotus corniculatus var. corniculatus*	Gazal boynuzu	Whole plant	Sedative [24].
53	Fabaceae	*Trifolium pratense var. pratense*	Çayır üçgülü	Flowers	Expectorant, antiseptic, sedative [24].
54	Fabaceae	*Trifolium repens var. repens*	Ak üçgül	Flowering branches	Tonic, rheumatism [24].
55	Fabaceae	*Vicia cracca subsp. stenophylla*	Kuş fiği	Seed, Aerial parts	Urinary tract diseases, tonic, epilepsy, jaundice [24], fodder [35].
56	Fagaceae	*Castanea sativa*	Anadolu kestanesi	Leaves, branch braks, Flowers	Constipation, antihypertensive [24], cough [25], shortness of breath, cardiovascular disease [26].
57	Fagaceae	*Fagus orientalis*	Doğu kayını	Brak	Constipation, antipyretic [24], stomache ache, intestinal parasites [25].
58	Gentianaceae	*Gentiana asclepiadea*	Sütlü güşad	Aerial parts	orexigenic, antipyretic [24].
59	Geraniaceae	*Geranium robertianum*	Dağ ıtırı	Whole plant	Hematischesis, diuretic, tonic, diabetes, constipation, stomache ache [24].
60	Hypericaceae	*Hypericum androsaemum*	Kamaniça	Leafy branches	Stomache ache, gall bladder [24].
61	Hypericaceae	*Hypericum olympicum f. olympicum*	Uludağ Kantaronu	Flowers	Stomache ache, wound healing, burn [39].

62	Hypericaceae	*Hypericum perforatum subsp. perforatum*	Kantaron	Flowering branches	Sedative, intestinal parasites, shortness of breath, wound healing, constipation [24], regulation of blood pressure, hemorrhoids, diuretic [25], women's diseases, rheumatism, stomache ache [28], ulcer [29]. Poisonous plant (if used overdose).
63	Lamiaceae	*Ajuga orientalis*	Dağ mayasılı	Aerial parts	Skin diseases [41].
64	Lamiaceae	*Clinopodium grandiflorum*	Kaba fesleğen	Leaves	spice, tea [25].
65	Lamiaceae	*Clinopodium vulgare subsp. arundanum*	Kamış fesleğen	Leaves, Flowers	spice [41].
66	Lamiaceae	*Origanum vulgare subsp. viridulum*	İstanbul kekiği	Flowering and Leafy branches	Diuretic, diaphoretic, carminative, sedative [24], cold [25], cholesterol, flu [29], tooth ache, headache, [30].
67	Lamiaceae	*Prunella vulgaris*	Erik otu	Flowering branches, Leaves	Expectorant, cold [24, 25], gastric ulcer [26], wound healings [41].
68	Lamiaceae	*Salvia glutinosa*	Yapışkan Adaçayı	Leaves	Wound healing, burn [25].
69	Lamiaceae	*Stachys annua subsp. annua var. annua*	Hacıosmanotu	Aerial parts	Somniferous, women's diseases [41].
70	Lamiaceae	*Teucrium chamaedrys subsp. chamaedrys*	Kısa mahmut	Aerial parts	Eczema [28], kidney diseases [30], pneumonia, swelling [33], stomache ache [30, 34], hemorrhoids [33, 41].
71	Lamiaceae	*Thymus pseudopulegioides*	Anzer çayı	Flowering branches	Sedative, intestinal parasites, blood regulator, stomache ache, throat ache [24], Cold [25].
72	Malvaceae	*Tilia rubra subsp. caucasica*	Kafkas Ihlamuru	Flowers, Leaves, bark	Diuretic, diaphoretic, sedative, somniferous, pectoral (softener) [24], cough, asthma, stomache ache, wound healings [25], cold, intestinal diseases [28].
73	Melanthiaceae	*Veratrum album*	Dokuztepeli	Rhizome	Skin diseases, itch [24]. Poisonous plant.
74	Orobanchaceae	*Euphrasia pectinata*	Göz otu	Flowering branches	Wound healings [24].
75	Oxalidaceae	*Oxalis acetosella*	Ekşi yonca	Leaves, Root	Astringent, diuretic, hematischesis, expectorant, wound healings [24].
76	Pinaceae	*Abies nordmanniana subsp. nordmanniana*	Doğu Karadeniz Göknarı	Cone resin, Leaves	Antiseptic, expectorant, constipation [24], wound healing, abscess [32].
77	Pinaceae	*Picea orientalis*	Doğu ladini	Resin, stamen	Muscle pain, stomache ache, lung diseases [24], anti-inflammatory [25].
78	Pinaceae	*Pinus sylvestris*	Sarıçam	Bud, Brak extract, resin, cone	Diuretic, expectorant [24], bronchitis, stomache ache, rheumatism [26], wound healing, snakebite [32], cold, tuberculosis [33], abdominal pain [34].
79	Plantaginaceae	*Digitalis ferruginea subsp. ferruginea*	Arıkovanı	Leaves, Root, Seed	Diuretic, expectorant, cardiac enhancer, itch, wound healings [24]. Poisonous plant.

80	Plantaginaceae	*Plantago lanceolata*	Damarlıca	Leaves	Constipation, diuretic, respiratory tract diseases [24], astringent, gastric ulcer, wound healings [29], abscess [30, 33], insect bite, apnea, bronchitis, varicose [39].
81	Plantaginaceae	*Veronica anagallis-aquatica*	Su gedemesi	Aerial parts	Stomache ache, rheumatism [33].
82	Poaceae	*Zea mays subsp. mays*	Mısır	Pistil, Seed	Diuretic [28], kidney stone [29, 32], hemorrhoids [33].
83	Polygonaceae	*Polygonum bistorta subsp. carneum*	Dağ lahanası	Rizom	Constipation, antiseptic, diuretic, hematischesis [24].
84	Polygonaceae	*Rumex acetosella*	Kuzu kulağı	Leaves, Root	Abscess, diuretic, gall bladder, antipyretic. rheumatism, Dangerous to people with gout or kidney disease [24], digestive system disaeses, tooth inflammation, stomache ache, sinusitis [25], hypertension, diabetes [29].
85	Polypodiaceae	*Polypodium vulgare var. vulgare*	Benli eğrelti	Whole plant	Kidney stone, gall bladder, headache, tonsillitis, carminative, stomache ache [30].
86	Primulaceae	*Primula veris subsp. columnae*	Tutya	Root, Leaves, Flowers	Expectorant, diuretic, sedative, pectoral (softener), abscess [24].
87	Primulaceae	*Primula acaulis subsp. acaulis*	Çuha çiçeği	Root, Leaves, Flowers	Expectorant, diuretic, pectoral (softener), abscess [24, 25], sedative, cough [29], rheumatism [41].
88	Ranunculaceae	*Caltha palustris*	Bataklık nergisi	Flowering branches, Root, Leaves	Sedative, blood enhancer. Poisonous plant [24], internal disease [37].
89	Ranunculaceae	*Helleborus orientalis*	Çöpleme	Aerial parts	Anesthetic, stimulant, heart diseases, parasites in animals. Poisonous plant [24], edema [33].
90	Ranunculaceae	*Ranunculus cappadocicus*	Yağlı çanak		Rheumatism [36].
91	Ranunculaceae	*Ranunculus repens*	Tiktakdana	Flowers, Aerial parts	Rheumatism [33, 36].
92	Rosaceae	*Cerasus avium*	Kiraz	Gövde, Fruit stem	Intestine diseases [26], diuretic [28].
93	Rosaceae	*Crataegus monogyna var. monogyna*	Adi alıç	Fruit, Flowers	Tonic [24], hypertension, bronchitis [28], cardiovascular disease [30].
94	Rosaceae	*Fragaria vesca*	Dağ çileği	Fruit, Root	Constipation, diuretic, orexigenic [24], diabetes [25].
95	Rosaceae	*Geum urbanum*	Meryem otu	Whole plant	Constipation, stomache ache, tonic [24]. Harmful if used overdose.
96	Rosaceae	*Laurocerasus officinalis*	Karayemiş, taflan	Seed, Leaves, Fruit	Goiter, asthma, bronchitis, burn, stomache ache, diabetes, cough [24], gastric ulcer [25] hemorrhoids, backache, diuretic [26], headache, hypertension [29], cardiac disorder [36].
97	Rosaceae	*Mespilus germanica*	Muşmula	Leaves, Fruit, Seed	Constipation, diarrhea, diuretic [24], kidney stone [26], diabetes, hypertension [29].
98	Rosaceae	*Pyrus communis subsp. communis*	Bey armudu	Fruit, Leaves	Foodstuff [29], diuretic [40].

99	Rosaceae	*Rosa canina*	Kuşburnu	Fruit, Seed, Flowers	Sedative, constipation, tonic [24), cold [25], cough [29], hemorrhoids [32, 33], diabetes [24, 40].
100	Rosaceae	*Rubus canescens var. glabratus*	Çoban kösteği	Fruit	Tonsillitis, anemia [29].
101	Rosaceae	*Rubus hirtus*	Akdiken	Leaves, Root	Wound healing, allergy, burn [24], hemorrhoids, diabetes [32].
102	Rosaceae	*Rubus idaeus subsp. idaeus*	Ahududu	Leaves, Fruit	constipation, orexigenic, diuretic, tonic [24], cancer, eczema [25], birth pangs [40].
103	Rosaceae	*Sanguisorba minor subsp. minor*	Küçük çayır düğmesi	Whole plant	Constipation, stomache ache, diuretic, orexigenic [24].
104	Rosaceae	*Sorbus aucuparia*	Kuş üvezi	Leaves, Fruit	Constipation, pectoral (softener) [24], hypertension [30].
105	Rubiaceae	*Galium odoratum*	Orman iplikçiği	Whole plant	Diuretic [37].
106	Solanaecae	*Atropa belladonna*	Güzel avratotu	Leaves, Fruit	Painkiller, antispasmodic. Poisonous plant [24], skin beauty [25].
107	Solanaecae	*Physalis alkekengi*	Güvey feneri	Fruit	Blood pressure, diabetes, cold [25], diuretic [41]. Poisonous plant.
108	Thymelaeaceae	*Daphne pontica subsp. pontica*	Sırımbağı	Bark	Diuretic, constipation, diaphoretic [24]. Poisonous plant.
109	Urticaceae	*Urtica dioica subsp. dioica*	Isırgan otu	Whole plant	Headache, stomache ache, kidney diseases, blood purifier, diabetes, diuretic, orexigenic, cancer, rheumatism, milk enhancer [24], hemorrhoids [25], bronchitis [26, 29], itch [28], eczema [24, 29], respiratory tract diseases [29] baldness [30], hypoglycemia, snakebite [33].
110	Violaceae	*Viola sieheana*	Çayır menekşesi	Flowers	Upper respiratory tract diseases, cough, bronchitis, sinusitis [38], skin beauty [37].

Table 3. Distribution of detected plant species in main disease groups

No	Main disease groups	Treatment	Quantity	Total
		Constipation	34	
		Stomache ache	30	
		Hemorrhoids	14	
		Carminative	6	
		Intestinal diseases Intestinal	5	
		parasitic	5	
1	Digestive system	Gastric ulcer	5	111
		Digestive	4	
		Gall bladder	4	
		Nausea	1	
		Diarrhea	1	
		Obesity	1	
		Emetic	1	
		Wound healing	27	
		Abscess	9	
		Eczema	8	
		Itch	8	
2	Skin Diseases	Burn	6	63
		Skin beauty	2	
		Curing warts	1	
		Allergy	1	
		Hairpin	1	
		Cough	10	
		Bronchitis	9	
		Expectorant	9	
		Shortness of breath	7	
		Pectoral (softener)	6	
3	Respiratory system	Tonsillitis (Anjin)	3	53
		Throat ache (Faranjit)	3	
		Asthma	2	
		Sinusitis	2	
		Lung diseases	1	
		Pneumonia	1	
		Diuretic	37	
		Kidney stone	6	
4	Urinary system	Kidney diseases	4	51
		Nephritis	3	
		Urinary system	1	
		Hypertension	13	
		Embolism	4	
		Astringent	3	
5	The circulatory system	Cardiac disorder	3	29
		Blood purifier Cholesterol	3	
		Varicosis	2	
			1	
		Rheumatism	16	
		Joint aches	4	
6	Musculo-skeletal System	Injury	2	24
		Backache	1	
		Gout	1	
7	Internal secretion system	Diabetes	15	16
		Guatr	1	
		Cold	10	
8	Infectious diseases	Flu	2	13
		Hepatit	1	
9	Reproductive organs	Women’s diseases	7	9
		Prostate	2	
10	Nervous system	Epilepsy	1	1

11	Tonic	14
12	Sedative	13
13	Diaphoretic	8
14	Orexigenic	8
15	Painkiller	7
16	Antipyretic	6
17	Headache	6
18	Antiseptic	4
19	Anemia	3
20	Hematischesis	3
21	Toothache	3
22	Cancer	2
23	Somniferous	2
24	Snake bite	2
25	Hair disorders	1
26	Insect bite	1
27	Wound purify	1
28	Dizziness	1
29	Earache	1
30	Vitamin supply	1
31	Apne	1
32	Milk enhancer	1

Table 4. Cover-abundance values of species.

No	Plant taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total cover	Weighted total cover
1	*Picea orientalis*	62,5	2,5	37,5	37,5	37,5	2,5	62,5	15,0	62,5	15,0	37,5	37,5	37,5	2,5	62,5	2,5	62,5	15,0	37,5	62,5	692,50	34,625
2	*Fagus orientalis*		0,1	0,1	0,1		62,5	15,0	15,0		37,5	15,0	0,1	0,1	62,5		0,1	2,5	15,0		0,1	225,70	11,285
3	*Rhododendron ponticum*			15,0		2,5	15,0	15,0	2,5		37,5	15,0		2,5				15,0	2,5	15,0		137,50	6,875
4	*Pinus sylvestris*		37,5		2,5								15,0		0,1	2,5	62,5				2,5	122,60	6,13
5	*Galium odoratum*	2,5		37,5			15,0	0,1		2,5		2,5	2,5			15,0				0,1		77,70	3,885
6	*Oxalis acetosella*	15,0				0,1		15,0		15,0	2,5	2,5				2,5					0,1	52,70	2,635
7	*Sanicula europaea*			2,5	15,0			0,1		2,5	2,5	2,5		2,5		15,0	2,5					45,10	2,255
8	*Carpinus betulus*						2,5		2,5		0,1			15,0		0,1		0,1	2,5	0,1	0,1	23,00	1,15
9	*Primula veris subsp. columnae*		15,0	0,1						0,1			2,5				0,1			0,1	0,1	18,00	0,9
10	*Vaccinium arctostaphylos*					0,1		15,0	0,1			0,1						2,5				17,80	0,89
11	*Rubus hirtus*	0,1			0,1		0,1	2,5	0,1	2,5	2,5	2,5				2,5					2,5	15,40	0,77
12	*Fragaria vesca*	2,5		0,1	0,1			0,1	2,5	0,1	0,1	0,1	0,1	0,1	2,5	0,1	0,1		0,1	2,5	2,5	13,60	0,68
13	*Clinopodium vulgare subsp. arundanum*	0,1	2,5		0,1			0,1	2,5				0,1	0,1	0,1	2,5	2,5	0,1		2,5	0,1	13,30	0,665
14	*Viola sieheana*	0,1	0,1		0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	2,5		0,1	2,5	0,1	2,5	2,5	0,1	0,1	11,40	0,57
15	*Phedimus stoloniferus*	0,1									0,1	2,5		2,5				2,5	2,5	0,1		10,30	0,515
16	*Pteridium aquilinum*			0,1			2,5	2,5	2,5	0,1				2,5								10,20	0,51
17	*Trifolium repens var. repens*		0,1										2,5	2,5	2,5		2,5			0,1		10,20	0,51
18	*Geranium robertianum*			0,1			0,1	0,1			0,1			0,1	0,1	2,5		0,1		2,5	2,5	8,20	0,41
19	*Petasites hybridus*	0,1		2,5		0,1	0,1	0,1			2,5	2,5		0,1		0,1						8,10	0,405
20	*Rubus idaeus subsp. idaeus*			2,5									2,5				2,5					7,50	0,375
21	*Dioscorea communis*				0,1		0,1	2,5	0,1		0,1					0,1	0,1	0,1	2,5		0,1	5,80	0,29
22	*Polypodium vulgare var. vulgare*					2,5					0,1					0,1		2,5		0,1	0,1	5,40	0,27
23	*Ranunculus cappadocicus*	0,1		2,5						0,1		2,5			0,1		0,1					5,40	0,27
24	*Castanea sativa*					2,5			0,1									0,1	2,5			5,20	0,26
25	*Securigera varia*								0,1						2,5				0,1		2,5	5,20	0,26
26	*Cardamine bulbifera*			2,5						0,1		2,5						0,1				5,20	0,26
27	*Dryopteris filix-max*	0,1		2,5							2,5											5,10	0,255

28	*Cirsium hypoleucum*				0,1	0,1				0,1	0,1				0,1	0,1	2,5		0,1		0,1	0,1	3,40	0,17
29	*Clinopodium grandiflorum*			0,1	0,1				0,1			2,5								0,1		0,1	3,00	0,15
30	*Valeriana alliariifolia*										0,1	2,5				0,1				0,1	0,1		2,90	0,145
31	*Prunella vulgaris*									0,1	0,1				0,1					0,1	2,5		2,90	0,145
32	*Gentiana asclepiadea*				0,1		2,5					0,1	0,1										2,80	0,14
33	*Origanum vulgare subsp, gracile*													0,1				0,1			2,5		2,70	0,135
34	*Rubus canescens var. glabratus*															2,5					0,1	0,1	2,70	0,135
35	*Sedum pallidum var. pallidum*														2,5			0,1			0,1		2,70	0,135
36	*Teucrium chamaedrys subsp. chamaedrys*			0,1						2,5												0,1	2,70	0,135
37	*Abies nordmanniana subsp. nordmanniana*					2,5												0,1					2,60	0,13
38	*Daphne pontica subsp. pontica*					2,5				0,1													2,60	0,13
39	*Hypericum olympicum f. olympicum*													2,5		0,1							2,60	0,13
40	*Primula acaulis subsp. acaulis*														0,1						2,5		2,60	0,13
41	*Sambucus ebulus*				0,1				2,5														2,60	0,13
42	*Sanguisorba minor subsp. minor*			2,5						0,1													2,60	0,13
43	*Salvia glutinosa*											2,5											2,50	0,125
44	*Tilia rubra subsp. caucasica*						2,5																2,50	0,125
45	*Digitalis ferruginea subsp. ferruginea*	0,1	0,1	0,1	0,1	0,1			0,1				0,1	0,1	0,1	0,1		0,1		0,1		0,1	1,20	0,06
46	*Corylus avellana var. avellana*					0,1	0,1		0,1			0,1			0,1		0,1			0,1	0,1	0,1	0,90	0,045
47	*Rosa canina*					0,1		0,1				0,1		0,1		0,1		0,1				0,1		0,70	0,035
48	*Leontodon hispidus var. hastilis*									0,1				0,1	0,1	0,1		0,1				0,1		0,60	0,03
49	*Tanacetum parthenium*				0,1				0,1			0,1				0,1	0,1						0,1	0,60	0,03
50	*Urtica dioica*				0,1			0,1			0,1						0,1					0,1		0,50	0,025
51	*Alnus glutinosa subsp. barbata*						0,1					0,1	0,1						0,1					0,40	0,02
52	*Asplenium trichomanes*						0,1					0,1	0,1				0,1							0,40	0,02
53	*Geum urbanum*				0,1						0,1						0,1				0,1			0,40	0,02
54	*Vicia cracca subsp. stenophylla*					0,1								0,1		0,1		0,1						0,40	0,02
55	*Anthriscus nemorosa*				0,1					0,1										0,1				0,30	0,015
56	*Asplenium adiantum-nigrum*														0,1				0,1	0,1				0,30	0,015
57	*Campanula rapunculus subsp. lambertiana*						0,1												0,1			0,1		0,30	0,015

58	*Euphorbia amygdaloides subsp. amygdaloides*	0,1							0,1		0,1					0,30	0,015
59	*Lotus corniculatus var. corniculatus*			0,1						0,1		0,1				0,30	0,015
60	*Atropa belladonna*						0,1						0,1			0,20	0,01
61	*Crataegus monogyna var. monogyna*		0,1		0,1											0,20	0,01
62	*Grammosciadium platycarpum*													0,1	0,1	0,20	0,01
63	*Hypericum perforatum subsp. perforatum*					0,1					0,1					0,20	0,01
64	*Nasturtium officinale*				0,1			0,1								0,20	0,01
65	*Plantago lanceolata*		0,1												0,1	0,20	0,01
66	*Pyrus communis subsp. communis*		0,1							0,1						0,20	0,01
67	*Ranunculus repens*		0,1		0,1											0,20	0,01
68	*Rumex acetosella*				0,1									0,1		0,20	0,01
69	*Sorbus aucuparia*				0,1				0,1							0,20	0,01
70	*Sorbus torminalis var. torminalis*					0,1					0,1					0,20	0,01
71	*Symphytum asperum*			0,1					0,1							0,20	0,01
72	*Trachystemon orientalis*	0,1		0,1												0,20	0,01
73	*Echium vulgare subsp. vulgare*										0,1				0,1	0,15	0,0075
74	*Achillea millefolium*										0,1					0,10	0,005
75	*Ajuga orientalis*									0,1						0,10	0,005
76	*Cota tinctoria*					0,1										0,10	0,005
77	*Anthyllis vulneraria subsp. pulchella*										0,1					0,10	0,005
78	*Bellis perennis*														0,1	0,10	0,005
79	*Calystegia silvatica*							0,1								0,10	0,005
80	*Centaurea jacea*					0,1										0,10	0,005
81	*Securigera orientalis subsp. orientalis*		0,1													0,10	0,005
82	*Euphrasia pectinata*														0,1	0,10	0,005
83	*Helianthemum nummularium subsp. nummularium*										0,1					0,10	0,005
84	*Hypericum androsaemum*					0,1										0,10	0,005
85	*Mercurialis perennis*		0,1													0,10	0,005
86	*Muscari neglectum*											0,1				0,10	0,005
87	*Oenanthe pimpinelloides*						0,1									0,10	0,005

88	*Physalis alkekengi*						0,1															0,10	0,005
89	*Polygonatum multiflorum*			0,1																		0,10	0,005
90	*Polygonum bistorta subsp. carneum*																0,1					0,10	0,005
91	*Scabiosa columbaria subsp. columbaria var. columbaria*		0,1																			0,10	0,005
92	*Stachys annua subsp. annua var. annua*		0,1																			0,10	0,005
93	*Euonymus europaeus*										0,1											0,10	0,005
94	*Microthlaspi perfoliatum*		0,1																			0,10	0,005
95	*Trifolium pratense var. pratense*													0,1								0,10	0,005
96	*Veratrum album*		0,1																			0,10	0,005
97	*Veronica anagallis-aquatica*			0,1																		0,10	0,005
98	*Anthriscus sylvestris*																0,1					0,05	0,0025
99	*Cerinthe minor subsp. minor*		0,1																			0,05	0,0025
	Total Number of Species in Sample Areas	**14**	**22**	**28**	**19**	**17**	**16**	**21**	**23**	**16**	**28**	**21**	**18**	**27**	**22**	**21**	**24**	**17**	**17**	**29**	**22**		

Conflict of Interests

Authors declare that there is no conflict of interests.

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Subjective Effects of Lemon Seed, Rose and Lavender Essential Oils on Humans: A Case Study from Two Different Age Groups

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Abstract: Plant volatile oils have been used for different purposes for years, especially in the scientific and commercial areas. Among the major areas of their uses were the cosmetic, pharmaceutical and food industries, and the aroma/phyto therapeutic applications. The lemon seed, rose and lavender oils were the most commonly used oils and their effects on humans also differed individually. In this study, effects of these essential (volatile) oils on two age groups, 17-24 and 25-40 were thus investigated. The study was conducted on 80 people for one hour and the effects were evaluated using questionnaires. We used SPSS method for statics. In 17-25 age group, lavender oil caused drowsiness and weakness in 60% and 50% of respondents respectively, and also some mild giddiness, nausea, headache and dizziness. In 25-40 age group it caused giddiness and weakness in 40% respondents, and also gave mild vigor and tranquility. Lemon seed oil caused drowsiness in 85%, weakness and headache in 50%, and clear sensation in 55% of the respondents from 17-24 age group, while in 25-40 age group it caused clear sensation in 80% of respondents, and gave the mood of tranquility in 70% and happiness in 60%. However, rose oil demonstrated more intense effects on people, causing drowsiness and nausea in 55%, weakness and headache in 50%, and also giddiness, face flush and palpitation in 17-24 age-group-respondents. In 25-40 age group it caused drowsiness and nausea in 40% while it gave happiness and tranquility in 60% of respondents. Study results indicated that effects of different types of volatile oils on humans vary based on the age groups. So, the preference of essential oils in our daily lives is implied to have great importance. Therefore, further studies on the subject should be conducted to better emphasize the choice and use of these oils based on the age groups.

Keywords: Lemon seed oil, Rose oil, Lavender oil, Subjective, Human

1. INTRODUCTION

The natural treatment methods based on the use of aromatic or essence oils from plants are called “aromatherapy”. The use of these oils covers wide range of areas such as from cosmetics industry to the medical applications. Their use also extend back to around 5000 BC [1]. The term 'aromatherapy' was first introduced by the French biochemist Renee-Maurice Gattefosse in 1937. Lately, more studies have been conducted on the essential oils and number of these studies is also ever-increasing [2].

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In our daily lives, we experience the effects of aromatherapy unconsciously, for instance, with soil smell after rain and smell of fresh bread etc. However, systematic aromatherapy application is based on the principle that plants are employed in treatment using their high-density essential oils and smells. In aromatherapy born from the ancient Egypt and Indian civilizations centuries ago, essential oils were often combined with the therapeutic effect of massage [3-4].

More than 60 different types of medicinal herbs have been used in aromatherapy. The most well-known aromatic plants include lavender oil, sweet orange oil, rose oil, jojoba oil, olive oil, geranium, sage, juniper, jasmine, sandalwood, eucalyptus and various incense.It has been reported that aromatherapy maintains the body and soul balance, provides the relaxation with pleasant odor, reduces the anxiety, fear, pain, nausea and vomiting, and increases the sense of well-being [5-7]. The only substance that able to pass through brain membrane is volatile oils. Thus, this transition has been very important in terms of treatment. For example, orange oil inhaled by smell gives cheerfulness, refreshes mind and relaxes depression, lavender oil relieves insomnia and headache, and rose oil removes postpartum depression, regulates menstrual period and relieves menopause complaints [8].

Pure essential oils have various effects on living organisms. They may aid in emotion and emotion-state control, function as anxiolytic [9] and antidepressant [10], and provide arousal [11], memory enhancement [12] and correction of cognitive impairment in dementia-related illnesses [13]. These effects are mediated by linkages extending to limbic system and hypothalamus through major olfactory tract, tractus olfactorius [9,14]. Natural rose oil includes ingredients like citranellol, geraniol, nerol, eugenol, methyl eugenol and phenylethyl alcohol [15]. Rose essence oil is also used for its calming effect and skin care in addition to its good fragrance [16,17]. Natural lavender oil contains linalool, linalyl acetate, lavandulyl acetate, Z-ocimene and caryophyllene. Lemon seed oil includes D-limonene as terpene [18].

Thus, effects of essential oils on humans have been important. In this regard, this work was attempted to investigate the effects of lavender, rose and lemon seed oils on two different age groups.

2. MATERIAL and METHODS

In this work, the effects of essential oils on two different age groups 17-24 and 25-40 were investigated. Study was performed on 80 people for one hour in the morning at 9:30-10:30 am. Each individual was tested on a separate day but at the same time. On the first day, rose oil was dropped in the room and subjects were exposed for one hour. Similarly, on the second and third days respectively, subjects were exposed to the lavender and lemon seed oils for one hour. Then, results were evaluated using questionnaires. Respondents were surveyed about 14 questions about fatigue, facial flushing, drowsiness, dizziness, giddiness, nausea, headache, palpitation, hallucinations, visual disturbances, happiness,clear sensation, peace and vigority. They were then asked to assess the questions on a scale of 1 to 10. Values 1-5 were classified as too low, values 5-7 were as medium strength and values 7-10 were as very strong for the tested effects. Subsequently, all questionnaires were re-evaluated based on the effects seen as either “with-impact” or “without-impact”. The surveyed questionnaire is given in Table 1.

Table 1. Surveyed Questionnaire

Essential oil	Lavandula			Lemon Seed				Rose		
Age	17-24						25-40			
Symptoms seen	RATING									
	1	2	3	4	5	6	7	8	9	10
Perception openness										
Fatigue										
Sleeping state										
Dizziness										
Drowsiness										
Nausea										
Headache										
The palpitation										
The hallucination										
Facial flush										
Happiness										
Peace										

3.RESULTS

3.1. Lavender Oil

Lavender oil is known to be a good antiseptic and it is also reported to maintain the body and soul balance, help the relaxation with pleasant odor, relieve the anxiety, fear, pain, nausea and vomiting, and increase the sense of well-being. Herein, effects of lavender oil on 17-24 and 25-40 age groups seemed to differ from each other. In questionnaires responded by 17-24 age group, from 40 people, %60 respondents reported for drowsiness and %50 respondents reported for fatigue. Besides, these effects were also seen in the same people. In addition, lavender oil also caused some mild giddiness, dizziness and headache in the subjects. No other adverse effects like facial flushing, palpitation, nausea, hallucinations and visual disturbances were observed, and young group did not also see positive effects such as peace, vigority and clear sensation. On the other hand, in 25-40 age group, the intensity of effects decreased, out of 40 respondents, %40 observed drowsiness and fatigue, and %23 reported for vigority and peace [Table 2].

Table 2. Analysis Results for Lavander Oil

	17-24				25-40			
	<5	%	>5	%	<5	%	>5	%
Perception openness	36	90	4	10	38	95	2	5
Fatigue	20	50	20	50	24	60	16	40
Sleeping state	24	60	16	40	20	50	20	50
Dizziness	40	100	0	0	38	95	2	5
Drowsiness	24	60	16	40	24	60	16	40
Nausea	36	90	4	10	32	80	8	20
Headache	32	80	8	20	34	85	6	15
The palpitation	36	90	4	10	40	100	0	0
The hallucination	36	90	40	10	40	100	0	0
Facial flush	32	80	8	20	40	100	0	0
Happiness	36	90	4	10	40	100	0	0
Peace	32	80	8	20	31	77	9	23
Vigor	32	80	8	20	31	77	9	23

Lavender essential oil contains 23.29% camphor, 10.87% phencon, 4.07% eucalyptol, and 1.5% linalool and linalyl acetate [19]. Thus, negative effects like dizziness, nausea and giddiness, particularly in the young age group could be caused by these oil ingreidents. On contrary, adverse effects in the middle age group are reduced and relaxing effects appear.

3.2. Rose Oil

Pure rose oils have various effects on the emotion and emotion-state control, anxiety, depression, arousal, memory enhancement and in amelioration of cognitive impairment in dementia. Some essential oils applied via respiration and orally were also reported to reduce the symptoms and strengthen the memory in neurodegenerative diseases like Alzheimer (7,14). In this work, different effects were observed in the studied age groups. In 17-24 age group, out of 40 people, %55 respondents had an intense nausea and drowsiness while %50 respondents reported for headaches and weakness. Besides, respondents also reported for some mild face flushing, giddiness and palpitations. On the other hand, in 25-40 age group, only %40 subjects -from 40 respondents- had these negative effects. Besides, rose oil essence also created happiness and peace in %60 subjects [Table 3].

Natural rose oil contains substances like citranellol, geraniol, nerol, eugenol, methyl eugenol and phenylethyl alcohol [15]. 85% of these substances fall under a group called citronellal, consisting of citronellal, neral and nerol. This group has allergic effect and also has soothing effect especially in the intense environments (environments with high temperature, humidity etc.). Thus, effect of citranellol has become a major cause of adverse effects especially in young people, whereas phenylethyl alcohol increased the peace and happiness in the middle age group.

Table 3. Analysis Results for Rose Oil

	17-24				25-40			
	<5	%	>5	%	<5	%	>5	%
Facial flush	28	70	12	30	40	100	0	0
Weakness	20	50	20	50	32	80	8	20
Sleeping state	18	45	22	55	24	60	16	40
Dizziness	22	55	18	45	32	80	8	20
Clear Sensation	26	65	14	35	32	80	8	20
Nausea	19	45	21	55	24	60	16	40
Headache	20	50	20	50	32	80	8	20
The palpitation	30	75	10	25	40	100	0	0
The hallucination	36	90	4	10	40	100	0	0
Disorder in sight	36	90	4	10	10	100	0	0
Happiness	40	100	0	0	16	40	24	60
Peace	40	100	0	0	16	40	24	60
Vigor	40	100	0	0	36	90	4	10

3.3. Lemon Seed Oil

Lemon seed oil has been a natural antioxidant source since it is rich in vitamin C. This effect is especially useful in eliminating mental and physical fatigue. In questionnaires responded by 17-24 age group, from 40 people, %85 respondents reported for drowsiness because of its relaxation effect while %50 respondents reported for fatigue. Besides, %55 respondents also reported for clear sensation and vigority. On the other hand, in 25-40 age

group, results were quite different; no negative effect was reported by respondents, on contrary, %72 people had clear sensation, %63 people had peace and %57 people had happiness [Table 4].

Lemon seed oil contains 34,34-43,59% linoleic, 20,65-28,48% oleic and 23,72-30,33% palmitic acids. These fatty acids serve as energy storage facility. Especially they help to improve cell functions; in case of deficiencies the depression and behavioral disorders can occur. Thus, effects of their odor on humans emerged quite clearly.

Table 4. Analysis Results for Lemon Seed Oil

	17-24				25-40			
	<5	%	>5	%	<5	%	>5	%
Clear Sensation	19	47	21	53	11	28	29	72
Weakness	20	50	20	50	40	100	0	0
Sleeping state	6	15	34	85	28	70	12	30
Dizziness	38	95	2	5	40	100	0	0
Perception openness	24	60	16	40	40	100	0	0
Nausea	32	80	8	20	40	100	0	0
Headache	20	50	20	50	32	80	8	20
The palpitation	40	100	0	0	40	100	0	0
The hallucination	40	100	0	0	40	100	0	0
Disorder in sight	40	100	0	0	20	50	20	50
Happiness	32	80	8	20	17	43	23	57
Peace	30	75	10	25	15	37	25	63
Vigor	19	47	21	53	32	80	8	20

4. CONCLUSION

Present study has demonstrated the effects of essential oils on different age groups and these effects on the subjects differed individually. The active ingredients of tested oils led more intense negative effects the on the 17-24 age group. In 25-40 age group, these oils caused negative effects as well as positive subjective effects like happiness, peace, vigority and clear sensation. The presence of lavender oil in the environment brought about its effects as dizziness and giddiness, while rose oil caused the effects of nausea, facial flushing and palpitation. Besides, lemon seed oil has no negative effects, other than drowsiness and fatigue, on the 17-24 age group, while in 25-40 age group the effects have been more positive, with responses about increased clear sensation, happiness and peace.

Thus, preference of essential oils being used in our daily lives is of great importance. So, further studies should be conducted to better elucidate the effects of those essential oils on individuals/age groups as well as to understand their health implications on humans.

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Quality Characteristics of Noodle Including Ground Yellow Poppy Seed

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Abstract: The objective of this study is the production of noodle including ground yellow poppy seed which has phenolic compounds. Phenolic compounds have antioxidant activity. According to the researches, these compounds reduce risk of many diseases like cancer. In this study, ground yellow poppy seed (purchased from Afyon) was added to the noodle formulation at the ratios of 5% and 10%. Flour used in the production was obtained by milling of two wheat variety (Kunduru and Altıntas). The control sample was noodle sample including no ground yellow poppy seed. After production, quality characteristics, total phenolic content and color values of noodle samples were analyzed. Quality characteristics of noodle samples made from Kunduru wheat flour were higher than that of noodle samples made from Altıntas wheat flour. Ground yellow poppy seed addition caused dark color at the noodle samples. Control samples had higher quality characteristics compared to noodle samples including ground yellow poppy seed. Higher phenolic content was observed for noodle samples including ground yellow poppy seed.

Keywords: Poppy seed, Noodle, Phenolic

1. INTRODUCTION

Poppy (*papaver somniferum*) is cultivated as an annual crop in some countries (China, India, Turkey) [1]. Ozcan and Atalay [1] determined palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid in poppy seed oils. Poppy seed oils had α-tocopherol, β-tocopherol and δ-tocopherol in the range of 26.8-37.2 ppm, 309.5-567.3 ppm, and 6.1-18.6 ppm, respectively. Poppy seeds had high amount of P, K, Ca, Mg, Na, and Fe [1]. Bozan and Temelli [2] demonstrated that total phenolic content of poppy seed was 930 mg/100 g. Emir et al. [3] investigated sensory properties, aromatic profiles and consumer preferences of cold pressed poppy seed oils and reported that roasting before cold pressing improved sweet aromatic values and roasted samples were more liked by consumers. The yellow (produced in Afyon) roasted sample was the most preferred sample (53.55%) by consumers in all samples (white, yellow, blue).

Noodles are classfied into different types on the basis of raw material, processing methods, composition and shape of strands [4]. There are researches for increasing of total phenolic content in noodle, pasta and spaghetti, because phenolics have antioxidant activity.

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Khan et al. 2013[5] reported that addition of red sorghum flour and white sorghum flour to durum wheat pasta formulation increased total phenolic content of pasta due to sorghum contains polyphenolic antioxidants. Total phenolic content of wheat pasta was increased with increasing of addition level of red sorghum flour and white sorghum flour. Wheat pasta had 0,77 mg GAE/g total phenolic content, while wheat pasta including red sorghum flour had 1,88 mg GAE/g total phenolics content and wheat pasta including white sorghum flour had 1,06 mg GAE/g total phenolic content. Total phenolic content of wheat pasta including red sorghum flour was higher than that of wheat pasta including white sorghum flour because red sorghum flour had higher total phenolic content (6,65 mg GAE/g) compared to white sorghum flour (2,17 mg GAE/g)

There are some studies about increasing of total phenolic content of noodle, pasta and spaghetti. But there is no study about the effect of utilization of ground yellow poppy seed in noodle formulation to increase total phenolic content. The objective of this study was to evaluate the effect of ground yellow poppy seed addition to wheat noodle on quality characteristics, color values and total phenolic content of noodle.

2. MATERIAL and METHODS

2.1. Materials

Flours of two Turkish wheat cultivars (grown in Afyon), Kunduru-1149 and Altıntas-95, were supplied from Tınaztepe factory (Afyon, Turkey). Ground yellow poppy seed was purchased from local producer (Afyon).

2.2. Grain characteristics

Grain vitreousness was assessed by analysis of kernel cross sections using Grobecker kernel cutter and expressed as percentage of vitreous grains. Hectoliter weight of wheat samples was determined according to the AACC approved method [6]. Thousand kernel weight of wheat samples was determined according to the method of TS 1136. The experiments were performed in three replications.

2.3. Flour characteristics

Moisture, ash, protein (Nx 5.70), wet gluten and dry gluten contents of wheat flours were determined according to the AACC approved methods [6]. Zeleny sedimentation and modified sedimentation values of wheat flours were measured according to the method of ICC (116/1) [7]. Farinograph characteristics of wheat flours were determined according to ICC method (115/1) [7] using Brabender Farinograph (Brabender OHG, Duisburg, Germany). The experiments were performed in three replications.

2.4. Poppy seed characteristic

Oil content of ground yellow poppy seed was determined according to the method of AOAC [8] and color values (L*, a*, b*) of sample were performed by using X-rite spectrophotometer (USA).

2.5. Noodle preparation and analysis

The wheat flour and ground yellow poppy seed (5%, 10%) were mixed with 60% water in a mixer (Essenso Professeur 216912, China) for 10 min at a speed 1. The dough was sheeted by a sheeting roller. The dough sheet was cut into strips by pasta machine (Essenso Professeur 981121, China). Dough strips were dried at room temperature (25ºC). The final dried noodle samples contained a maximum of 9% moisture. The noodles were packed into plastic bags and stored until analyses. The samples were evaluated in terms of cooking, color properties and total phenolic content.

2.5.1. Cooking analysis

Noodle (25 g), cut into pieces of 2.5 cm, was cooked in boiling water (100 ml). The optimum cooking time of noodle was determined as the time required for disappearance of white core when compressed between two glass slides. Cooking loss is the amount of solid substance lost to cooking water. For determining of cooking loss, the cooking water was collected in a tarred beaker and evaporated to dryness in an oven at 98°C. The residue was weighted and reported as percentage of the starting material. Water absorption (%) and swelling volume (%) of the noodle samples were calculated as follows;

$$\text{Water absorption (\%)} = \frac{\text{weight of cooked noodle} - \text{weight of uncooked noodle}}{\text{weight of uncooked noodle}} \times 100$$

$$\text{Swelling volume (\%)} = \frac{\text{volume of cooked noodle} - \text{volume of uncooked noodle}}{\text{volume of uncooked noodle}} \times 100$$

The cooking time, cooking loss, water absorption and swelling volume analysis were carried out in duplicate.

2.5.2. Color analysis

The color measurement (L*, a*, b*) of noodle samples was carried out in duplicate using the L*a*b* color system, where L* is lightness, a* is redness, and b* is yellowness. The instrument used was a X-rite (USA).

2.5.3. Total phenolic content

Extracion of samples was carried out according to the method reported by Awika et al. [9]. Samples (1 g) were mixed with 10 mL of 1% HCL in methanol for 1 h and centrifuged at 3000 rpm for 20 min (DAIHAN Scientific Co., Ltd., WiseSpin® CF-10 Microcentrifuge, Korea). The supernatant was used for determination of total phenolic content.

Total phenolic content of noodles was determined by the Folin-Ciocalteu method of Li et al. [10]. Sample extract (0.2 mL) was mixed with 0.8 mL diluted Folin-Ciocalteu reagent (1/10) and waited for 3 min. Then 2 mL of sodium carbonate solution (15%, w/v) was added to mixture. After addition of 2 mL distilled water, the mixture was vortexed and kept at room temperature for 1 h in the dark. The absorbance was measured at 760 nm using UV-Vis spectrophotometer (Optizen pop, Korea). Gallic acid (0-0.5 mg/mL) prepared in methanol, was used as standard. The results were expressed as mg gallic acid (GAE) /g sample (dry basis). The analysis was carried out in duplicate.

2.6. Statistical analysis

Data related to cooking properties color, properties and total phenolic content of the noodle samples were statistically evaluated by one-way analysis of variance procedure. Duncan test was applied to compare mean values.

3. RESULTS and DISCUSSIONS

3.1. Grain characteristics

Physical properties of wheat samples (Kunduru-1149 and Altıntas-95) are given in Table 1. Kunduru-1149 had 59.3% vitreous kernels, while Altıntas-95 had 82.7% vitreous kernels. According to Dexter et al. [11], semolina yield was reduced with decreasing of vitreous kernels. Vitreous durum wheat is harder than starchy durum wheat and gives higher yield of semolina and lower yield of flour. The thousand kernel weight is associated with average kernel size.

Larger kernel causes higher milling yield [12]. The thousand kernel weights of Kunduru-1149 and Altıntas-95 were 41.7 g and 37.7 g, respectively. Kunduru-1149 had higher thousand kernel weight and higher milling/semolina yield than Altıntas-95. Hectoliter weight of Kunduru-1149 was higher than that of Altıntas-95. Hectoliter weight exhibits a linear relation with kernel weight and therefore milling/semolina yield [11].

Table 1. Physical and Chemical Properties of Wheat Grain

Physical and Chemical Properties of Wheat Grain	Kunduru -1149	Altıntas - 95
Hectoliter weight (kg/hl)	78.3	77.1
Thousand kernel weight (g)	41.7	37.7
Vitreousness (%)	59.3	82.7
Ash content (%)	1.7	1.6
Protein content (%)	12.2	14.5

3.2. Flour characteristics

The moisture contents of wheat flours (Kunduru-1149 and Altıntas-95) were 12.8% and 14.2%, while their ash contents were 1.7% and 1.6%, respectively. The protein contents of wheat flours (Kunduru-1149 and Altıntas-95) were 12.2% and 14.5% (d.b.), respectively. Physicochemical characteristics of durum wheat samples (Kunduru-1149 and Altıntas-95) are given in Table 1.

Altıntas-95 had higher protein content and also higher wet gluten content (27.3%) than those of Kunduru-1149 (22.7%). Altıntas-95 wheat flour had higher zeleny sedimentation volume than Kunduru-1149 wheat flour due to having higher protein content. The protein quality according to zeleny test was higher for the Altıntas-95 wheat flour (29.7 ml) compared to that for the Kunduru-1149 wheat flour (21.3 ml).

Farinograph results of wheat flours are given in Table 2. water absorption of kunduru-1149 wheat flour was 61.0%, while that of Altıntas-95 wheat flour was 61.5%. Kunduru-1149 wheat flour had lower dough development time and maximum consistency than Altıntas-95 wheat flour. Tolerance index of Kunduru-1149 wheat flour was higher than that of Altıntas-95 wheat flour.

Table 2. Gluten and Farinograph Properties of Wheat Flour

Farinograph and Gluten Properties of Wheat Flour	Kunduru -1149	Altıntas - 95
Development time (min)	9.4	14.3
Max. Consistency (B.U.)	400	445.0
Mixing Tolerance (B.U.)	28.3	6.7
Zeleny Sedimentation (ml)	21.3	29.7
Modified Zeleny Sedimentation (ml)	24.7	32.3
Gluten	22.7	27.3

3.3. Poppy seed characteristics

Oil content of ground yellow poppy seed was found as 49.85%. L*, a*, b* color values of sample were 60.08, -2.78 and 12.52, respectively.

3.4. Analysis of noodle

3.4.1. Cooking time

Cooking time values of the noodles are found as 20 min.

3.4.2. Cooking loss

Cooking loss values of noodles are given in Table 3. Addition level of ground yellow poppy seed to noodle caused significant changes in cooking loss values of noodles (Table 3). Noodle samples including 10% ground yellow poppy seed had the lowest cooking loss in noodle samples tested. Cooking loss values of noodles decreased significantly, as the level of ground yellow poppy seed in noodle decreased. Noodle samples prepared from Kunduru-1149 wheat sample were found to have significantly lower cooking loss values as compared to their respective samples prepared from Altıntas-95 wheat sample, indicating better quality. The higher cooking loss of noodles including ground yellow poppy seed might be due to decreasing of gluten content in noodle. Because ground yellow poppy seed have not gluten protein. But the results of noodle samples including ground yellow poppy seed were also acceptable due to lower than 8% [5]. The similar effect was observed for wheat pasta incorreperating with sorghum flour [5].

Table 3. Properties of noodles

Samples	Cooking loss (%)	Water absorption (%)	Swelling volume (%)	L*	a*	b*	Total phenolic content (mg GAE/ g)
K1	1.64 ^{e}	177.5 ^{a}	226.5 ^{a}	89.55 ^{a}	5.09 ^{e}	15.92 ^{d}	0.50 ^{f}
K2	2.84 ^{c}	146.0 ^{c}	194.5 ^{b}	83.83 ^{c}	5.67 ^{d}	16.92 ^{c}	0.69 ^{d}
K3	3.58 ^{b}	107.0 ^{d}	131.5 ^{c}	82.66 ^{d}	7.14 ^{a}	19.88 ^{a}	0.89 ^{f}
A1	2.12 ^{d}	172.5 ^{b}	222.0 ^{ab}	89.49 ^{a}	5.01 ^{e}	16.18 ^{d}	0.55 ^{e}
A2	3.62 ^{b}	96.0 ^{e}	133.0 ^{c}	85.25 ^{b}	6.00 ^{c}	18.16 ^{b}	0.79 ^{c}
A3	4.65 ^{a}	81.5 ^{f}	92.0 ^{d}	84.04 ^{c}	6.91 ^{b}	20.21 ^{a}	0.93 ^{a}

Note: K1: control noodle (Prepared from Kunduru -1149 flour); K2: noodle including 5 % ground yellow poppy seed (Prepared from Kunduru -1149 flour); K3: noodle including 10 % ground yellow poppy seed (Prepared from Kunduru -1149 flour); A1: control noodle (Prepared from Altıntas – 95 flour); A2: noodle including 5 % ground yellow poppy seed (Prepared from Altıntas – 95 flour); A3: noodle including 10 % ground yellow poppy seed (Prepared from Altıntas – 95 flour).

3.4.3. Water absorption

Water absorption values of noodles are given in Table 3. Addition level of ground yellow poppy seed to noodle caused significant changes in water absorption values of noodles (Table 3). Noodle samples including 10% ground yellow poppy seed had the lowest water absorption in noodle samples tested. Water absorption values of noodles decreased, as the level of ground yellow poppy seed in noodle increased. The lower starch content is a major reason for the lower water absorption of noodles including ground yellow poppy seed. Noodle samples prepared from Kunduru-1149 wheat sample resulted in significantly higher water absorption values when compared with those of noodle samples prepared from Altıntaş-95 wheat sample indicating better quality.

3.4.4. Swelling volume

Swelling volume values of noodles are given in Table 3. Addition level of ground yellow poppy seed to noodle formulation caused significant changes in swelling volume values of

noodles (Table 3). Noodle samples including 10% ground yellow poppy seed had the lowest swelling volume in noodle samples tested. Swelling volume of noodle increased, as the level of ground yellow poppy seed in noodle decreased. Noodle samples prepared from Kunduru-1149 wheat sample had significantly higher swelling volume values than that of noodle samples prepared from Altıntas-95 wheat sample indicating better quality.

3.4.5. Color properties

The colors of noodles are given in Table 3. The color of noodle is an important quality factor which influences consumer preference [13]. The level of ground yellow poppy seed in noodle significantly affected the L*, a* and b* color values of noodles. Noodles became darker with the increased level of ground yellow poppy seed in noodles. Noodle samples prepared from Kunduru-1149 wheat sample had L*, a* and b* color values comparable to their respective samples prepared from Altıntas-95 wheat sample.

3.4.6. Total phenolic contents

Total phenolic contents of noodles are given in Table 3. Ground yellow poppy seed significantly affected total phenolic content of noodles. Total phenolic content of noodles increased with increasing of ground yellow poppy seed in noodles. Noodles including 10% ground yellow poppy seed had significantly highest antioxidant activity due to having highest total phenolic content. Noodle samples prepared from Altıntas-95 wheat flour had significantly higher total phenolic content than that of noodle samples prepared from Kunduru-1149 wheat flour. The increase in total phenolic content was observed for wheat pasta after addition of sorghum flour [5].

4. CONCLUSIONS

In this study, ground yellow poppy seed was added to wheat noodle formulation for increasing of total phenolic content of noodle. Then the effect of utilization of ground yellow poppy seed in noodle on quality characteristics of noodle was studied. Quality characteristics of noodles including no ground yellow poppy seed were found better than those of noodles including ground yellow poppy seed. Ground yellow poppy seed enhanced the total phenolic content of noodle. Noodle samples including 10% ground yellow poppy seed had the highest total phenolic contents. According to literature, phenolics can prevent some diseases So noodles including ground yellow poppy seed were healthier compared to wheat noodle. Ground yellow poppy seed in noodles resulted in decrease in L*color values and increases in a* and b* color values.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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