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Prof. Dr. Ramazan MAMMADOV
Editor in Chief
International Journal of Secondary Metabolite

Pamukkale University, Art & Science Faculty,
Department of Biology,
20070, Denizli, Turkey

Phone : +90 258 296 3575
Fax : +90 258 296 3535
E-mail : ijsm.editor@gmail.com rmammadov@pau.edu.tr

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Support Contact

Prof. Dr. İzzet KARA
Journal Manager & Founding Editor

Phone : +90 258 296 1036
Fax : +90 258 296 1200
E-mail : ikara@pau.edu.tr

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

Investigation of Bioactive Compounds on Relict Endemic *Ajuga relicta* P. H. Davis (Lamiaceae) from Turkey

Nazan Comlekcioglu [1,*], **Yusuf Ziya Kocabas** [2], **Ashabil Aygan** [1]

[1] Kahramanmaras Sutcu Imam University, Faculty of Science and Letters, Department of Biology, Kahramanmaras, Turkey
[2] Kahramanmaras Sutcu Imam University, Turkoglu Vocational High School, Medicinal Plants Department, Kahramanmaras, Turkey

Abstract: Species belonging to genus *Ajuga* (Lamiaceae) have been used to treat many diseases in traditional medicine. The plants of the genus *Ajuga* have been reported to have antifungal, antibacterial, antimycobacterial, antihypertensive, antiplasmodial, hypoglycaemic, and larvae and insect activity. *Ajuga relicta* is a relict endemic plant which grows only in Kahramanmaras. The total phenolic contents of the extracts have been quantified with Folin Ciocalteu colorimetric method, and the antioxidant activities of the extracts have been tested with DPPH, and FRAP. Antimicrobial activities of plant extracts were determined by the well-diffusion method against seven bacteria and four yeasts. Besides, the fatty acid composition was determined in GC-MS. As a result of GC-MS analysis of the oil obtained from the *A. relicta* extracts, 21 different fatty acids were identified. The highest contents of these fatty acids were palmitic acid (29.50%), oleic acid (23.51%), stearic acid (9.13%) and linoleic acid (7.18%). Total phenolic value of plant extract was 11.94 mg/g, total flavonoid amount 2.28 mg/g, FRAP value 43.53 µg/g and DPPH value 1.63 mg/g. Antimicrobial activity experiment on a total of 11 microorganisms (seven bacteria and four yeast) showed that *Ajuga* extracts inhibited the growth of tested microorganisms except *Enterobacter cloacae*, *Candida glabrata* and *Saccharomyces cerevisiae*. *A. relicta* was found to have high bioactive content and antimicrobial activity. The plant extracts are rich in constant fatty acids and similar to olive oil (palmitic-oleic-linoleic), which is well known for its health benefits in terms of major fatty acids.

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1. INTRODUCTION

Plants are generally unique sources of new drugs that play an important role in the treatment of human diseases. Infectious diseases are one of the most important problems the communities face all over the world. Because of the adverse side effects of synthetic drugs and the emergence of antibiotic-resistant bacteria, new natural compounds with broad activity against bacterial strains are required. Plant secondary metabolites are excellent candidates for developing new phytopharmaceuticals with various biological activities. However, herbal

*CONTACT: Nazan Comlekcioglu ✉ noktem80@gmail.com Kahramanmaras Sutcu Imam University, Faculty of Science and Letters, Department of Biology, Kahramanmaras, Turkey

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medicines have not been scientifically researched enough. It is often applied on the basis of knowledge and experimental observations of traditional healers [1]. In this context, new investigations on medicinal plants or herbal products, which traditionally used but not scientifically researched, will greatly, contribute to the development of new herbal medicines or herbal formulations [2].

Species belonging to Lamiaceae family are known for their biological activities and especially their antioxidative properties. Lamiaceae is represented by 236 genera and 7200 species worldwide [3]. Lamiaceae is the third largest plant family in Turkey, 46 genera and 580 species are represented. Of these species, 260 are endemic and the endemism rate is approximately 45% [4, 5]. The *Ajuga* genus, a member of the Lamiaceae family, has more than 300 species of annual and perennial herbaceous flowering plants distributed in the temperate regions of Asia, Europe, Australia, North America and Africa [6]. *Ajuga* has 13 species (6 endemic) and 10 subtypes (1 endemic) in Turkey [4].

Several species of the genus *Ajuga* (Lamiaceae) are used in African and Asian folk medicine. These plants are used as folk remedies in the world for effective antihelminthic, tightening, antifungal, and anti-inflammatory agents as well as rheumatism, fever, toothache, dysentery, malaria, hypertension, diabetes and gastrointestinal disorders [7]. In addition to these features, this plant is used against eczema, tonic, menstrual diuretic, wounds and as an antidote against the bites of venomous animals in Turkey [8]. This study was done with *A. relicta*, which is known very little about it. The plant was first collected in 1907 from Kahramanmaraş Ahırdağı. But the plant is no longer seen in Ahırdağı. It is estimated that *A. relicta* has disappeared as a result of the elimination of moist habitats around 1830 m and heavy grazing [9]. *A. relicta*, an herbaceous perennial plant, is an endemic species. It is a plant belonging to the old glacial period and it is only grown in the world in Kahramanmaraş, Çimendağı/Yavşan Plateau. Recently, the demand for *Ajuga* species, which is considered to be an ornamental plant, has increased significantly in addition to its medical and pharmacological properties. Although the genus *Ajuga* has been widely studied, there is very little literature about *A. relicta*. There is no study about *A. relicta* in the literature other than systematic, steroids and terpenoids, morpho-anatomical study and antioxidant activity [5, 10-12].

The aim of this study was to investigate the antioxidant and antimicrobial activities, total phenol and flavonoid content of aerial parts of endemic *A. relicta*, whose taxa are in restricted areas and exhausted over the years. Fatty acid content of plant extracts was also investigated by using GC-MS. Although the antioxidant activity and total phenolic content of *A. relicta* has been previously investigated, but total flavonoid content, antimicrobial activity, fatty acid content has been investigated for the first time.

2. MATERIAL and METHODS

2.1. Plant materials

The plants used in this study were collected from openings in the forested areas of Çimendağı-Yavşan Plateau at an altitude of approximately 1500 m, on 20 July 2015 (Figure 1). Plants were identified according to Flora of Turkey [4]. A voucher specimen was deposited in the Herbarium of the KSU [YZK-980].

2.2. Sample Preparation and Extraction

The plants have been dried for a week (in a room temperature) and then powdered by grinding in a Waring blender. The extraction was performed by using Soxhelet apparatus at 60 °C for 6 hours with the addition of methanol (100 ml) on to 10 g of the plant material. After elimination of the solvent in a vacuum rotary evaporator at 40 °C, the extract was stored at -20 °C for further analysis [13]. Total phenolics, total flavonoids, antioxidant activity and

antimicrobial activity were analyzed using these obtained extracts. Total phenolics, total flavonoids, antioxidant activity and antimicrobial activity were also analyzed on this extracts.

Fig 1. Appearance of *A. relicta* in nature (a), flower (b).

2.3. Determination of ash and protein content

The ash content was analyzed according to the European standard method UNIEN 14775 [14]. Protein content of the samples was assayed by using AOAC method [15]. All experiments were done in triplicate.

2.4. Antioxidant Assay

2.4.1. *Determination of Total Phenolic and Flavonoid Content*

Total phenolic contents of the fractions were determined using the Folin–Ciocalteau colorimetric method [16]. The total flavonoid content in leaf extracts was determined spectrophotometrically [17]. All experiments were performed in triplicate.

2.4.2. *DPPH and FRAP Analysis*

Scavenging free radical potentials were analyzed using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) [18]. Ascorbic acid was used as positive control. The results were indicated as IC50 value which is the concentration of sample required to scavenge 50% of DPPH free radicals. The FRAP assay was carried out according to Benzie and Strain [19]. All experiments were done in triplicate.

2.5. Antimicrobial Assay

The antimicrobial activities were researched using the well-diffusion method. The test microorganisms were *Enterobacter cloacae* ATCC 13047D, *Escherichia coli* ATCC 39628, *Klebsiella pneumonia*, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538P, *Sarcina lutea* ATCC 9341NA, *Candida albicans*,

Candida parapsilosis, *Candida glabrata* and *Saccharomyces cerevisiae*. Mueller Hinton agar plates were cultured with standardized inoculums (10^8 cfu/ml) of each bacterial strain and also Sabouraud dextrose agar were cultured with each of yeast strains (2.1 x 10^3 cfu/ml) [20]. Extracts (50 µl) were added into wells and the plates were incubated at 37 ^oC for 16-18 h. After incubation, the diameter of inhibition zones was measured by a compass. DMSO was used as solvent control since it was used as a solvent for extraction. The plant extracts showing antimicrobial activity were then tested to determine the MIC values in a microwell plate [20].

2.6. Determination of Fatty Acid Content

Total 0.1 g of plant extract was mixed with 1 ml of KOH solution prepared with 2 N methanol and then vortexed for 2 min. After 15 minutes, 10 ml of hexane was added and the mixture was stirred well. After centrifugation at 7000 rpm for 10 min, 1 microliter of the upper phase was injected into the GC-MS device [21]. GC-MS analyses were quantified using a Shimadzu 2025 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu AOC-20i automatic sampler (Shimadzu, Kyoto, Japan). The condition of GC analysis was as follows: flame ionization detector (FID) 250 °C; column TR-CN100, 60 m × 0.25 mm × 0.20 mm (Teknokroma); carrier gas He with a flow rate of 1.5 mL/min. Fatty acid peaks were identified against the chromatogram of a mixed fatty acid methyl ester standard (37 Comp. FAME Mix 10 mg/mL in CH_2Cl_2; Supelco, USA). The injector and detector temperatures were kept at 250 °C. The column oven temperature was programmed at 80 °C for 2 min initially, then 5 °C/min up to 140 °C (maintained for 2 min at 140 ^oC), and then 3 °C/min up to 240 °C (maintained for 5 min at 240 ^oC). The injection and detector temperatures were set at 240 and 250 °C, respectively. The fatty acids were expressed as percentage of the total fatty acids, calculated with peak areas.

3. RESULT and DISCUSSION

3.1. Protein, ash, fatty oil content and fatty acid composition of *A. relicta*

The results of protein, ash and oil content in leaves of *A. relicta* ranged to 6.36%, 8.64% and 4.53%, respectively. Fatty acid composition in leaves of *A. relicta* is given in Table 1 and GC MS chromatogram is given in Figure 2.

21 fatty acids were found as a result of fatty acid analysis of *Ajuga* plant extract (Table 1). The major components in oil were palmitic acid (29.50 %), oleic acid (23.51 %), stearic acid (9.13 %), and linoleic acids (7.18 %). The researchers indicated that the need for saturated fats for energy, hormone production, cellular membranes and organs [22]. Total 50% of the fatty acids of *Ajuga* are saturated fats. Some saturated fatty acids are also necessary for essential signalling and stabilization processes in the body. Saturated fatty acids that play an important role in these processes are known as palmitic acid, myristic acid and lauric acid [23]. *A. relicta*, which contains all three fatty acids, has palmitic acid predominantly. The most commonly found and produced fatty acids in animal fats are palmitic, stearic and oleic acids [24, 25]. Additionally, C16:0, C18:0, and *cis*-9 C18:1 are typically the most abundant FA found in commercial fat supplements commonly fed to dairy cows. The increase in the the level of palmitic and oleic acid gave better results in terms of energy quality [25]. These two fatty acids are also the major fatty acids in *Ajuga* oil. Essential fatty acids that animals cannot produce and must take from outside are linoleic acid (LA) (omega-6), arachidonic acid (AA) (omega-6), gamma linolenic acid (GLA) (omega-6), alpha linolenic acid (LNA) (omega-3), eicosapentaenoic acid (EPA) (omega-3) and docosahexaenoic acid. (DHA) (omega-3). According to our result, *A. relicta* contains all of these fatty acids in different proportions.

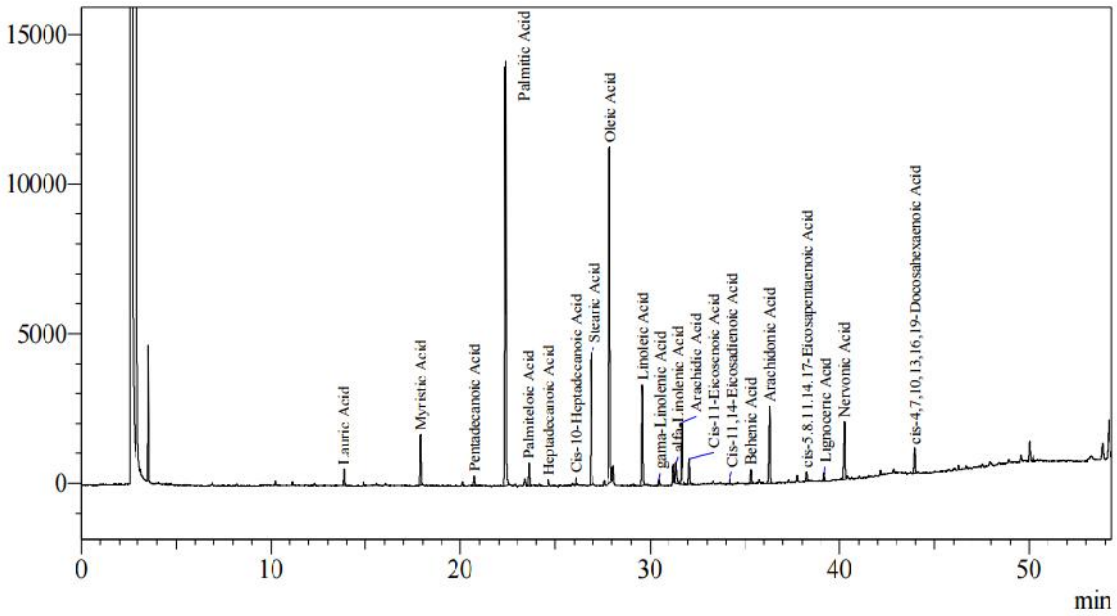

Figure 2. GC-MS chromatogram analysis of *A. relicta* extract

Table 1. Fatty acid compositions (%) of the *A. relicta*.

No	Carbon Number	Fatty Acids	Content (%)
1	C12:0	Lauric Acid	0.89 ± 0.02
2	C14:0	Myristic Acid	3.27 ± 0.01
3	C15:0	Pentadecanoic Acid	0.68 ± 0.01
4	**C16:0**	**Palmitic Acid**	**29.50 ± 0.03**
5	C17:0	Heptadecanoic Acid	0.38 ± 0.01
6	**C18:0**	**Stearic Acid**	**9.13 ± 0.01**
7	C20:0	Arachidic Acid	4.73 ± 0.01
8	C21:0	Behenic Acid	0.92 ± 0.00
9	C24:0	Lignoceric Acid	0.50 ± 0.00
10	C16:1	Palmitoleic Acid	1.60 ± 0.01
11	C17:1	Cis-10-Heptadecanoic Acid	0.48 ± 0.00
12	**C18:1**	**Oleic Acid**	**23.51 ± 0.01**
13	C20:1	Cis-11-Eicosenoic Acid	1.86 ± 0.00
14	C24:1	Nervonic Acid	4.46 ± 0.01
15	**C18:2**	**Linoleic Acid**	**7.18 ± 0.01**
16	C20:2	Cis-11,14-Eicosadienoic Acid	0.38 ± 0.00
17	C18:3	Gamma-Linolenic Acid	0.49 ± 0.00
18	C18:3	Alfa-Linolenic Acid	1.90 ± 0.00
19	C20:4	Arachidonic Acid	5.66 ± 0.01
20	C20:5	cis-5,8,11,14,17-Eicosapentaenoic Acid	0.69 ± 0.00
21	C22:6	cis-4,7,10,13,16,19-Docosahexaenoic Acid	1.79 ± 0.00
		SFA	50.0
		MUFA	31.91
		PUFA	18.09

3.2. Total phenolic, flavonoid contents and antioxidant activity of *A. relicta*

One of the most important points to be considered when working on the biological activities of plants is to elucidate the phytochemical composition. Herbal extracts consist of a mixture of very different phytochemicals. There is a strong relationship between phytochemical content and pharmacological potential of plants. One of the most studied and important phytochemical components of plants are phenolic compounds [26]. Various studies have shown that phenolic compounds are common in *Ajuga* species and may contribute to their antioxidant activity [2]. In this study, the total phenolic and flavonoid amounts of *A. relicta* extracts were determined in order to establish a relationship between the phenolic components and the biological activities of extracts. Phenolic, flavonoid and antioxidant activity properties were studied in *Ajuga iva* plant and methanolic extract showed the highest performance against hexane, chloroform and water extracts [27]. The researchers concluded that methanol is the most suitable solvent for the extraction of phenolic compounds and determination of antioxidant activity. Therefore, methanol was preferred as extraction solvent in this study. The results of antioxidant activity, total phenolic and flavonoid contents in *A. relicta* species are listed in Table 2.

Table 2. Total phenolic and flavonoid contents with antioxidant activity in the extracts of *A. relicta*

Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)	IC_{50} of DPPH% (mg dw/ml)	FRAP (µg AAE/g)
11.94 ± 0.66	2.28 ± 0.64	1.63 ± 0.13	43.53 ± 0.48

The total phenolic content of *A. relicta*'s methanolic extracts was 11.94 mg GAE/g dw and the total flavonoid content was 2.28 mg QE/g dw. In the literature, the total phenolic content and IC_{50} value of the methanolic extract of *A. relicta* reported as 34.6 mg GAE/g and 0.205 mg/ml, respectively [12]. Although plants were collected from the same location and in the same year, we have obtained a significant decrease compared to Sönmez and Köse [12] in terms of phenolic content and IC50 value. The decrease may be due to seasonal differences and vegetative maturity. Collection time of the plant in their study [12] was at the beginning of June while our collection time was at the end of July. Since the June is flowering stage, while the July is seeding stage of plant. The total phenolic content of *A. relicta* was found to be lower than the methanolic extract of *A. laxmannii* (56.76 mg GAE/g dw) [2]. The total phenolic content in flower portions of *A. reptans* and aerial fragments of *A. chamaecistus* subsp. *scoparia* were 20.86 mg GAE/g dw and 20.32 mg GAE/g dw, respectively [28, 29]. The total phenolic content of *A. relicta* was found be higher than *A. reptans* and *A. chamaecistus* subsp. *scoparia*. In previous studies, Toiu et al. [28] found a TFC value of 12.38 ± 0.22 mg RE/g dw for a methanol extract of *A. reptans* flowers. The flavonoid content of *A. laxmannii* was 36.14 ± 0.53 mg RE/g dw; water, ethyl acetate, methanol and acetone extract of *A. chamaepitys* L. Schreb TFC value 9.32 ± 0.33, 91.76 ± 0.81, 63.87 ± 0.66, and 61.77 ± 0.51 mg RE/g dw, respectively [2, 30].

Numerous test systems have been developed to determine the antioxidant activity of plant extracts. Each test system evaluates the antioxidant activity of the test material from a different perspective [31]. The best way to measure the antioxidant activity of a plant extract is to combine two or more complementary test systems. In this study, antioxidant activity of *A. relicta* extracts was evaluated using radical scavenging and reducing power analysis. DPPH analysis was used to determine the radical scavenging activity of the extract and FRAP analysis was used to determine the activity of reducing power (Table 2). According to the data presented in the table, the clearance activity of the extract on DPPH radicals was calculated as IC_{50} value

and was determined to be 1.63 mg dw/ml. In FRAP analysis, the FRAP activity of *Ajuga* extract was obtained as 43.53 µg AAE/g.

Phenolic compounds are considered to be functional bioactive compounds. According to the results of many studies, these compounds are also the main compounds that contribute to the antioxidant activities of plants [32]. The high antioxidant activity of *A. relicta* extracts can be explained by the high phenolic compound contents.

3.3. Antimicrobial activity of *A. relicta*

Most of the therapeutic agents used in disease treatment are obtained from plant sources. The number of plant species that are under investigation to reveal their therapeutic potential, have been increasing day by day [26]. Despite all these efforts, there are many plant species that have not yet been investigated for their biological and/or pharmacological potential. *A. relicta*, which is the subject of this study, is one of these plants. According to the results of our literature review, no data is available in the literature concerning the antimicrobial activity of *A. relicta*.

The antimicrobial effect of the methanolic extract of *A. relicta* was examined against seven bacteria and four yeasts. The results showed that the methanolic extracts of *A. relicta* have significant inhibitory activity against all tested bacteria except *E. cloaca*. The inhibition zones of the methanolic extract of *A. relicta*, which were obtained against all test bacteria, were in the range of 10-12 mm and 9 mm for tested fungi (Table 3). When the MIC values were examined, it was seen that *A. relicta* extract had higher inhibitory effect on *E. coli* than other bacteria. The highest inhibitory activity was determined against *E. coli*. On the other hand, the weakest inhibitory activity was determined against *E. faecalis, K. pneumonia* and *S. lutea*.

Table 3. The antimicrobial activity of *A. relicta* against test microorganisms

Test bacteria	Inhibition Zone (mm)	Gentamicin (mm)	MIC (mg/ml)
Bacillus subtilis ATCC6633	10	21	12.5
*E. Cloaca**	-	16	NT
*Enterococcus faecalis**	12	26	25
Escherichia coli 309628	12	24	6.25
*Klebsiella pneumonia**	11	28	25
*Staphylococcus aureus**	10	25	12.5
Sarcina lutea ATCC 9341NA	12	28	25
Test fungus		Nystatine (mm)	
*Candida albicans**	9	18	1.562
*Candida glabrata**	-	18	NT
*Candida parapsilosis**	9	18	12.5
Saccharomyces cerevisia	-	24	NT

*Clinical isolate, NT: Not tested, -: No inhibition zone

Among the 4 yeast strains, *C. parapsilosis and C. albicans* was inhibited by *A. relicta* extracts, while *Candida glabrata* and *Saccharomyces cerevisiae* were not affected. It could be an important property having an inhibitory and non-inhibitory activity against pathogenic and non-pathogenic strains, respectively, for food and pharmaceuticals. In terms of extraction method, although various the most promising results were obtained with the activity of *A. relicta* against *C. albicans* (MIC: 1.562 mg/ml). Around the world, so many plants were screened by many researchers with different methods against different microorganisms. Here in this study, the methanolic extract of *A. relicta* obtained was tested against common

microorganisms. As a result, these extracts seem to be reasonably effective against test organisms including clinical isolates. Plant phenolic compounds are known to be responsible for a variety of biological properties, including antimicrobial properties [27]. Therefore, it is thought that the antimicrobial activity of *A. relicta* extracts is related to the phenolic and flavonoid compounds of the plant.

4. CONCLUSION

In this study, phenolic and flavonoid content, antioxidant, antimicrobial activity and fatty acids of *A. relicta*, which is endemic and a relict species were evaluated. Other species belonging to the genus *Ajuga* spreads in 5 continents around the world and used for medical purposes among the population. *A. relicta* is a forgotten plant because of its naturally spread in a very narrow environment and also narrowing with various pressures. With this study, *A. relicta* was found to have high bioactive content and antimicrobial activity, as well as other members of the genus. It has been found that plant extracts show profile rich in constant fatty acids and show a profile similar to olive oil (palmitic-oleic-linoleic), which is well known for its health benefits in terms of major fatty acids.

Orcid

Nazan Çömlekcioğlu https://orcid.org/0000-0001-7729-5271
Yusuf Ziya Kocabaş https://orcid.org/0000-0003-2831-8910
Ashabil Aygan https://orcid.org/0000-0003-4936-9872

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

Quantitative analysis of Polydatin in a Turkish oak: *Quercus coccifera* L. with HPLC-DAD

Merve Yüzbaşıoğlu Baran [1,*], **Didem Şöhretoğlu** [1], **Ayşe Kuruüzüm Uz** [1]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, Sıhhiye 06100 Ankara, Turkey

Abstract: In this study, a new, simple, rapid and sensitive HPLC-DAD method was used for analysing polydatin contents of *Quercus coccifera* (Fagaceae) woody parts extracted with methanol and water. Our results showed that methanol and water extracts of *Q. coccifera* had high polydatin contents: 14.898±0.147 and 5.574±0.112 mg/g dry extracts, respectively. This is the first developed analytical method for qualitative and quantitative analysis of polydatin in *Quercus* L. species.

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Quercus coccifera L., Oak, Fagaceae, HPLC-DAD, Polydatin

1. INTRODUCTION

Plants have been a very important source of traditional medicine for years and continuously provided new therapies for humankind. Great efforts have been made to identify and quantify natural active ingredients from plants using various techniques. Resveratrol is now gaining much attention because of its antiaging, anticancer and antioxidant effects. Polydatin (PL), also named piceid, is a resveratrol glucoside (*trans*-resveratrol 3-*O*-glucoside) (Figure 1) isolated from *Polygonum cuspidatum* and other genera such as *Rosa*, *Rumex*, *Picea*, *Arachis*, *Malus* and *Quercus*. It is also detected in hop cones, red wines, hop pellets, cocoa-containing products, chocolate products and many daily diet components [1,2]. Enzymatic, microbiologic or chemical methods are used for transforming PL to resveratrol. *cis*-Resveratrol and *cis*-polydatin are typically found at lower concentrations and are often less biologically active than their *trans* forms. When compared to resveratrol, PL demonstrates better antioxidant activity and higher bioavailability. Therefore, PL stands out as an attractive compound to conduct more research on [3-5]. It has various biological activities such as antiinflammatory, antioxidant, antishock, anticancer, antimicrobial, neuroprotective, lung protective and hepatoprotective effects [1]. Chinese FDA approved PL for multiple phase II clinical trials mainly for antishock

*CONTACT: Merve YÜZBAŞIOĞLU BARAN merve.yuzbasioglu@hacettepe.edu.tr Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, Sıhhiye 06100 Ankara, Turkey

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applications [6]. In connection with promising results from bioactivity studies, interest in this compound increased.

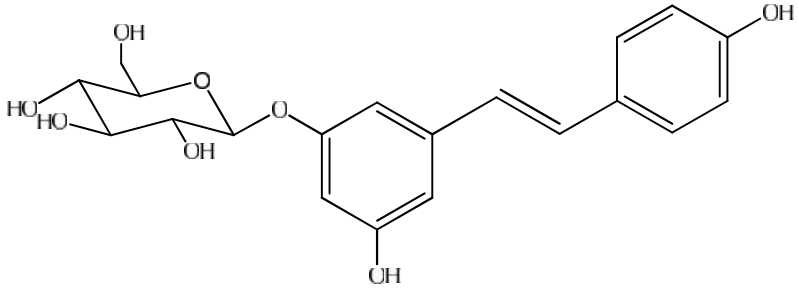

Figure 1. Chemical structure of Polydatin

The *Quercus* L. (Fagaceae) genus has 23 species naturally distributed in Turkey, of which four are endemic [7]. Intense research has been focused on *Quercus* sp. since it is used not only by humans and animals as food but also for its contribution to different industries including winemaking and wood. Barrels made from oak are usually used for wine-maturation process. Oak is used for colouring wood and protection against fungal decay. In Turkish traditional medicine, *Quercus* plants have been used as antiseptic, antidiarrheal, hemostatic, wound healing, and stomachic agent as well as remedy for poisoning from alkaloids, copper, lead and heavy metal salts [8,9]. So far, flavonoids, tannins, triterpenes, ionones, phenols, lignans and catechin derivatives were isolated from *Q. coccifera* L., which is native to the Mediterranean region of Anatolia and locally called as “kermes mesesi and pinar”. It is used for the treatment of diabetes and diarrhea. Furthermore, the decoction of this plant is used for burns is used for burns [9-11]. [9-11]. Previously, we also reported the isolation and structure elucidation of phytochemicals such as an ionone derivative, polydatin, lignans and a catechin derivative from the methanolic extract of *Q. coccifera.* Polydatin was one of the major compound in this extract and it was found for the first time in *Quercus* L. genus [11]. The aim of the current study is to determine the PL content of the *Q. coccifera* woody parts, which have already been identified as a new source of PL.

2. MATERIAL and METHODS

2.1. Chemicals

HPLC-grade acetonitrile was purchased from Merck Millipore, Germany. HPLC grade water was obtained from Milli-Q water purification system (18.2 MΩ/cm, Millipore), and analytical grade phosphoric acid was purchased from Merck, Germany. Polydatin (PL), which was used as the standard compound, was purchased from Sigma (15721)

2.2. Plant Material

Quercus coccifera L. (Fagaceae) was collected from Sertavul-Akçeşme between Mut and Konya (Turkey), near roadway at 1600 m in August 2008. It was identified by Prof. Zeki Aytaç (Department of Biology, Faculty of Sciences, Gazi University). A voucher specimen is deposited in the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 10003).

2.3. Extraction

Air dried and powdered woody parts (50 g) of *Q. coccifera* were extracted in two different ways. In the first method, same with the traditional usage, plant material was heated in distilled water (2 x 500 mL) at 100 ˚C for 3 hours to prepare a decoction and in the second one, it was extracted in MeOH (2 x 500 mL) at 45 ˚C for 3 hours under reflux. Both of the extracts were

dried in vacuo and lyophilized (Virtis-6KBTES) to obtain the water extract (4 g, 8%) and MeOH extract (3.58 g, 7.2%). Lyophilized extracts were kept at -20 ˚C.

2.4. Equipment and Chromatographic Conditions

The high performance liquid chromatography was performed using Dionex P680 HPLC system with photo-diode array detector (DAD). Samples were injected into the column filled with the stationary C18 (25 cm x 4.6 mm, 5 µm particles, I.D, ACE) with a pre-column (10x4 mm) and a Dionex ASI-100 autosampler at 30 °C. The data were acquired and processed by using Chromoleon 7.2, Thermo Fischer Scientific software. All the calculations concerning the quantitative analysis were performed with external standardization method by measuring the peak areas. Flow rate was set at 1 mL/min and column temperature was set to 30 °C. All the solvents were filtered through a 0.45 µm membrane filter before use and de-gassed in an ultrasonic bath. The injection volume was 20 µL. The mobile phase was a mixture of H_3PO_4 in pH:2.4 buffer system (solution A) and acetonitrile (solution B) with gradient elution. The composition of the gradient was (A:B), 95:5 at 0 min, 90:10 at 5 min, 80:20 at 10 min, 75:25 at 20 min, 70:30 at 30 min and 0:100 at 40 min. The chromatograms were recorded from 200 to 400 nm. Quantification was performed by measuring the areas under curve for PL in both of the extracts at 307 nm using a photo-diode array detector. The chromatographic run time was 40 min.

2.5. Method Development

Various gradient and isocratic methods were tested to detect PL using different literature data [16-20]. Isocratic 17% acetonitrile in 0.5% water formic acid [18] did not give baseline separation but peak splitting of compounds. The HPLC conditions were similar to those described by Lamuela-Ravento´s et al. [16] and Romero-Pérez et al. [17], however, a mixture of H_3PO_4 in pH:2.4 buffer system (solution A) and acetonitrile (solution B) with gradient elution was tried as mobile phase: 0 min, 95.0% A, 5.0% B; 5 min, 90.0% A, 10.0% B; 10 min, 80.0% A, 20.0% B; 20 min, 70.0% A, 30.0% B; 25 min, 60.0% A, 40.0% B; 35 min, 100% B. At the end of all trials, we decided to use our new method given in the HPLC conditions part above.

Sample Preparation: 5 mg water and methanol extracts were weighed and dissolved in 1 mL mobile phase.

Standard Stock Solution: 1 mg PL was dissolved in 1 mL mobile phase.

2.6. Method Validation

Linearity, accuracy and precision of the analysis were determined by using the ICH guidelines [21].

2.6.1. Linearity

An external standard method was utilized to construct the calibration curve. Six different concentrations of PL (1-100 µg/mL, *n*=6) were prepared in mobile phase. 20 µL of each standard solution was injected into the system for 6 times, and then the peak areas obtained from the injections were plotted against the concentrations to establish the calibration graph (Table 1).

2.6.2. Precision

The precision was evaluated by using intra-day and inter-day results. Standard PL was injected to the column at three different concentrations (25-50-100 µg/mL, *n*=6) on the same day for determining the intra-day precision. The same procedure was applied on two different days for inter-day precision.

2.6.3. Limits of Detection (LOD) and Limits of Quantification (LOQ)

The LOD and LOQ values were determined by the signal-to-noise (S/N) method, where an S/N ratio of 3 was used for LOD and 10 for LOQ. LOD and LOQ were experimentally verified by the six injections of reference compound at three different concentrations (25-50-100 µg/mL) [16].

2.6.4. Accuracy

Accuracy was evaluated by adding three increasing concentrations of standard PL (25-50-100 µg/mL) to both water and methanol extracts. Nonspiked extract samples (blanks) were used for calculating the percentage recovery at each concentration.

3. RESULTS and DISCUSSION

In this study, methanol and water extracts of the woody parts of *Quercus coccifera* L. (Fagaceae) which is used as water decoction in folk medicine, are investigated for PL content. This is the first report of HPLC-DAD analytical method developed for quantitative analyse of PL in *Quercus* L. species. This study reports the development of a new RP-HPLC method for the determination of PL in *Q. coccifera*. In conclusion, the quantitative evaluation of PL in *Q. coccifera* is improved with our simple, low-cost, sensitive HPLC method.

Polydatin demonstrated a good linear response (R^2>0.9987), and low LOD (6.8 µg/mL) and LOQ (20.8 µg/mL) values for the analyses within the linear range at 1-100 µg/mL concentration (Table 1). Retention time for PL in these conditions was determined as 16.5 min for HPLC-DAD chromatograms of methanol extract and water extract as given in Figures 2-5. The results of the precision analysis which were found about 1% for both intraday and interday indicated that the good reproducibility of our method (Table 2). The percentage of the mean recoveries of the extracts were in the range of 98.59-103.70% (water extract) and 100.87-102.36% (methanol extract) as seen in Table 3. The identification of PL in the extracts was based on the retention time and the comparison of UV spectra (Figure 6) with those of authentic standard. This method can be used for quantifying this compound in plant extracts and the products. Our results demonstrated that methanol and water extracts of *Q. coccifera* had high PL content: 14.898±0.147 and 5.574±0.112 mg/g (in dry extract), respectively. Compounds in water extract may have been hydrolyzed by this preparation method during boiling with water for 3 hours as used in folk medicine. The preparation method can explain the low amount of the compound (PL) in the water extract. Our results are the first records on the quantity of PL in *Quercus* L. genus. A number of studies reported determination of PL in different genus and food and/or products. For instance, in a previous study, the level of PL was highest (7.14 µg/g) in the cocoa powders [12].

Polydatin is a natural precursor of resveratrol, well known as an antioxidant. According to Hollman et al. [13], Paganga and Rice-Evans [14] absorption of some phenols from diet is enhanced by conjugation with glucose. Furthermore, PL content of *Quercus* is probably responsible for the high radical scavenging activities of *Q. coccifera* [15]. Due to its high PL content, *Q. coccifera* can be a potentially good source of antioxidant effect for food, pharmaceutical and cosmetic industries.

Table 1. Calibration parameters for the detection of polydatin

Linear equation	R^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
y=2.593x + 4.8678	0.9987	1-100	6.8	20.8

Table 2. Precision values of polydatin

Concentration (µg/mL)	*Intra-day precision (RSD, %)**	*Inter-day precision (RSD, %)**
25	0.225	0.179
50	0.019	0.248
100	0.909	1.222

*RSD %, percentage of relative standard deviation (*n*=6)

Table 3. Accuracy of the HPLC method for determination of PL in both *Q. coccifera* water and methanol extracts

Extract	*Initial content (µg/mL)*	*Recovery after 25 µg/mL added (%)*	*Recovery after 50 µg/mL added (%)*	*Recovery after 100 µg/mL added (%)*
Water	31.22	99.46	103.70	98.59
Methanol	95.35	100.87	102.00	102.36

4. CONCLUSION

Besides promising results obtained from bioactivity tests and clinical trials, being an option to overcome the bioavailabity problem of resveratrol, it is important to find new sources and quantification methods for polydatin. The novelty of the present study was to apply of HPLC techniques to find its quantification in a Turkish oak: *Quercus coccifera* L. We developed a rapid, simple, sensitive HPLC method for determination of polydatin.

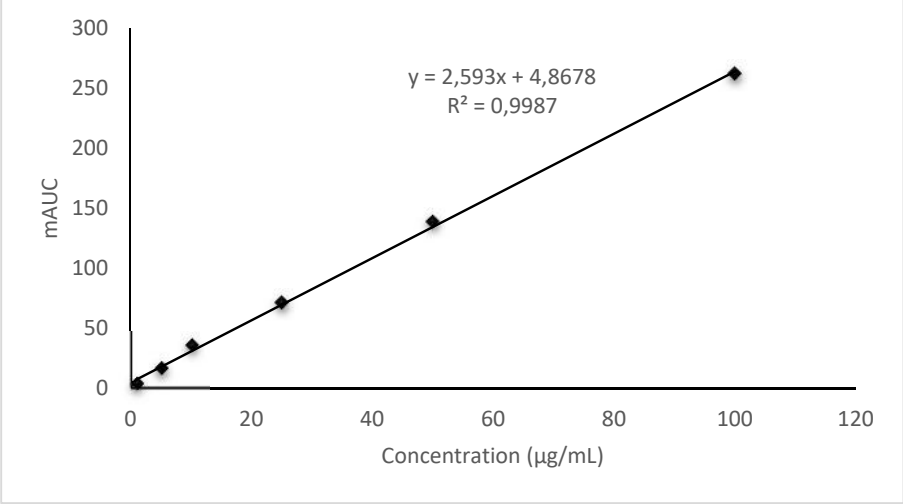

Figure 2. Calibration curve for polydatin

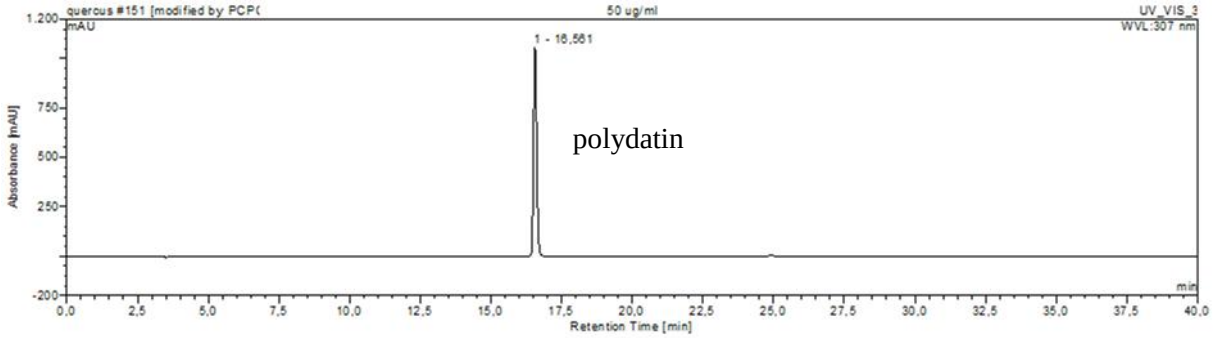

Figure 3. HPLC chromatogram of polydatin

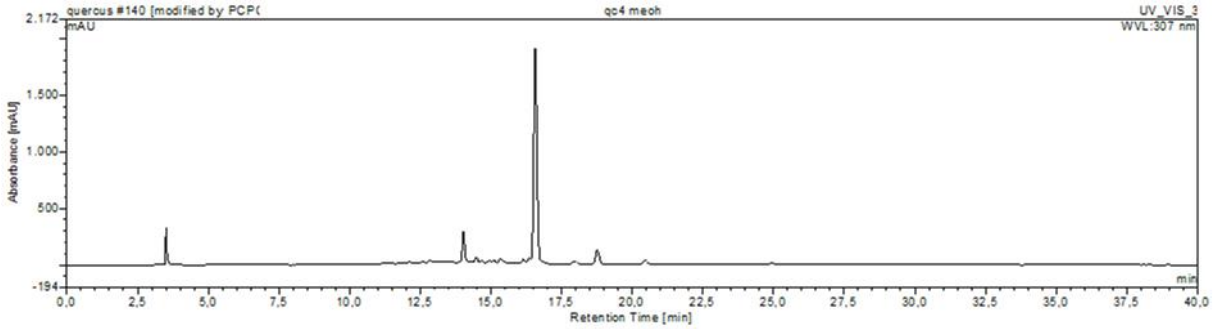

Figure 4. HPLC chromatogram of *Q. coccifera* MeOH extract

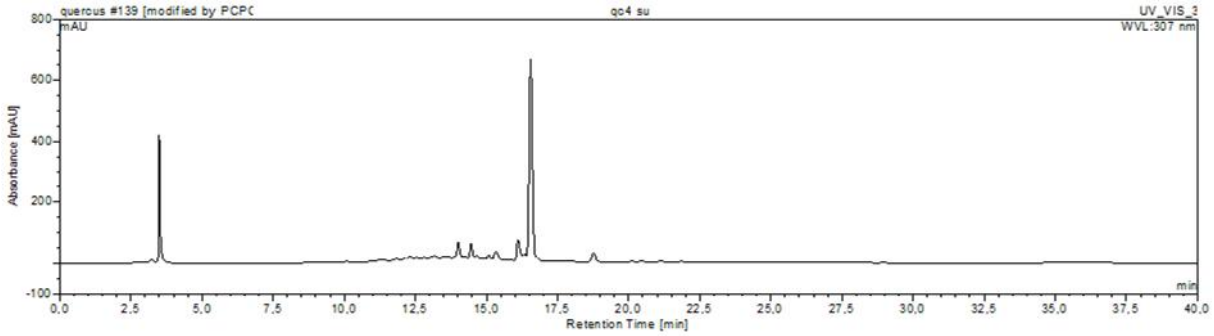

Figure 5. HPLC chromatogram of *Q. coccifera* water extract

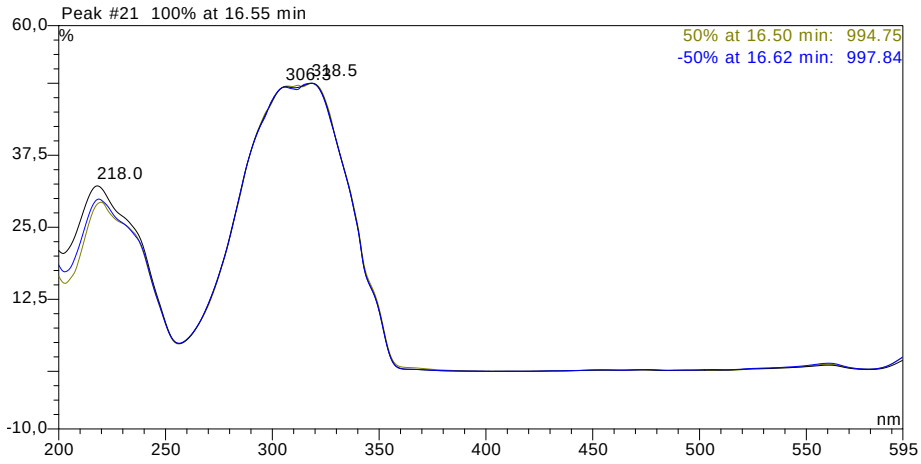

Figure 6. UV spectrum of polydatin in both *Q. coccifera* MeOH and water extracts

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Orcid

Merve YÜZBAŞIOĞLU BARAN: https://orcid.org/0000-0002-7482-0429
Ayşe KURUÜZÜM UZ https://orcid.org/0000-0003-3373-4759

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Effects of Methyl Jasmonate and Putrescine on Tryptanthrin and Indirubin Production in *in vitro* Cultures of *Isatis demiriziana* Mısırdalı

Özgür Karakaş *1

[1] Department of Agricultural Biotechnology, Faculty of Agriculture, Sirnak University, 73300, Idil, Sirnak, Turkey

Abstract: Tryptantrin and indirubin are pharmacologically active compounds used in treatment of disseases such as cancer and Alzheimer's. In this study, we investigated the influences of different concentrations of methyl jasmonate (MeJa) and putrescine (Put) on tryptanthrin and indirubin production in leaf explants and development of *Isatis demiriziana* Mısırdalı grown in vitro. In all media treated with methyl jasmonate, tryptanthrin production in leaves of plantlets showed an increase. The highest increase in tryptanthrin production was observed in solid Murashige-Skoog (MS) medium containing 1.0 mM MeJa (154.026 ± 0.11 µg g-1), about 2.85-fold higher than the control (untreated plantlets) (40.017 ± 0.031 µg g-1). Production of tryptanthrin decreased about 2.56-fold in the leaves of plantlets treated with Put, when compared to control. The highest indirubin production was obtained in the leaves of plantlets grown in the MS medium containing 0.1 mM MeJa (11.274 ± 0.035 µg g-1) but treatments with Put didn't show any positive affect on the indirubin production. Analysis of tryptanthrin and indirubin were performed using high performance liquid chromatography (HPLC).

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Isatis demiriziana

1. INTRODUCTION

Isatis demiriziana Mısırdalı is a biennial member of *Isatis* genus and an endemic to South-Eastern Anatolia Region of Turkey [1]. *Isatis* is herbaceous plants belonging to the family Brassicaceae (Cruciferea). The roots and leaves of *Isatis* have anti-viral, anti-inflammatory and anti-tumor effects and the leaves of these plants are used as a source of indigo (blue dye) [2-5]. Recently, a number of active constitutients have been isolated from plants belonging to the *Isatis* genus mainly *Isatis tinctoria* and *Isatis indigotica* such as terpenoids, phenylpropanoids and alkaloids. The indole alkaloids in *Isatis* have significant biological activities. Among these compounds, many reports are available about indole alkaloids such as indirubin and tryptanthrin [6,7]. Tryptanthrin has a wide range of biological activities and this natural product is a yellow and basic alkaloid. Moreover, tryptanthrin has been suggested as both a component of many dyes and medicinal herbal treatments [8]. Tryptanthrin has anti-cancer [9,10], anti-inflamatory [11] and anti-bacterial effects [12,13]. Indirubin is a red

CONTACT: Özgür KARAKAŞ okarakas80@gmail.com Department of Agricultural Biotechnology, Faculty of Agriculture, Sirnak University, Idil, 73300, Sirnak, Turkey

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coloured compound and isomer of indigo. In addition to the dye properties, due to its biological and pharmaceutical activities, indirubin has been used for the treatment of various diseases such as chronic myelocytic leukemia (CML) [14,15] and Alzheimer's [16].
Plant secondary metabolites are of a major importance as raw materials of drugs and dyes. In recent years, these products has gained great attention for their commercial importance and, many of these raw materials are produced from leaves and roots of the plants [17]. *In vitro* plant cultures have potential for large scale production of secondary metabolites. To increase the production of secondary metabolites via *in vitro* plant culture techniques have been used different ways. Among these ways, using of various biotic and abiotic elicitors has been effective to stimulate production of a series of important secondary metabolites [18-20]. Of these elicitors, Jasmonic acid (JA) and MeJa are important signalling agents in terms of promoting morphological and physiological changes in plants [21]. MeJa treatment can give rise to the accumulation of several classes of alkaloids [22], flavonoids and phenolics in some plants grown *in vitro* [23,24]. Polyamines (putrescine, spermine and spermidine) play an important physiological roles in plant growth and development [25]. Put is a precursor and stimulating agent for important alkaloids such as the pyrrolidine alkaloids (nicotine and nomicotine) in tobacco plants, pyrrolizidine alkaloids (retronecine) and some tropane alkaloids (hyoscine, meteloidine and hyoscyamine) possibly phenanthroindolizidines (tylophorine) [26,27].

To the best of our knowledge, this is the first report on the influences of MeJa and Put on tryptanthrin and indirubin production in *Isatis* species grown under *in vitro* conditions. In the current study, it was investigated the influences of different concentrations of MeJa and Put on tryptanth

2. MATERIAL and METHODS

2.1. Plant material and in vitro culture conditions

The mature seeds of *I. demiriziana* were collected in june 2014 from Diyarbakır-Ergani (1482 m above sea level), Turkey. Voucher specimens were deposited at the Herbarium of Dicle University, Faculty of Science (voucher no. DUF-6050). Specimens were identified by Prof. Dr. Ömer SAYA, from the same institution. Specimens were identified by. The seeds were washed thoroughly in tap water for 3 min and surface sterilized by dipping in a 70% ethanol solution for 60 sec, followed by immersion in a 6% sodium hypochlorite (NaOHCl) for 8 min, and then rinsed with sterile distilled water five times. After the sterilization stage, the seeds were placed on MS solid medium [28] supplemented with 0.6% agar and 3% sucrose prior to autoclaving at 1 atm, 121ºC for 20 min. The cultures were incubated under a photoperiod of 16 h light and 8 h darkness in a growth chamber at 25 ± 2 ºC. After 3 weeks initiation of cultures, plantlets produced from the germinated seeds were transferred to solid MS basal medium containing 0.6% agar and 3% sucrose for plant proliferation. Different concentrations of MeJa (0.05, 0.1, 0.25, 0.5 and 1.0 mM) and Put (0.5, 1.0 and 2.0 mM) were applied to three-week-old plantlets.

2.2. Reagents and chemicals

Tryptanthrin (≥98%), indirubin (≥98%), N,N-Dimethylformamide (DMF, ≥99.9%), MeJa (≥95%) and putrescine dihyrochloride (Put≥97%) were purchased from Sigma (St. Louis, Mo, USA). Methanol (≥99.9%) and acetonitrile (ACN, ≥99.9%) were purchased from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA, ≥99%) was purchased from Merck (Hohenbrunn, Germany).

2.3. Treatment and preparation of MeJa and Put

MeJa and Put were dissolved in methanol and then their stock solutions were prepared as 1 mg mL^{-1} and 5 mg mL^{-1} in methanol, respectively. 3-week-old plantlets were transferred to MS medium containing different concentrations of MeJa (0.05, 0.1, 0.25, 0.5 and 1.0 mM) and Put (0.5, 1.0 and 2.0 mM). The plantlets treated with MeJa and Put were harvested to determine influences of MeJa and Put on tryptanthrin and indirubin production in the leaves of *in vitro* grown plants after 12 days of treated with elicitors.

2.4. Calibration curves of tryptanthrin and indirubin

Standarts of tryptanthrin and indirubin were dissolved in DMF (1 mg mL^{-1}). The standard solutions of tryptanthrin and indirubin were prepared at seven different concentrations (0.05, 0.1, 0.25, 0.5, 1.0, 2.5; 5.0 µg mL^{-1}) by diluting their stock solutions with DMF. To generate calibration curves (Figure 1A-B), each standard solution was injected in triplicate. The concentrations of tryptanthrin and indirubin in the extracts were calculated using calibration curves of the compounds.

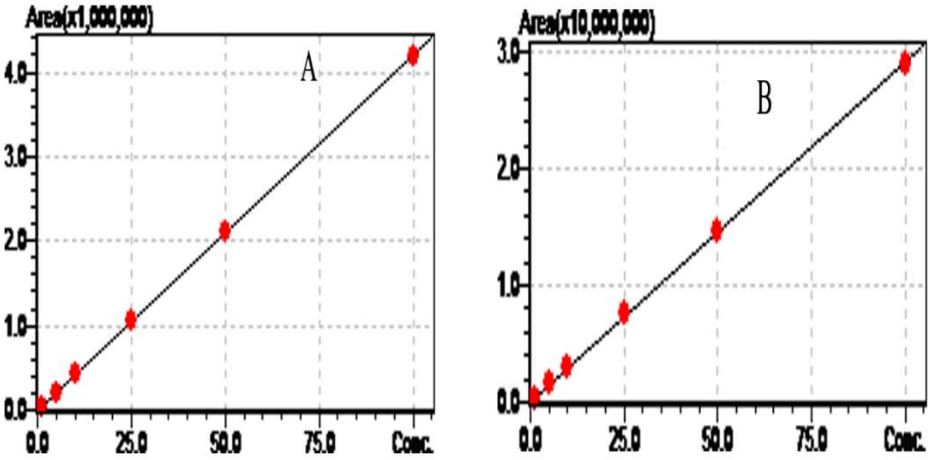

Figure 1. Calibration curves of tryptanthrin and indirubin. (A) Tryptanthrin; (B) Indirubin

2.5. Extraction of tryptanthrin and indirubin

It was used method developed by Liau et al., [29] for extraction. The leaves of plants were ground into powder using a laboratory blender. 0.2 mg of leaf sample was accurately weighed and extracted with 10 mL of methanol by ultrasonication (Jeiotech, US-05, Korea) for 5 min at 45 ^{0}C, in triplicate (10 mL x 3). Methanol in extracts was removed in vacuo (Labtech EV311, Evaporator), following metanolic extracts were combined. The final samples were dissolved in methanol of 5 mL and the final volume was adjusted to 40 mL with methanol. The solution was filtered through a 0.45 mm nylon filter membrane (Merck-Millipore® syringe filter) prior to analysis.

2.6. Analysis of tryptanthrin and indirubin using HPLC

Analysis was performed by using an HPLC system (Shimadzu Corporation, Japan) equipped with Inertsil ODS-3 C18 coloumn (5µm x 4.6 mm x 250 mm), LC-20AT pump, DGU 20A5R degaser, SIL 20A-HT autosampler and SPD M-20A PDA detector. The modified method of Zou et al. [4] was used to analysis of tryptanthrin and indirubin. The mobile phase consisted of water/acetonitrile 40/60 with 0.1% TFA for tryptanthrin and indirubin compounds. The mobile phase was filtered through a 0.45 mm filter and then degassed by ultrasonication. For both compounds, an isocratic elution profile was used, coloumn temperature was adjusted

to 30 ^{0}C and flow rate was 0.5 mL min^{-1}. Separation process was carried out at room temperature. Tryptanthrin and indirubin were detected at wavelengths of 305 and 275 nm, respectively. Retention times of reference compounds of tryptanthrin and indirubin were 14.9 and 13.4 min, respectively (Figure 2A-B). Injection volume was set as 20 µl. The correlation coefficients (*R*) of the standards were 0.9999 for tryptanthrin and 0.9997 for indirubin. Quantification of the two compounds were performed by comparing the retention time of the standards.

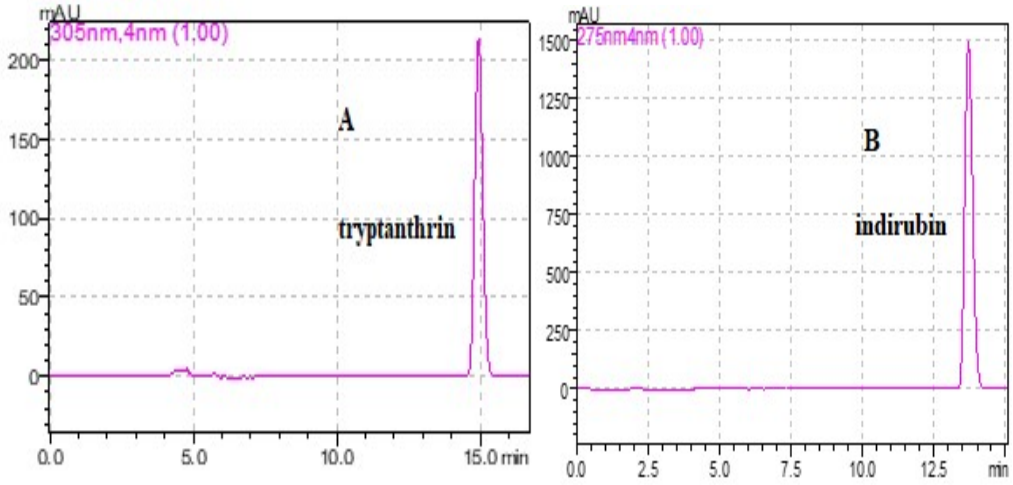

Figure 2. HPLC chromatograms of reference (standard) compounds. A. Tryptanthrin (100 ppm), B. Indirubin (100 ppm)

2.7. Statistical analysis

Statistical analyses were performed using SPSS Software Version 16.0 for Windows. Analysis of variance was performed using ANOVA. Duncan's multi-way range test was used for comparing of data. All data are expressed as the mean values ± standard deviation (SD). Differences were considered statistically significant at $p \leq 0.05$.

3. RESULTS and DISCUSSION

3.1. Influences of MeJa and Put on tryptanthrin and indirubin production

Table 1 shows the influences of MeJa and Put on tryptanthrin and indirubin production in the leaves of *I. demiriziana* grown *in vitro*. In the present study, I investigated the influences of MeJa and Put treatment on tryptanthrin and indirubin production in the leaf explants and development of *I. demiriziana* grown under *in vitro* conditions. Moreover, plantlets treated with MeJa and Put were compared to control plantlets (untreated plantlets). MeJa and Put treatment showed significant differences in production of tryptanthrin and indirubin. MeJa treatments were found to be more effective in production of tryptanthrin and indirubin. The retention times of tryptanthrin and indirubin compounds in the extracts were 14.9 and 13.4 min, respectively (Figure 3A-B, Figure 4A-B). The highest increase in tryptanthrin production was observed in the leaf explants of plantlets treated with 1.0 mM MeJa (154.026 ± 0.11 µg g^{-1}) and this value was about 2.85-fold higher than in the control samples (40.017 ± 0.031 µg g^{-1}). Tryptanthrin content of leaf explants in medium treated with 0.05 mM MeJa (43.922 ± 0.035 µg g^{-1}) was close to the *in vitro* control (40.017 ± 0.031 µg g^{-1}) but the amounts of tryptanthrin in MS solid media containing 0.5, 0.25 and 0.1 mM MeJa were 1.59, 1.06 and 0.97 times higher than that of the control, respectively.

All the MeJa treatments enhanced indirubin production, and the highest indirubin production was found in the leaf explants of plantlets treated with 0.1 mM MeJa (11.274 ±

0.035 µg g^{-1}) and this quantity was about 3.32-fold higher than in *in vitro* control plantlets (2.607 ± 0.027 µg g^{-1}). It was found that MeJa treatment enhanced about 6.5-fold vindoline (an indole alkaloid) production in shoot cultures *of Catharanthus roseus* compared to the control cultures [30]. The similar study showed that production of stemofoline alkaloids enhanced 1.42-fold in root extracts of *Stemona* sp. treated with 1.0 MeJa, when compared with control plants [31]. MeJa treatment induced the accumulation of dihydrosanguinarine alkaloid about 1.5-fold higher than in control [32]. Similarly, the another research was showed that adding of MeJa to cell cultures of *Catharanthus roseus* enhanced the production of ajmalicine alkaloid [33]. The results of our study were consistent with previous reports which indicated that MeJa induced the production of some important alkaloids in *in vitro* cultures.

As shown in Table 1, Put treatments did not show any affects on indirubin production compared to *in vitro* control plants and the differences between the quantities of indirubin in the leaf explants of plantlets treated with Put were statistically insignificant. Tryptanthrin production in leaf explants of plants treated with Put showed a decrease in all treatments but the highest decrease was observed in the medium treated with 0.5 mM Put (2.56-fold less than the control) compared to the control (Table 1). It was reported that Put treatment enhanced the content of capsaicin alkaloids in cell suspension cultures of *Capsicum frutescens* [34]. Similarly, it was found that Put treatment increased content of betalains in hairy root culture of *Beta vulgaris* [35]. However, Put adding to medium of transformed root cultures of *Datura stramonium* decreased the production of some tropane alkaloids [36]. As mentioned in the previous studies, Put treatment to the *in vitro* cultures revealed different results in the production of some important alkaloids. In the present study, we found that Put treatments significantly decreased the tryptanthrin production in *in vitro* shoot cultures of *I. demiriziana*. However, Put treatment did not affect on the indirubin production (Table 1).

Table 1. Influences of MeJa and Put on tryptanthrin and indirubin content (µg g-1) of leaf samples and survival percentage of *I. demiriziana* grown *in vitro*.

Treatment (mM)	Tryptanthrin content	Indirubin content	Survival percentage
In vitro control	40.017 ± 0.031f	2.607 ± 0.027e	100
0.05 MeJa	43.922 ± 0.035e	4.881 ± 0.093d	92.5
0.1 MeJa	78.947 ± 0.034d	11.274 ± 0.035b	87.5
0.25 MeJa	82.750 ± 0.032c	6.939 ± 0.019a	47.5
0.5 MeJa	103.751 ± 0.15b	4.047 ± 0.041c	20
1.0 MeJa	154.026 ± 0.11a	4.806 ± 0.026d	5
0.5 Put	11.226 ± 0.092g	2.481 ± 0.018e	100
1.0 Put	12.853 ± 0.010i	2.598 ± 0.025e	100
2.0 Put	15.572 ± 0.020h	2.657 ± 0.072e	100

Values expressed mean of three different experiments. A P-value of less than 0.05 were significant in terms of differences between data according to Anova multi-way Range Test. Meja; methyl jasmonate, Put; putrescine

3.2. Influences of MeJa and Put on appearance of plantlets

The treatment of different concentration of MeJa and Put had remarkable effects on development and survival percentage of plantlets in shoot cultures of *I. demiriziana*. The effect of elicitation on plant development was eveluated both morphologically and in terms of survival percentage.

As seen from the Figure 5A-B, plantlets in culture media supplemented with 0.05 mM MeJa and control medium were healthy and normal but in the remaining media had deformations in leaves and/or stems of plants in terms of morphological appearances (Figure 5C-F). Morphological appearances of plants treated with Put and grown in control medium

were normal and healthy at the end of 12 th day (Figure 5A). At the end of 12th day, significant differences were observed in both survival percentage and morphological appearance of plantlets grown in the media containing MeJa. Survival percentage of plantlets in medium containing 0.05 mM MeJa was 92.5%, in 0.1 mM MeJa was 87.5%. Plantlets grown in 0.25 and 0,5 mM MeJa were 47.5% and 20%, respectively and the majority of stems and leaves of plantlets grown in both media was observed drying. Among the media tested, the maximum loss was appeared in media containing 1.0 mM MeJa (5%) in terms of survival percentage of plants (Table 1). It was reported that the root biomass of *Panax ginseng* grown in suspension cultures treated with MeJa decreased 10% by day 9 [23] and MeJa treatment declined 20% on day 5 in hairy root culture of *Rehmannıa glutinosa* when compared with control cultures [37].

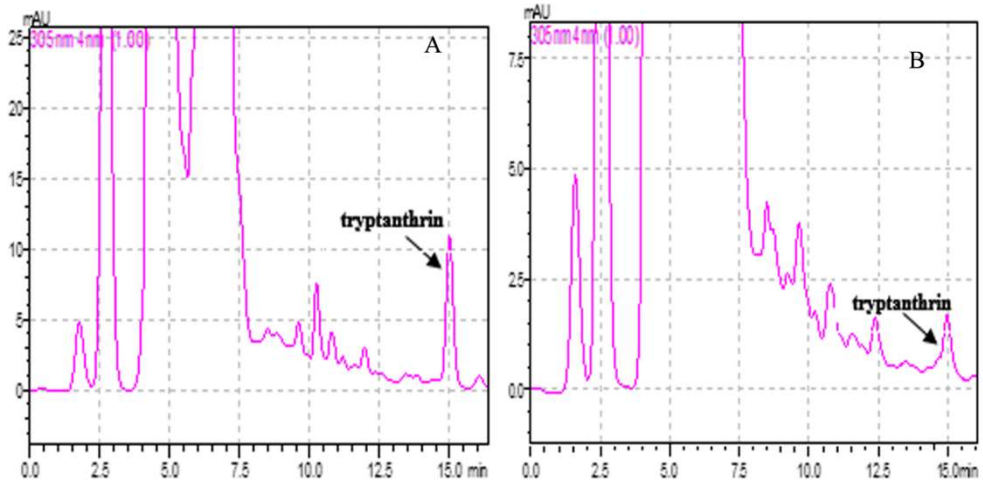

Figure 3. HPLC chromatogram of tryptanthrin. A. Treatment with 1.0 mM MeJa; B. Treatment with 2.0 mM Put. Peaks were enlarged for clarity

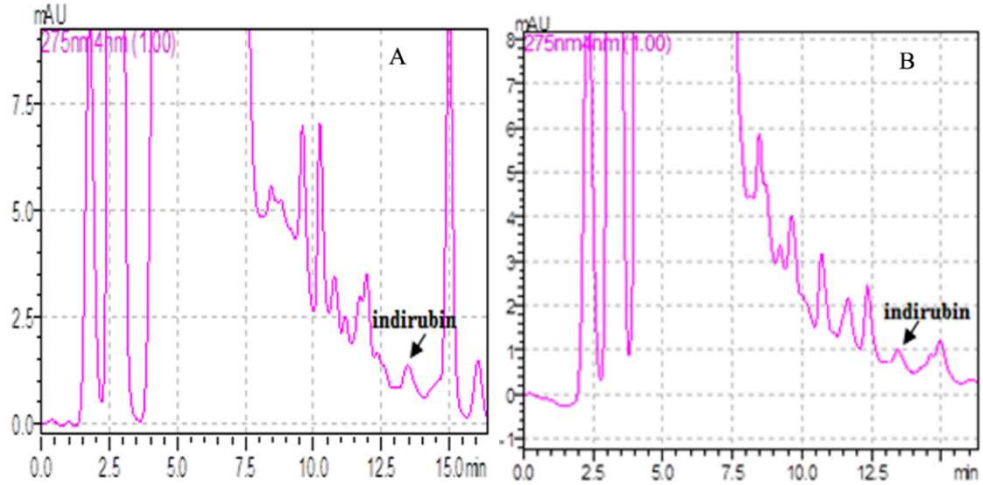

Figure 4. HPLC chromatogram of indirubin. A. Treatment with 1.0 mM MeJa; B. Treatment with 2.0 mM Put. Peaks were enlarged for clarity

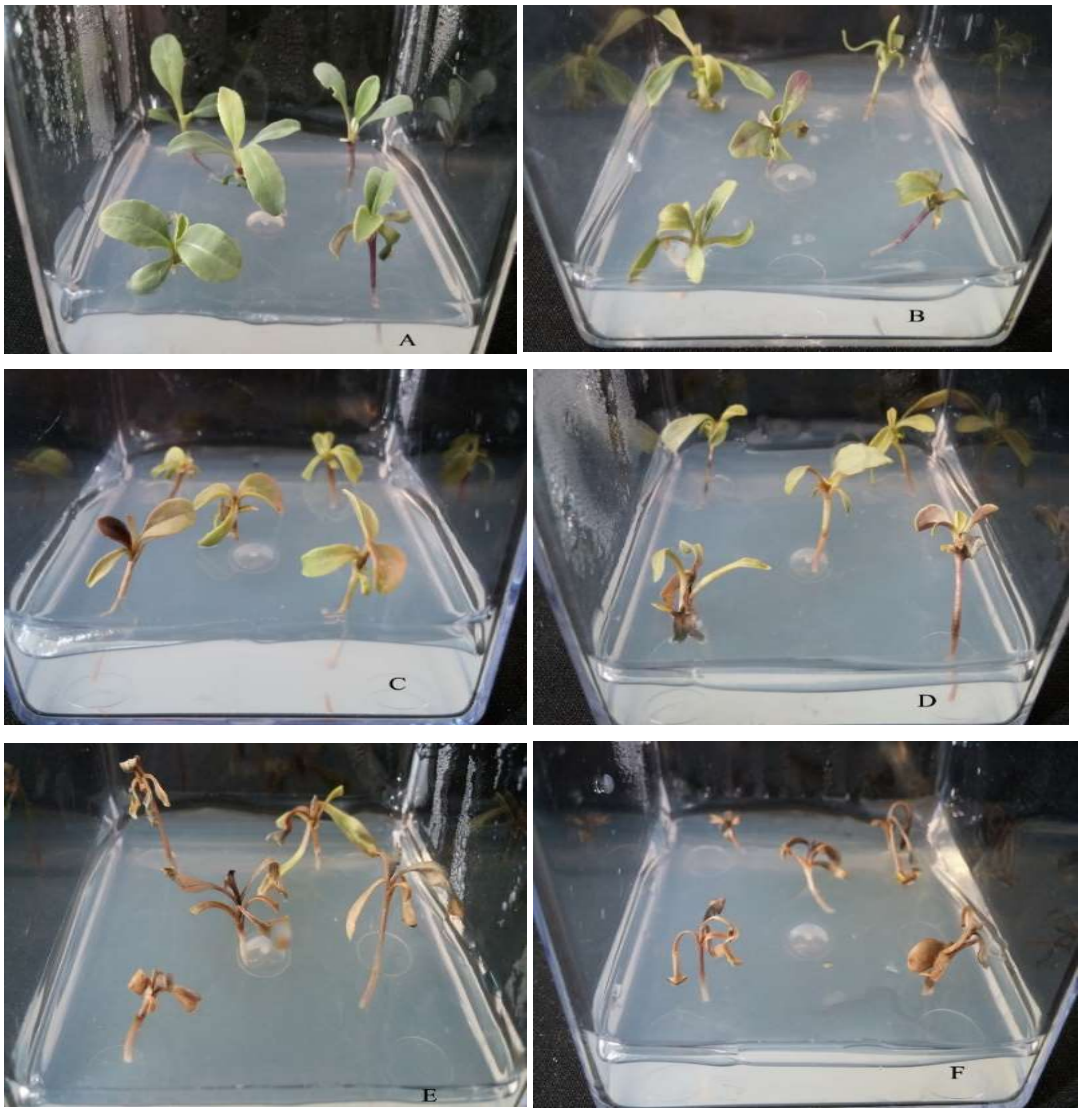

Figure 5. In vitro cultures of I. demiriziana treated with MeJa at different concentrations A. Control plantlets grown in vitro, B. 0.05 mM MeJa, C. 0.1 mM MeJa D. 0.25 mM MeJa, E. 0.5 mM MeJa, F. 1.0 mM MeJa

4. CONCLUSION

The present study demonstrated that the MeJa treatments significantly enhanced both tryptanthrin production (about 2.85 times more than that of control) and indirubin production (3.32 times more than that of control) in leaf explant of *I. demiriziana in vitro* grown. Put treatment reduced tryptanthrin production (1.57-2.56 times more less that of control) but it did not show any affects on indirubin production. Our study showed that adding of MeJa to *in vitro* culture media was an efficient way for the production of tryptanthrin and indirubin in *in vitro* conditions.

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Orcid

Özgür KARAKAŞ https://orcid.org/0000-0003-3339-4811

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Investigation of Physicochemical Properties of Some Monofloral Honeys in South Western Anatolia

Sukru Karatas [*1], **Abdurrahman Aktumsek** [2], **Mehmet Emin Duru** [3]

[1] Koycegiz Vocational School, Mugla Sıtkı Kocman University, Mugla, Turkey
[2] Department of Biology, Science Faculty, Selcuk University, Konya, Turkey
[3] Department of Chemistry, Science Faculty, Mugla Sıtkı Kocman University, Mugla, Turkey

Abstract: In this study, fourty monofloral honeys belonging to four sources (Chaste, thyme, citrus and heather) were obtained from South West Anatolia (Mugla, Aydin, Denizli and Antalya provinces). Firstly, pollen species of honeys were identified and categorized according to pollen concentrations. Then, physicochemical analyses of honey samples were carried out in terms of moisture, pH, free acidity, conductivity, diastase, proline and sugar profile. Physicochemical results of moisture values 15.04-19.52 %, density values 1.32-1.43 (g/cm^3), viscosity values 5.81-11.49 25 ^{0}C/Pa.s, ash content 1.32-1.43 %, pH values 3.74-4.78, free acidity values 8.96-33.92 meq/kg, conductivity values 0.15-1.41 µS/cm, diastase numbers 3.44-17.26 g/100g, proline contents 204.06-1588.93 mg/kg and sugar contents (glucose+fructose) 62.02-74.90 %. It was shown that the results obtained when compared to TFC (Turkish Food Codex) and CODEX Alimentarius Commission Honey Standards were in line with the standards.

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1. INTRODUCTION

Honey is probably known as the oldest natural sweetener product produced by honeybee (*Apis mellifera*) from different plants. Honey, a food product of natural sugars, is produced in almost all countries in the world and is widely consumed as a food source. Honey is highly preferred by consumers because of its wide range of uses such as nutritional quality, high energy values, sensory properties, and medical properties. However, honey cannot be considered to be exactly a food in the diet, but it can take part in nutrition as an additional nutritional supplement [1]. Honey is divided into the two main groups (flower and blossom), according to the source of nectar collected by honey bee. The general source of flower honey is the nectar of flowers and the source of blossom honey is from excretions of plant sucking insects (Hemiptera) in the living parts of plants or secretions of living parts of plants [2]. Honey has a high proportion of carbohydrates, 85-95% of which is composed of glucose and fructose. The fructose ratio of honey is higher than glucose. There are minerals, vitamins, enzymes and aroma in honey as well.

CONTACT: Sukru KARATAS ✉ sukrukaratas@mu.edu.tr Koycegiz Vocational School, Mugla Sıtkı Kocman University, Mugla, Turkey

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Melissopalynology analysis is the most widely used way to determine the honey's geographical and botanical origin [3]. If the honey consists of a single plant source or contains dominant pollen (>45%), it is called monofloral honey [1, 4, 5]. Monofloral honeys are characterized by their inclusion of parameters such as pH, water content, sugar, color and electrical conductivity [6-8]. The determination of these parameters gives us information about the origin of monofloral honeys, botanical type, freshness of honey and honey source [2].

South West Anatolia region, with high potential for production and export levels increasing day by day, is the biggest location in production of flower honey in Turkey. However, the physicochemical information which can give information about the commercial value of this region's trade and its medicinal use is very insufficient in Turkey.

The aim of this study is to determine the botanical origins and physicochemical qualities of the monofloral species supplied from the South West Anatolia region in Turkey.

2. MATERIAL AND METHODS

In this study, fourty monofloral honey samples belonging to four sources (chaste, thyme, citrus and heather) produced in South West Anatolia (Mugla, Antalya, Aydin and Denizli province) were collected from members of Honey Producer Association. The collected honey samples were coded in 850 mL glass jars and stored in dark and room conditions until the analyses were done. The codes of used honey samples in this study were given in Table 1. Firstly, pollen analysis of honey samples were made and categorized according to pollen concentration. Then, physicochemical analysis of honey samples was carried out in terms of moisture, pH, free acidity, conductivity, diastase enzyme, proline and total sugars in honey by following the standard methods of the Association of Official Analytical Chemists [9].

2.1. Determination of Water Content (Moisture)

The moisture content of the honey was determined by using the refraction index obtained at 20 ºC on the digital type refractometer (Mettler Toledo - RM 40) and using the moisture calculation chart [9].

2.2. Density

Honey samples' density was determined according to Bogdanov (1995) [10]. Consequently, it was calculated by comparing the density of the tape measure filled with 10 mL of honey to the density obtained by filling the same tape with distilled water.

2.3. Viscosity

50 g of honey sample was weighed and incubated for one hour at 25 °C in a water bath. The viscosity of honey samples was measured at 25 ± 0.5 ºC with digital thermostat rotational viscometer [11-13].

2.4. Ash content

Ash content in honey samples (AOAC, 1995) [9] was made according to the method. Accordingly, approximately 3 g of honey from each sample was weighed and burned in an ash oven at 900 °C, the residue was weighed on a sensitive balance and the ash content of the samples was calculated as a percentage.

Table 1. The codes, origin, region and harvest year of honey samples

Code No	Origin	Province	Town	Harvest Year
HC1	Chaste	Aydin	Cine	2017
HC2	Chaste	Aydin	Cine	2017
HC3	Chaste	Aydin	Cine	2017
HC4	Chaste	Aydin	Cine	2017
HK1	Chaste	Aydin	Kocarli	2017
HK2	Chaste	Aydin	Kocarli	2017
HS1	Chaste	Aydin	Soke	2017
HS2	Chaste	Aydin	Soke	2017
HS3	Chaste	Aydin	Soke	2017
KD1	Thyme	Mugla	Datca	2017
KD2	Thyme	Mugla	Datca	2017
KD3	Thyme	Mugla	Datca	2017
KD4	Thyme	Mugla	Datca	2017
KD5	Thyme	Mugla	Datca	2017
KK1	Thyme	Denizli	Tavas	2017
KK2	Thyme	Denizli	Tavas	2017
KU1	Thyme	Mugla	Ula	2017
KU2	Thyme	Mugla	Ula	2017
KU3	Thyme	Mugla	Ula	2017
NF1	Citrus	Antalya	Finike	2017
NF2	Citrus	Antalya	Finike	2017
NF3	Citrus	Antalya	Finike	2017
NF4	Citrus	Antalya	Finike	2017
NF5	Citrus	Antalya	Finike	2017
NK1	Citrus	Mugla	Koycegiz	2017
NK2	Citrus	Mugla	Koycegiz	2017
NK3	Citrus	Mugla	Koycegiz	2017
NK4	Citrus	Mugla	Koycegiz	2017
NK5	Citrus	Mugla	Koycegiz	2017
NK6	Citrus	Mugla	Koycegiz	2017
NK7	Citrus	Mugla	Koycegiz	2017
NK8	Citrus	Mugla	Koycegiz	2017
PC1	Heather	Aydin	Cine	2017
PC2	Heather	Aydin	Cine	2017
PD1	Heather	Mugla	Datca	2017
PD2	Heather	Mugla	Datca	2017
PD3	Heather	Mugla	Datca	2017
PK1	Heather	Aydin	Kocarli	2017
PK2	Heather	Aydin	Kocarli	2017
PK3	Heather	Aydin	Kocarli	2017

2.5. Pollen Analysis

The pollen experiment gives important information about the origin of honey. Pollen analysis was applied with the method adopted by International Beekeeping Authorities [16, 17, 18]. When pollen species of honey sample are examined, it is classified according to percentages. If the pollens concentration of a plant is more than 45% [15, 24], it is assumed that the examined honey consists of a single source [7].

The honey kept in a glass jar was first mixed with a glass bag and dispersed homogeneously. 10 g of this honey sample was transferred to a centrifuge tube and 20 mL purified water was added. This solution was centrifuged for 30 minutes at 4000 rpm and the solution pollen in the solution in the tube was thoroughly precipitated. The bottom of the precipitated honey was then transferred onto a slide. The slide was heated to 30-40 ºC and homogenous distribution of the pollen in the basic fucsin glycerin gelatin was achieved using a needle after the gelatin melt. The amount of pollen in the honey was determined with Olympus CX-31 brand microscope and the number of pollen in each preparation was calculated as a percentage. The fact that the pollens used in this study contain pollen densities of 45-94% indicates that they are monofloral [15].

2.6. Determination of pH and Free Acidity

pH measurement of honey samples was made with Mettler Toledo Seven Multi pH Meter. The titrimetric method was used to determination of free acidity. The titrimetric method: 10 g honey sample was dissolved in 75 mL of carbondioxide free-distilled water in a beaker and pH was recorded by means of immersing the pH electrodes in the solution. The obtained solution titrated with 0.1 N NaOH to pH 8.30 and also blind trial was made under the same conditions. The results were given as meq/kg [9].

2.7. Determination of Electrical Conductivity

The determination of the electrical conductivity of honey is principally based on the measurement of the electrical resistance. Measurements were made with the Mettler Toledo brand, Seven Multi model conductor. For the measurement of the conductivity, 20 g of honey was weighed in a 100 mL beaker. After dissolving with some distilled water, it was taken into a 100 mL volumetric flask and completed with distilled water. After taking 40 mL of this honey solution to a beaker, the temperature was adjusted to 20 ºC and measured by a conductor [9].

2.8. Diastase Activity

To determine the diastase number, 1 g of honey was put into 100 mL volumetric flask and 5 mL of samples were put into each of test tubes. The first test tube was placed in the acetate buffer only as a blind test and the Phadepas tablet was placed in each test tube and allowed to stay in a water bath at 40 ºC for 15 minutes. At the end of this period, 1 mL of a 0.5 M NaOH solution was added and the absorbance value of each tube was measured at 620 nm wavelength with UV-Spectrophotometer (Agilent, Cary-60 model) in 1 cm light path cuvettes [9].

2.9. Proline

The protein content of honey is usually determined by the proline specification and the spectrophotometric method is used to determine the amount of proline [9]. The reaction of proline was made with 3% ninhydrin in a formic acid medium and absorbance of the result product was measured spectrophotometrically at 510 nm wavelength (Agilent, Cary-60 model).

2.10. Sugar Analysis

Honey's glucose and fructose ratio and amount of sucrose were determined by means of sugar analysis. The sugar analysis was applied according to method specified by AOAC, 2005 [9]. Measurements were made on HPLC (Agilent-1200 Infinity) instrument. In accordance with

the method, 5 k silica gel filled column was used. Refractive Index Detector (RID) was used as the detector. HPLC operating conditions are shown below.

Column	: Zorbax (NH_2) 4.6x250mm 5µm (Agilent 880952-708)
Mobile Phase (Isocratic system)	: (CH_3CN:H_2O) (83:17,v/v) for analytical chromatography
Flow rate	: 1 mL/min
Column temperature	: (30 ºC)
Detector (RID) temperature	: (35 ºC)
Injection volume	: 10 µL

3. RESULTS and DISCUSSION

3.1. Moisture Content

The amount of moisture in honey is the best indicator of the honey's maturity and shelf life. The amount of moisture in honey depends on environmental conditions and manipulation during the harvesting period. It may also vary with season and year [21]. If the moisture value of honey is high, it indicates that honey is harvested before maturation [2]. In this study, the values of moisture content were found between 15.04% and 19.00% range in chaste honey samples, between 15.16% and 19.12% range in thyme honey samples, between 16.12% and 18.76% range in citrus honey samples and between 16.24% and 19.52% range in heather honey samples (Table 2a, 2b, 2c and 2d). When the total of 40 honeys was considered, the minimum moisture value was found to be 15.04% and the maximum moisture value was found to be 19.52%.

The values of moisture content of our samples were found to be under the upper limit of 20%, previously reported for different kinds of floral honeys [23].

3.2. Density

The density of honey is an important physical property affecting the stratification in honey. The honey's density is slightly higher than that of water, although it depends on the moisture content of the honey [24]. The core densities of 4 different honey subject to this study are in the range of 1.32-1.43 g/cm^3. When the average of the core density of each monofloral honey is calculated; respectively, 1.37 in chaste honey, 1.38 in thyme honey, 1.36 in citrus honey and 1.36 (g/cm^3) in heather honey. These values meet the 1.43 (g/cm^3) limit set by the TFC and CODEX Alimentarius Standards (2001) [4, 30].

3.3. Viscosity

Viscosity is defined as the resistance of liquid molecules to flow due to frictional force. Honey is a food with high viscosity due to its components and low moisture content. As the temperature increases, the viscosity of honey decreases [11, 12]. In a study investigating the physicochemical properties of honey, it was reported that the viscosity of pine, fir, cotton, orange, sunflower and thyme honey ranged between 2.54-23.4 (P.as) [13]. In another study of the various condiments produced in Turkey, viscosity values of results obtained for samples of 1.77-(11:38) to range Pa.s range, the lowest of clover honey, while the cedar honey has been reported to have the highest viscosity [12]. In this study, the viscosity results of the honeys we studied are in agreement with the results given in the literature.

3.4. Ash Content

As can be seen Table 2a, 2b, 2c and 2d, the ash content of the honey we work on varies between 0.09% and 0.72% by weight. The lowest amount was calculated in citrus honey, while the highest ash content was calculated in purple honey. These values are consistent with the data in the literature [14].

3.5. Pollen Analysis

In this study, density of pollen were found between 47% and 85% range in chaste honey samples, between 48% and 94% range in thyme honey samples, between 48% and 92% range in citrus honey samples and between 50% and 90% range in heather honey samples.

According to the obtained results has been proved that all the used honey in the study shows the monofloral honey feature (more than 45% dominant pollen). The pollen concentrations of the honey samples and microscopic images of pollen types in this study are given in Table 1 and in Figure 1.

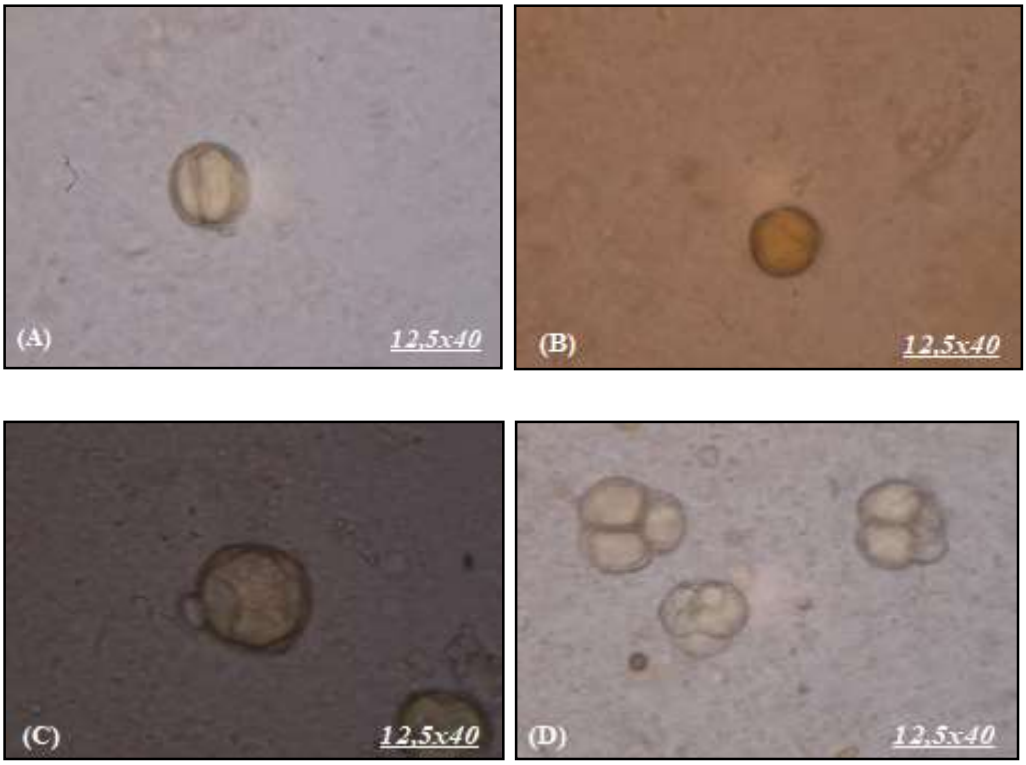

Figure 1. Microscopic Images of Pollen Types (A) Chaste (Verbenaceae-*Vitex agnus-castus* L.); (B) Thyme (Lamiaceae-*Thymus subs*.); (C) Citrus (Rutaceae-*Citrus subs*.); (D) Heather (Ericaceae-*Erica subs*.)

3.6. pH and Free Acidity

The amount of acid contributes particularly to the unique taste of monofloral honeys. Having a low pH of honey is important because it prevents the presence and growth of microorganisms in the honey. Having a low pH allows the honey to blend easily with other known food products [26, 27].

In this study, the pH values of chaste honey samples were found to be between 3.80 and 4.23 range, between 3.96 and 4.75 range in thyme honey samples, between 3.74 and 4.59 range in citrus honey samples and between 3.83 and 4.78 range in heather honey samples (Table 2a, 2b, 2c, 2d). The pH values of the studied honey samples ranged from 3.74 to 4.78 range. These values are in accordance with acceptable range for honey [1] and similar to those obtained with others Turkish honeys [28].

The free acidity of chaste honey samples was ranged from 14.83 to 26.91 meq/kg, thyme honey samples from 19.88 to 33.92 meq/kg, citrus honey samples from 8.96 to 27.92 meq/kg, heather honey samples from 15.95 to 31.82 meq/kg. According to CODEX Alimentarius, the

free acidity value of a honey cannot be more than 50 meq/kg of the high-quality honey, indicating the absence of undesirable fermentation. The obtained results of free acidity are given in the Table 2a, 2b, 2c and 2d and found to be compatible with CODEX Alimentarius [4] and agreement with data reported for Silva et al. [9].

3.7. Electrical Conductivity

Electrical conductivity has an important role in determining the source of honey. The electrical conductivity of honey is related with organic acids, proteins, sugars and mineral substances in the honey [7, 27]. According to the TFC, the conductivity values of the honeydew honey should be above 0.8 µS/cm and the conductivity values of the flower honeys should be below 0.8 µS/cm. If the flower honey is mixed with the honeydew honey, the conductivity values may be above 0.8 µS/cm.

The electrical conductivity results of analyzed honey samples are given in Table 2a, 2b, 2c and 2d. The electrical conductivity of chaste honey samples were ranged from 0.27 to 0.76 µS/cm, thyme honey samples from 0.82 to 1.50 µS/cm, citrus honey samples from 0.15 to 0.71 µS/cm, heather honey samples from 0.83 to 1.41 µS/cm. When the obtained results are evaluated, chaste and citrus honeys are found below 0.8 µS/cm and above thyme and heather honeys 0.8 µS/cm. The reason why thyme and heather honeys are greater than 0.8 µS/cm, is that there are thyme species of *Thymbra spicata, Origanum onites* and *Thymus cilicicus* in the area where thyme samples are collected. These three types of thyme are also consumed as spices and tea. There are intense pine trees in the area where thyme and heather plants are found. Since the collection of samples coincided with the early pine bark period, the sporophyte obtained by the Marcellina bug was observed in the palynological analysis carried out in small quantities except pollen. The mixing of these secretions with thyme and heather honeys caused an increase in the conductivity level.

3.8. Diastase Number

The diastase number is important for the quality of honey because the diastase enzyme is the honey bee transits from its body to the nectar during processing. Diastase is an enzyme secreted from the bottom of the honey bee and plays a role in the digestion of starch [29]. According to CODEX Alimentarius standards, the minimum diastase number should be at least 8. Despite this, the minimum diastase number of low natural enzyme content e.g. citrus honeys should be at least 3 according to the TFC [30] and Kuc et al. [31].

The results of the lowest diastase number chaste, thyme, heather and citrus honey were found to be 8.06, 8.11, 8.07, 3.44, respectively. When the results obtained are evaluated, it was found that these values comply with previous studies [31, 32] and worldwide accepted standards (CODEX Alimentarious and TFC). Diastase numbers of honey samples were measured and the values found are given in Table 2a, 2b, 2c and 2d.

3.9. Proline

The honey contains 20 kinds of amino acids. Proline is the most widely present amino acid in honey, in comparison with other amino acids. The amount of proline in the honey is indicative of the purity of honey. The level of the proline is more closely related to the performance of the bees [33, 27].

The values of proline content are given in Table 2a, 2b, 2c, 2d and it is found that thyme honeys are 1588.93 mg/kg higher than other monofloral honey samples in terms of proline amount and the citrus samples were found to have the lowest proline content of 204.06 mg/kg. According to TFC and Bogdanov et al. [34], minimum acceptable value proline content for genuine honey is 180 mg/kg in control laboratories. The obtained data of our studies are in accordance with the TFC and the other studies.

Table 2a. Physicochemical Results of Chaste Honey Samples

Code No	Moisture (%)	pH	Free acidity (meq/kg)	Conductivity (µS/cm)	Diastase	Proline (mg/kg)	Polen density (%)	Viscosity (25°C/Pa.s)	Ash content (%)	Density (g/cm^3)
HC1	18.08	4.02	18.89	0.76	12.51	859.59	55	7.39	0.31	1.34
HC2	17.16	4.23	22.85	0.73	14.77	772.36	47	8.06	0.27	1.36
HC3	16.90	3.86	17.87	0.28	17.26	816.95	72	8.42	0.19	1.38
HC4	18.56	3.87	20.95	0.51	8.06	643.89	80	7.15	0.34	1.33
HK1	15.40	4.16	24.71	0.32	9.38	515.29	85	8.83	0.28	1.42
HK2	19.00	3.97	26.91	0.49	9.18	460.44	60	6.62	0.36	1.32
HS1	18.52	3.99	16.80	0.38	10.80	457.11	68	7.11	0.23	1.33
HS2	16.40	3.80	14.83	0.27	10.05	508.77	48	8.56	0.35	1.39
HS3	15.04	4.03	16.92	0.35	10.97	523.39	51	9.07	0.22	1.43

Table 2b. Physicochemical Results of Thyme Honey Samples

Code No	Moisture (%)	pH	Free acidity (meq/kg)	Conductivity (µS/cm)	Diastase	Proline (mg/kg)	Polen density (%)	Viscosity (25°C/Pa.s)	Ash content (%)	Density (g/cm^3)
KD1	17.16	3.96	21.84	0.82	8.11	682.50	60	9.88	0.27	1.37
KD2	16.76	4.31	27.92	1.22	8.76	912.47	78	10.45	0.18	1.38
KD3	16.04	4.04	27.82	0.81	8.94	1110.08	90	10.63	0.31	1.39
KD4	15.53	4.57	21.82	1.07	11.82	588.65	70	10.97	0.20	1.42
KD5	16.87	4.74	19.88	1.50	15.40	470.13	55	10.37	0.26	1.38
KK1	15.60	4.17	28.68	0.92	11.22	1244.16	50	10.95	0.33	1.42
KK2	17.68	4.55	22.91	1.17	9.73	744.80	85	9.15	0.19	1.36
KU1	17.68	4.31	28.89	0.96	11.74	810.66	53	9.18	0.21	1.36
KU2	19.12	4.75	21.88	1.27	8.66	761.65	94	8.35	0.17	1.32
KU3	15.16	4.43	33.92	1.31	8.25	1588.93	48	11.49	0.25	1.43

Table 2c. Physicochemical Results of Citrus Honey Samples

Code No	Moisture (%)	pH	Free acidity (meq/kg)	Conductivity (µS/cm)	Diastase	Proline (mg/kg)	Polen density (%)	Viscosity (25°C/Pa.s)	Ash content (%)	Density (g/cm^3)
NF1	17.24	3.74	12.88	0.23	7.44	340.58	48	6.92	0.11	1.36
NF2	17.04	3.75	15.88	0.24	7.01	435.05	55	7.03	0.17	1.37
NF3	16.12	3.99	10.88	0.20	3.60	205.09	95	8.14	0.15	1.40
NF4	16.20	4.59	8.96	0.71	5.02	300.76	60	8.09	0.09	1.39
NF5	17.26	4.07	10.99	0.19	3.62	216.38	92	6.93	0.19	1.36
NK1	16.97	4.32	11.92	0.45	4.28	315.47	80	7.72	0.13	1.37
NK2	16.97	4.45	11.91	0.56	4.10	325.05	77	7.74	0.15	1.37
NK3	18.24	4.24	23.72	0.68	6.29	541.13	50	5.96	0.12	1.34
NK4	17.16	4.16	10.87	0.27	4.01	357.33	87	6.95	0.14	1.36
NK5	18.72	4.23	11.97	0.26	3.44	231.84	48	5.87	0.18	1.33
NK6	17.60	3.93	20.91	0.29	6.24	633.26	65	6.75	0.20	1.36
NK7	18.76	4.13	27.92	0.58	11.22	984.96	72	5.81	0.14	1.33
NK8	17.26	4.14	10.88	0.15	5.45	204.06	60	9.94	0.16	1.38

Table 2d. Physicochemical Results of Heather Honey Samples

Code No	Moisture (%)	pH	Free acidity (meq/kg)	Conductivity (µS/cm)	Diastase	Proline (mg/kg)	Polen density (%)	Viscosity (25°C/Pa.s)	Ash content (%)	Density (g/cm^3)
PC1	17.26	3.83	31.82	0.83	8.07	758.53	56	9.28	0.39	1.37
PC2	17.60	4.22	25.93	0.99	14.33	787.82	66	8.75	0.54	1.36
PD1	18.18	4.78	15.95	1.41	10.15	496.73	82	8.41	0.43	1.34
PD2	16.24	4.05	17.82	0.80	12.33	789.46	50	10.48	0.72	1.39
PD3	16.52	4.72	21.82	1.39	10.02	569.53	70	10.15	0.69	1.38
PK1	18.86	4.50	18.87	0.88	9,81	647.16	77	7.34	0.41	1.33
PK2	19.52	4.47	19.89	0.92	11.13	843.47	90	7.11	0.47	1.32
PK3	17.68	4.62	21.78	1.25	14.02	664.01	60	8.29	0.36	1.36

3.9. Sugar Analysis

The content of honey sugar depends on the botanical origin. The sucrose in the form of nectar is produced by the bees and is formed by enzymatic hydrolysis to provide glucose and fructose formation [35]. Glucose and fructose are the major constituents of honey. Fructose is always the most important sugar because fructose is sweeter than glucose. In our study, fructose concentration was higher than glucose concentration in all honey samples studied and also fructose/glucose ratio affects the taste of honey. According to CODEX Alimentarious and TFC, it was determined that the amount of sucrose should be at most 5 g / 100 g and the concentration of fructose and glucose in honey samples should be at least 60 %.

The results of our honey samples, the amount of sucrose was found to match the determined limits and it was determined that the glucose and fructose concentrations of all honey samples were higher than 60% and the sugar content (glucose + fructose) was between 62.02 and 74.90 % and the F/G ratio in the honey samples evaluated ranged between 1.1 and 1.5 (Table 3), which is close to the ratio found by de Sousa et al. [36] and TFC in honey samples.

Table 3. Results of Sugar Profile

Code No	Fructose	Glucose	F + G	F/ G	Sucrose
HC1	39.71	27.91	67.62	1.42	N.D.
HC2	41.82	28.21	70.03	1.48	N.D.
HC3	40.94	28.86	69.80	1.42	1.63
HC4	40.16	26.98	67.14	1.49	N.D.
HK1	41.20	28.61	69.81	1.44	1.83
HK2	40.33	27.82	68.15	1.45	N.D.
HS1	38.35	30.77	69.12	1.25	1.61
HS2	40.14	28.63	68.77	1.40	N.D.
HS3	40.19	27.78	67.97	1.45	N.D.
KD1	38.90	26.49	65.39	1.47	1.08
KD2	39.79	31.54	71.33	1.26	N.D.
KD3	35.72	28.62	64.34	1.25	N.D.
KD4	38.56	26.28	64.84	1.47	N.D.
KD5	37.39	24.63	62.02	1.52	N.D.
KK1	38.56	26.15	64.71	1.47	1.73
KK2	38.39	29.23	67.62	1.31	N.D.
KU1	39.25	26.44	65.69	1.48	N.D.
KU2	39.34	27.98	67.32	1.41	1.65
KU3	39.08	29.36	68.44	1.33	1.42
NK1	36.74	35.32	72.06	1.04	N.D.
NK2	37.25	32.32	69.57	1.15	N.D.

Table 3. Continues

NK3	37.89	32.23	70.12	1.18	2.17
NK4	37.19	33.27	70.46	1.12	1.99
NK5	39.43	32.89	72.32	1.20	N.D.
NK6	38.17	33.47	71.64	1.14	N.D.
NK7	37.29	33.31	70.60	1.12	1.47
NK8	39.47	34.32	73.79	1.15	1.65
NF1	38.45	33.92	72.37	1.13	N.D.
NF2	40.35	34.55	74.90	1.17	1.02
NF3	37.26	32.28	69.54	1.15	N.D.
NF4	38.16	29.89	68.05	1.28	N.D.
NF5	38.56	28.34	66.90	1.36	N.D.
PC1	34.23	28.71	62.94	1.19	1.99
PC2	38.57	27.34	65.91	1.41	1.39
PD1	39.19	26.34	65.53	1.49	1.08
PD2	40.78	27.57	68.35	1.48	1.61
PD3	40.47	27.63	68.10	1.46	1.73
PK1	38.63	26.59	65.22	1.45	1.67
PK2	39.56	26.81	66.37	1.48	1.81
PK3	37.91	28.42	66.33	1.33	1.83

4. CONCLUSION

According to the obtained results has been proved that all the used honey in the study shows the monofloral honey feature (more than 45% dominant pollen). Moisture content of the honey samples analyzed was found at the expected values. The moisture content of thyme honeys was found to be less than the other honey's moisture content. This value shows that the thyme honey will last longer than the other honey. Because the moisture content, in time, causes the acidity of honey to increase and therefore increases the hydrolysis event in honey.

pH and acidity values of honey samples were found within TFC limits. According to these results, it was determined that citrus honey had the least acidity value compared to other honey. In addition, the acidity value of honey honeys was higher. Honey's pH and acidity values provide important information about its quality and condition.

As briefly described in section 3.4, the reason for the conductivity value of thyme and Heather honey is greater than 0.8 µS/cm due to the geographical position and time of collection of the region. There are thyme species such as *Thymbra spicata, Origanum onites* and *Thymus cilicicus* in the area where thyme honey are collected. There are intense pine trees in the area where thyme and heather plants are found. Since the collection of samples coincided with the early pine bark period, the sporophyte obtained by the Marcellina bug was observed in the results of palynological analysis carried out in small quantities except pollen. The mixing of these secretions with thyme and heather honeys caused an increase in the conductivity level.

As a result of the analyzes, it was determined that the honeys included in the study had monofloral properties. The physicochemical results of all honey samples were found to be compatible with the Turkish Food Codex and CODEX Alimentarious Commission Honey Standarts.

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Orcid

Sukru KARATAS https://orcid.org/0000-0003-0348-1076

Abdurrahman AKTUMSEK https://orcid.org/0000-0002-5151-2650
Mehmet Emin DURU https://orcid.org/0000-0001-7252-4880

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Promoting effect of foliar silicon on steviol glycoside contents of *Stevia rebaudiana* Bertoni under salt stress

İlkay Yavaş [*1], **Fatih Mehmet Yılmaz** [1,2], **Aydın Ünay** [3]

[1] Department of Plant and Animal Production, Kocarli Vocational High School, Adnan Menderes University, Aydın, Turkey
[2] Department of Food Engineering, Faculty of Engineering, Adnan Menderes University, Aydın, Turkey
[3] Department of Field Crops, Faculty of Agriculture, Faculty of Agriculture, Adnan Menderes University, Aydın, Turkey

Abstract: This study investigated the effect of foliar silicon application on fresh weight and dry matter contents as well as steviol glycoside contents of stevia (*Stevia rebaudiana* Bertoni) grown under salt stress. In this respect, an experimental research with factorial arrangement on a Completely Randomized Design with four replications was conducted in plastic pots under greenhouse conditions. Four different salinity levels (0, 25, 50, and 75 mM NaCl) were used for three weeks old seedling and two different foliar silicone concentrations (12.5 and 25 mM) were applied in the form of sodium silicate which was launched after 4 weeks of planting. Both NaCl concentrations and foliar silicone application had statistically significant effect ($p \leq 0.05$) on fresh weight and dry matter characteristics and also on rebaudioside-A and stevioside contents of stevia leaves which were harvested before flowering period. Results showed that salt stress decreased both rebaudioside-A and stevioside contents, but foliar silicone application improve negative influences of NaCl treatments with even increasing the steviol glycoside contents. Rebaudioside-A content did not change, but stevioside content decreased with foliar silicone application in control conditions. However, highest rebaudioside-A and stevioside accumulation were recorded in 25 mM foliar silicone application under 50 mM NaCl stress. In these conditions, rebaudioside-A and stevioside contents were 112 and 26% higher than control, respectively. It could be concluded that the application of silicon significantly enhanced the plants ability to withstand salt stress conditions through increased silicon content, increased steviol glycoside production. Silicon application could therefore improve crop production under salt stress.

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1. INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a perennial medicinal plant belonging to Asteraceae family. *Stevia rebaudiana* contains high concentrations of steviol glycosides which give stevia a great importance, as an alternative source of table sugar [1-3]. Its leaves contain high sweetness (250-300 times greater than sucrose) due to the presence of glycosides diterpene [1,

CONTACT: İlkay YAVAŞ ✉ iyavas@adu.edu.tr Department of Plant and Animal Production, Kocarli Vocational High School, Adnan Menderes University, Aydın, TURKEY

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4]. Despite several subunits of steviol glycosides have been identified in the literature as stevioside (SV), rebaudioside A (RbA), rebaudioside B (RbB), rebaudioside D (RbD), rebaudioside F (RbF), steviolbioside (Stb), rubusoside (Rub), rebaudioside C (RbC) and dulcoside A (DuA) [5, 6, 7], stevioside and rebaudioside A emerge as the most dominant steviol glycosides among others. Stevia leaves also contain considerable amounts of other components including vitamin C, beta-carotene, niacin, magnesium, phosphorus, selenium and iron. Due to the non-caloric and sweetening properties, it is alternative to table sugar [8]. Stevia can be cultivated in semiarid climates like in Mediterranean region. Growth and yield reduction in stevia plants occur when salts accumulate in the roots due to the water stress [9]. Stevia is known to be very sensitive plant to salinity stress which affects stevia yield directly [2].

Salt stress is considered as one of the major abiotic stresses that can limit the plant growth and productivity similar to other abiotic stresses which lead to oxidative stress through the increase in reactive oxygen species [6, 10]. Salt stress affects several important processes such as growth, protein synthesis, lipid metabolism and photosynthesis by altering the ultrastructure of the organelles, chlorophyll content and enzyme activities involved in these processes [11]. Salinity stress, which suppresses plant growth and limits crop productivity, has been continually increasing worldwide.

Silicon (Si) is accumulated by plant roots and is transported to the stems and leaves, thus exhibits mitigating effects of abiotic stresses especially salinity and drought [12]. Plants belonging to Cyperaceae and Gramineae family accumulate silicon and a better development is provided by external application [13].

Based on all the given information, the objective of this research is to evaluate the effect of NaCl induced salt stress (1), to investigate a possible mitigating effect of silicon under salinity conditions on the dry matter contents (2) and the percentages of stevioside and rebaudioside A of *Stevia rebaudiana* Bertoni.

2. MATERIAL AND METHODS

2.1. Determination of fresh weights and dry matter contents

The fresh weights of the plants were measured directly after harvesting by using sensitive balance. Dry matter contents were determined using an oven operated at 50 °C until a constant weight was obtained (~48 hours) [14].

2.2. HPLC Analysis of Steviol Glycosides Extraction

A 2 g dried and milled stevia leaf was weighed in a covered extraction vessel and completed up to 100 ml with 50% EtOH solution. The mixture was extracted at 55 °C within a water bath for 60 min. After extraction, the mixtures were filtered with Whatman no:1 filter paper and supernatant was collected in falcon tubes. Finally, the supernatants were filtered through 0.45 µm PVDF filter and transferred to vials before injection into HPLC.

2.3. HPLC Analysis

HPLC analysis was carried out using the method suggested by Jentzer et al. [15] with some modifications. Sample extracts and standards (rebaudioside A and stevioside; 10 – 200 ppm) were injected into HPLC (Shimadzu Prominence LC-20A).

Column: Inertsil ODS-3 column; 250 mm x 4.60 mm x 5 µm (HICHROM. UK)
Detector: UV/VIS detector (Shimadzu SPD-20A)
Wavelength: 210 nm
Mobile phase: Acetonitrile (A) and LC-grade water with %0.1 formic acid (B)
Column temperature and flow rate: 40 °C and 1 ml/min. (%31: A - %69: B; isocratic flow)

Identification was achieved by considering the retention times of both standards and samples on the chromatogram. The amounts of steviol glycosides were then determined using the standard curves of various known concentrations of standards.

2.3. Statistical Analysis

All the data were statistically analysed by analysis of variance (ANOVA) using the SPSS [16]. The differences between the means were compared by least significant difference (LSD) ($p \leq 0\ 05$).

3. RESULTS and DISCUSSION

This study was initially planned at the salinity doses of 25, 50 and 75 mM concentrations. However, plants with 75 mM salinity did not grow and plants died. For this reason, 75 mM was omitted and the results of control group (0 mM), 25 mM and 50 mM salinity levels were evaluated.

The main effects of salinity (A) and silicon (B) and also the interactions of A and B (AxB) were statistically significant ($p<0.05$) on rebaudioside A and stevioside contents (Table 1).

Table 1. ANOVA (mean squares) results of the effect of silicon under salt stress conditions on rebaudioside A and stevioside contents

Source of Variance	df	Rebaudioside A	Stevioside
Salt (A)	2	359.78 **	912.06 **
Error	9	8.31	3.58
Silicon (B)	2	466.16 **	281.14 **
A x B	4	262.01 **	376.67 **
Error 2	18	5.681	4.94
General	35		

**, significant at α levels of 0.01.

Rebaudioside A contents were significantly affected by salt stress. Silicone application had no significant effect on rebaudioside A contents in the control pots. The effectiveness of silicone was apparent at higher salinity condition. The use of higher doses of silicon revealed higher rebaudioside A accumulation under salinity stress (Table 2). These results corroborated the Pandey and Chikara [5], who observed an increase in the rebaudioside A under higher concentration of NaCl. Cantabella et al. [17] reported that low salt levels did not affect steviolbioside and rebaudioside A contents. Pandey and Chikara [5] demonstrated that amount of stevioside and rebaudioside A significantly increased at 50, 75 and 100 mM NaCl but not at 25 mM NaCl. A study carried out in Egypt showed that saline water irrigation decreased rebaudioside A contents at the second harvest [2].

Table 2. Interaction of salt stress and silicon on RebaudiosideA (g kg-1)

	Control	Silicon (12.5 mM)	Silicon (25.0 mM)
Control	20.54 Aa	20.14 Aa	20.99 Ab
Salinity (25 mM)	15.77 Bb	16.94 Bb	17.97 Bb
Salinity (50 mM)	15.18 Cb	20.89 Ba	43.58 Aa
LSD 0.01=3.54			

Means followed by the same lower case letters in a column and capital letters on the lines do not differ significantly by the LSD test ($p < 0.05$).

Stevioside contents decreased significantly in salinity conditions compared to control conditions but increased with silicone doses in higher salinity conditions (Table 3). Fallah et al.

[18] found that the highest amounts of both stevioside and rebaudioside A were observed in control conditions (with NaCl-free). In other research, it was showed that 60-90 mM salt concentration decreased the content of stevioside and rebaudioside A [19]. On the contrary, El-Housini et al. [20] stated that the increasing salinity level in irrigation water from enhanced stevioside contents in all cutting. Besides, Shahverdi et al. [7] showed that low level of NaCl concentration (30 mM) caused increasing steviol glycosides and concluded that stevia is a moderate NaCl tolerant plant. According to the results of other research [17], stevioside and rebaudioside A contents did not exhibit statistically significant changes in the plants treated with 2 g/L NaCl, whereas 5 g/L NaCl decreased the contents of steviolbioside and rebaudioside A by 21% and 34%, respectively.

Table 3. Interaction of salt stress and silicon on Stevioside (g kg-1)

	Control	Silicon (12.5 mM)	Silicon (25.0 mM)
Control	50.91Aa	41.65 Ca	47.03 Bb
Salinity (25 mM)	32.92 Bb	31.76 Bb	29.83 Bc
Salinity (50 mM)	35.90 Cb	39.93 Ba	64.21 Aa
$LSD_{0.01}$=3.30			

Means followed by the same lower case letters in a column and capital letters on the lines do not differ significantly by the LSD test ($p < 0.05$).

The main effects of salinity (A) and silicon (B) and also the interactions of A and B (AxB) were statistically significant (Table 4).

Table 4. Analysis of variance (mean squares) of the effects of silicon under salt stress conditions on fresh weight and dry weight

Source of Variance	df	Fresh weight	Dry weight
Salt (A)	2	208.92 **	2.23 **
Error	6	1.09	0.013
Silicon (B)	2	90.41 **	1.15 **
A x B	4	18.68 **	1.29 **
Error 2	12	1.091	0.018
General	26		

**, significant at α levels of 0.01.

High doses of salinity caused a significant reduction in fresh weights in plants. The highest values were obtained with 12.5 mM silicone application in high salinity conditions. The lowest value was observed at highest salinity application with silicone free treatment (Table 5). These results are consistent with Shahverdi et al. [7] stating that NaCl stress caused reduction fresh and dry weights of leaf. Noori Akandi et al. [21] reported that fresh weight of leaf and dry weight of root decreased linearly when NaCl concentration increased in the growing media. According to Pandey and Chikara [5], NaCl application significantly decreased fresh weight of leaf at 100 mM concentration. Reis et al. [9] reported that increasing concentration of NaCl declined total fresh weight of leaf.

They concluded that only one harvest of stevia plant is possible even if the irrigation is well applied at high salinity. However, twice harvesting would be possible in low salinity conditions because of the lower salinity accumulation in soil compared to high salinity treatment.

Table 5. Interaction of salt stress and silicon on fresh weight (g)

	Control	Silicon (12.5 mM)	Silicon (25.0 mM)
Control	25.87 Bb	29.62 Ab	28.05 Ab
Salinity (25 mM)	29.70 Ca	34.91 Aa	31.12 Ba
Salinity (50 mM)	15.83 Bc	26.02 Ac	25.10 Ac
LSD 0.05=1.86			

Means followed by the same lower case letters in a column and capital letters on the lines do not differ significantly by the LSD test ($p < 0.05$).

The high level of salinity caused significant decreases in dry matter contents of the plants. The highest dry matter contents were observed in 12.5 mM silicone inclusion under 25 mM salinity conditions. The lowest value was obtained from high salinity conditions without silicone (Table 6). Salt stress has been reported to decrease fresh and dry weight of Cape gooseberry plants. The use of silicon (1.0 g L^{-1}) has alleviated the influence of salinity (0.5% NaCl) on chlorophyll, carotenoids, stomatal density and leaf blade thickness. When salt stress was about 1.0%, silicon was not effective [10]. Similarly Zeng et al. [19] reported that stevia plants treated with 90 and 120 mM NaCl showed significantly lower leaf fresh/dry weight and shoot dry weight than control conditions. The total dry weight decreased by 40% at 120 mM NaCl treatment. Pandey and Chikara [5] stated that *Stevia Rebaudiana* responds least resistant to increasing salinity conditions but also reported the optimum NaCl concentrations as 50 and 75 mM for healthy plant growths.

Table 6. Interaction of salt stress and silicon on dry weight (g)

	Control	Silicon (12.5 mM)	Silicon (25.0 mM)
Control	4.90 Aa	5.08 Ac	4.93 Aa
Salinity (25 mM)	5.11 Ba	6.06 Aa	5.07 Ba
Salinity (50 mM)	3.55 Cb	5.45 Ab	4.26 Bb
LSD 0.05=0.240			

Means followed by the same lower case letters in a column and capital letters on the lines do not differ significantly by the LSD test ($p < 0.05$).

4. CONCLUSION

The use of silicone adapted the negative effects of salinity stress applied to stevia and also increased stevioside and rebaudioside A contents significantly. Generally, the prominent conditions for the fresh weight and dry matter contents were 12.5 and 25 mM, silicone and NaCl concentrations, respectively. On the other hand, the lowest values were observed under 25.0 mM salinity concentration without silicon application. The results of this research showed that 25.0 mM silicon treatment had the desired influence on mitigation of the negative influences of 50 mM salinity on stevia. The use of exogenous silicon could be an alternative to ameliorate the generated stress. Therefore, the increase of salt tolerance was incorporated with the Steviol glycoside that allowed stevia plants to cope better with salt stress. Finally, it is suggested that salt-affected soils with high doses of silicon are more suitable for growth of stevia plant with higher stevioside and rebaudioside A contents.

ORCID

İlkay YAVAŞ https://orcid.org/0000-0001-7805-519X
Fatih Mehmet YILMAZ https://orcid.org/0000-0002-1370-1231
Aydın ÜNAY https://orcid.org/0000-0002-7278-4428

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Identification of Morphological and Pomological Characteristics of Iraq Pomegranate (*Punica granatum* L.) Variety Salakhani and Comparing with Variety Zivzik

Khabbat H. Al-Jabbarı [1], Mine Pakyürek [*,1], Adnan Yaviç [2]

[1] Department of Horticulture, Faculty of Agriculture, Siirt University, Siirt, Turkey
[2] Department of Horticulture, Faculty of Agriculture, Yüzüncü Yıl University, Van, Turkey

Abstract: In this study, morphological and pomological characteristics of Salakhani, local variety of Iraq and Zivzik (*Punica granatum* L.), local variety of Siirt, were compared. Salakhani variety, which is one of the well-known pomegranate cultivars in Halabja region, has been cultivated for centuries in this region, North of Iraq. Zivzik variety is considered to be one of the important local varieties in Siirt province in the South East of Turkey. Salakhani variety was collected from their natural area in Halabja and Zivzik variety was collected from Zivzik Village in Şirvan. This is the first study comparing both varieties in point of pomological and morphological features. Our study has shown that two varieties are similiar on account of some features on the other hand are also different with regard to some properties. In addition to that the study showed Salakhani has more bigger fruit size and with soft seed than Zivzik pomegranate; conversely, Zivzik cv. has also very juicy fruit and high quality of fruit juice compare with Salakhani. This study has shown the superior characteristics of these two varieties can be the subject of different breeding studies in developing new pomegranate varieties.

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1. INTRODUCTION

Pomegranate tree is noted to be one of the oldest ancient and sacred fruit trees in the Mediterranean region. *Punica granatum* L. is considered to be one of the most important horticultural products and is known in *Punicaceae* family, which comprises *Punica* genus includes two species *Punica granatum* L. and *Punica proptopunica* Balf [1-4].

In recent years, pomegranate is cultivated and grown naturally and is well adapted to the regional climate in Afghanistan, China, Morocco, Palestine, India, Iraq, Iran, Israel, Italy, Cyprus, Egypt, Syria, Saudi Arabia, Thailand, Tunisia, Turkey as well as being grown in some other countries. Iran is considered to be the main producer country of pomegranate fruit followed by India, Turkey and Spain [5].

Pomegranate species need little chilling. Pomegranate is not damaged by late frost in late spring, but fruit in late varieties can be influenced by early autumn frosts. The annual rainfall of 500 mm is considered to be sufficient for the growth of pomegranate, especially in the fall and spring months. This information is taken into consideration for the pomegranate growing

CONTACT: Mine PAKYÜREK ✉ mine.pakyurek@siirt.edu.tr Department of Horticulture, Faculty of Agriculture, Siirt University, Siirt, TURKEY

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in the appropriate region of Turkey; Mediterranean, Aegean and South Eastern Anatolia Region. However, partial air-conditioning can also be done for pomegranate growing in certain regions [6].

Salakhani variety, which is one of the well-known pomegranate cultivars in Halabja region, has been cultivated for centuries in Halabja is located in the North of Iraq, 15 km west of the Iranian border, 61 km southeast of Sulaymaniyah and 241 km northeast of the capital Baghdad. It is a local pomegranate genotype of high quality grown naturally and well adapted to the regional climate and cultivation in residential areas is an important source of income and survival lihood for regional residents. Villages and districts, which surround Halabja region, are famous for the pomegranate fruit, called as Hanary Hawraman (Hawraman pomegranate).

Zivzik variety is registered and commercially produced pomegranate cultivar in villages (Zivzik, Kapılı, Sarı Dana and Pirinçli) have microclimate areas and belongs to Şirvan district in Siirt. Zivzik pomegranate, registered in 2008 by the Pistachio Research Institute, by virtue of with big aril, extremely good in terms of taste, aroma and fruit juice quality and it can be stored for 6 months when stored under suitable conditions, is a commonly produced variety.

The aim of this study was to characterize and compare two local pomegranate varieties Salakhani, in Halabja province in north of Iraq, and Zivzik, in southeast of Turkey, selected as the study area, as well to determine pomological and morphological characteristics of both varieties and find out the similarities and differences between them.

2. MATERIAL and METHODS

2.1. Material

2.1.1. Geographical structure and climate features of the study area

The study was carried out in 2015-2016, at the Department of Horticulture, Faculty of Agriculture in Siirt University. Siirt province is located between east longitude 41˚-57' and north latitude 37˚-55". Siirt province is surrounded by Şırnak and Van in the east, Batman and Bitlis in the north, Mardin and Şırnak provinces in the south. A large part of the territory of the province is covered with mountains. The province has a total area of 6.186 km^2 [7]. In Siirt province, that experiences the most significant features of the four seasons, the continental climate prevails. The summers are hot and dry, between June and October precipitation is not observed. More precipitation has been observed in the spring, the amount of moisture with 40% in normal has reached the value over this rate. In the eastern and northern regions of Siirt province that has much difference between day and night temperatures, the winters are rainy and frosty and the southern and western regions are warm. The wind blowing from the east and northeast at night blows from the south and southwest during the day [7]. The altitude of the city centre of Pervari district is 1.380 m, and agricultural production is performed over 7.300 acres [8]. The 90-year average of Siirt province climate data as shown in Table 1 [9].

Halabja province is located in the southeast of the Sulimanni province. It is situated near the border of Iranian with the city of Halabja and is surrounded by the Hawamian and Sorine mountains in the northeast and north, the Sirwan river in the west, Penguin district in the north, Sharazore district in the northwest, Derbandikhan Lake in the west and southwest. Halabja lies between east longitude 45°59'03.4"E and north latitude 35°10'32.8"N. The elevation is about 695 m above sea level (MSL) and the average wind speed is 2.3 m/s in summer, and 1.4 m/s in the winter season. The estimated cultivation area of fruit trees in the region is approximately 2245 hectares [10]. 10 year average (2006-2016) climate data of Halabja province was shown in Table 2 [11].

Table 1. The average of climatic data for 90 years (1926-2016) of Siirt province [5].

Months	Average Temperature (˚C)	Average High Temperature (˚C)	Average Low Temperature (˚C)	Highest Temperature (˚C)	Lowest Temperature (˚C)	Average Precipitation (mm)	Average Relative Humidity (%)
January	2.6	6.5	-0.6	19.7	-19.3	97.5	77
February	4.2	8.7	0.5	20.6	-16.5	98.2	69
March	8.2	13.2	4	28.5	-13.3	111	65
April	13.7	19.1	8.9	32.9	-4.1	104.4	59
May	19.3	25.2	13.5	36.2	2	61.8	52
June	25.9	32.1	18.9	40.2	8.2	8.8	36
July	30.5	37	23.4	44.4	13.1	1.6	30
August	30.1	36.9	23.1	46	14.4	0.9	29
September	25	32.1	18.7	39.9	8.5	5	34
October	17.9	24.3	12.7	36.6	0.3	49.6	47
November	10.4	15.4	6.3	26	-4.1	81.4	64
December	4.7	8.7	1.6	24.3	-14.6	95.2	72
Total	16	21.6	10.9	46	-19.3	715.4	52.83

Table 2. The average of climatic data for 10 years (2006-2016) of Halabja Province [6].

Months	Average Temperature (˚C)	Average High Temperature (˚C)	Average Low Temperature (˚C)	Highest Temperature (˚C)	Lowest Temperature (˚C)	Average Precipitation (mm)	Average Relative Humidity (%)
January	7.42	11.04	2.77	16.04	-2.82	97.13	56.78
February	9.18	13.69	4.89	19.89	-1.24	110.43	54.6
March	13.5	18.6	8.38	25.7	3.02	78.04	47.55
April	18.35	23.92	12.68	31.62	6.6	80.62	45.31
May	23.97	31.69	18.88	39.51	12.54	29.71	35.7
June	32.28	39.73	25.26	39.66	20.65	1.02	23.24
July	35.31	42.73	28.16	45.96	24.31	0.35	27.13
August	35.26	42.77	27.9	46.29	24.21	0.12	26.98
September	30.24	37.51	22.94	42.8	17.48	0.77	33.3
October	23.28	28.92	17.5	36.4	11.02	67.16	35.73
November	14.66	19.22	9.18	25.23	4.67	88.29	51.22
December	9.19	13.71	8.79	19.4	1.65	86.91	53.17
Total	21.05	26.96	15.61	32.38	10.17	640.08	40.89

2.1.2. Geographical structure and climate features of the study area

In this experiment, a private orchard of pomegranate Salakhani and Zivzik were selected. Plant age ranged between 20-25 years old trees were selected on the basis of their uniformity in appearance, growth habits and vigour. Salakhani variety is a local pomegranate genotype grown naturally, specifically in Halabja province. Zivzik variety is considered to be one of the local varieties in Siirt province in the southeast of Turkey. Salakhani variety was collected from their natural area by Khabbat H. Al-Jabbari and Zivzik variety was collected from Zivzik Village in Şirvan, Siirt. Salakhani fruits are medium to large size, the peel is thick with reddish to yellow colour, aril full of juice with pink to red colour, they had a good flavour sour-sweet

taste and fruits can be used for fresh consumption or local production of the concentrated pomegranate juice [12]. Zivzik fruits are small to medium size, the peel is thick with pink to green colour, aril full of juice with pink to dark pink colour, they had a good flavour sour to sour-sweet taste with semi easy in aril separating and seed hard [13].

2.2. Methods

2.2.1. *Pomological properties*

Pomegranate genotypes with superior properties were determined according to the selection criteria of fruit and some pomological characteristics were examined. Genotype, fruit weight (g), fruit length (mm), fruit width (mm), fruit juice volume (mL), fruit volume (mL, calyx diameter (mm), calyx length (mm), peel thickness (mm), aril colour, peel colour, number of chamber, appearance of calyx, easiness for aril separating, the taste of fruit, fruit pulp weight (g), fruit shape index and aril yield (%) were determined. Moreover, Total Soluble Solids (T.S.S) (%), pH and titratable acid ratio (%) contents also were measured. Some pomological properties are shown in Figure 1 [14-16].

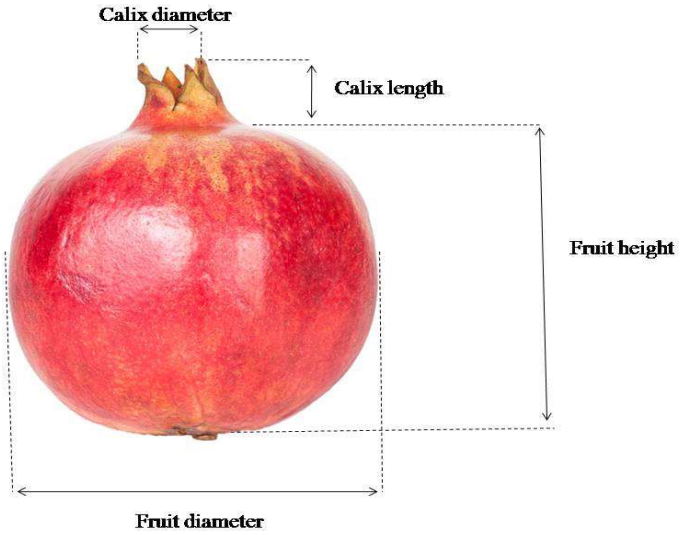

Figure 1. Fruit and calyx sizes: fruit width, fruit height, diameter of calyx, calyx length.

2.2.2. Field study

The flowering time of pomegranate fruit in Siirt province starts at the month of May, and the harvested date starts in the middle of the month of October. In order to ensure the best quality the fruit should be picked at the fully ripened stage and it depends on the local climate and pomegranate variety. Whereas, the flowering time of Salakhani variety in Halabja Province begins in the middle of the month of April, and the harvest time starts at the beginning of the month of October, depends on climatic conditions and variety of pomegranate (Figure 2). On each tree, the following morphological and phenological characters were determined: pomegranate local name, tree location, altitude (m), crown height (cm), crown width (cm), trunk number, trunk girth (cm), intensity of branching, cold damage, date of first leafing, flowering date and harvest date.

Figure 2. Salakhani pomegranate fruit and appearance of its orchard.

The fruits at commercially ripe stage from two main pomegranate variety 'Salakhani' from North Iraq and 'Zivzik' from Southeast of Turkey were harvested from 20 year-old trees in October 2016. The trees were spaced 4 and 3 m between and within rows (Zivzik trees are seen in Figure 3). Both cultivars were grown under the different geographical conditions. 15 fruits were picked up randomly from each cultivar as a sample were randomly collected at morning and then put in cooler bags to be quickly transferred to the laboratory. The fruit samples were collected during the maturity stage and the analyses were performed in the laboratory of the Faculty of Agriculture, Department of Horticulture in Siirt University. All fruits were first flushed by tap water before the peel, pulp and seed fractions were carefully separated. The peel and pulp were separated manually after measurement of fruit fresh mass and volume. 15 fruits were maintained for each analysis and the results were presented average of the fruits values.

Figure 3. Zivzik pomegranate fruit and appearance of its orchard.

3. RESULTS and DISCUSSION

3.1. The Results Obtained from The Field

Studies were carried out in the private orchard in Halabja province. The information were obtained from the questionnaires filled in for Salakhani variety is summarised below. Pomegranates local variety grown in the main production area of Halabja Province and surrounding area. It covers a wide areas of this region, Halabja Province is also well known for pomegranate production and the high quality of its fruits and some other fruits due to appropriate climatic conditions and these areas are ideal places for pomegranate growing.

This area is characterised by a highly fertile and non-stressful conditions. Furthermore, it is considered to be rich in nutrient elements. For this reason, commercial fertilisers are rarely used, while, that the farmyard manure (organic fertilizers) is generally used as a plant fertiliser. Nevertheless, a common fertilizer application is also performed. Fertilizers are commonly used for growing all the plants, with application rates depending on soil fertility and economic conditions of the farmers. In orchards, plants are normally trained to a multiple trunks, in order to reduce risk of total tree loss. Most of the pests deployed in the region such as aphids and trunk borers. Fruit cracking and splitting phenomenon were observed in the region.

Table 3. Physical properties of pomegranate fruit.

Fruit Features	Salakhani variety	Zivzik variety
Fruit weight (g)	389.43 -578.51	129.8- 379.5
Fruit height (mm)	87.70- 99.49	59.50- 82.50
Fruit width (mm)	88.28-103.11	66.70 - 90.50
Fruit volume (mL)	350 – 600	250 – 400
Calyx radius (mm)	6.4- 8.74	7.7- 8.9
Calyx length (mm)	21.56- 23.45	15.7- 22.4
Fruit juice volume (mL)	180- 250	60- 85
Fruit pulp and peel weight (g)	205- 287	85-220
Fruit taste	sour-sweet	sour-sweet
Aril colour	Pink	dark pink
Seed hardness	Hard	semi-hard
Aril yield (%)	59.10- 69.04	51.17 – 61.12
Upper fruit peel colour	Light pink- pink	Red
Bottom fruit peel colour	pink	Yellow
Peel thickness (mm)	2.11 – 2.99	2.75 – 4.00
Upper chamber number	5 to 6	5 to 7
Bottom chamber number	3 to 4	4 to 5
Appearance of calyx	Clear	semi-clear
Easiness of aril separating	Easy	semi-easy
Fruit shape index	0.96- 0.99	0.89- 0.91

3.2. Pomological Properties

In this study, two types of pomegranate fruit were selected to examine the physical features, and the averages of the results obtained are shown below. Fruit weight of Salakhani fruit was found to be between 389.43-578, 51 g, while, that the weight of Zivzik fruit ranged between 129.8-379.5 g (Table 3). As demonstrated in Table 3 fruit height of Salakhani variety was found to be between 87,70-99,49 mm. Whereas, the fruit height of Zivzik variety ranged between 59.50-82.50 mm. Fruit width of Salakhani variety was found to be between 88.28-103.11 mm, while; the fruit width of Zivzik variety ranged between 66.70-90.50 mm (as shown in Table 3). Fruit volume of Salakhani variety was found to be between 350-600 mL, while the fruit volume of Zivzik variety was found to be between 250-400 mL (as shown in Table 3). Calyx radius of Salakhani variety ranged between 6.4-8.74 mm, while in Zivzik variety it ranged between 7.7- 8.9 mm. Calyx length of Salakhani variety ranged between 21.56-23.45 mm and in Zivzik variety it ranged between 15.7-22.4 mm. Fruit juice volume of Salakhani variety ranged between 180-250 mL, while in Zivzik variety it was found to be between 60-85 mL. Fruit pulp of Salakhani variety ranged between 205-287 g, while the fruit pulp of Zivzik variety ranged between 85-220 g (Table 3). Fruit taste of Salakhani and Zivzik varieties were

sour sweet (Table 3). Aril colour of Salakhani pomegranate is pink, while, the aril colour of Zivzik pomegranate is dark pink. As demonstrated in Table 3 seed hardness of Salakhani variety is hard, while the seed hardness of Zivzik variety is semi-hard. Aril yield of Salakhani variety ranged between 59.10-69.04 %, while the aril yield of Zivzik variety was found to be between 51.17-61.12 %. Upper side colour of Salakhani variety is light pink into pink, while the upper side colour of Zivzik variety is red. Bottom side colour of Salakhani variety is pink, while the bottom side colour of Zivzik variety is yellow. Peel thickness of Salakhani variety is found to be between 2.11-2.99 mm, while the peel thickness of Zivzik variety is found to be between 2.75-4.00 mm. Upper chamber number of Salakhani variety is found to be between 5-6, while the upper chamber number of Zivzik variety is between 5-7. Bottom chamber number of Salakhani variety is found to be between 3-4, while bottom chamber number of Zivzik variety ranged between 4-5. The appearance of calyx of Salakhani variety is clear and the appearance of calyx of Zivzik variety is semi-clear (as shown in Table 3). Aril separating in Salakhani variety is easy, while the aril separating in Zivzik variety is semi-easy. Fruit shape of Salakhani variety was found to be between 0.96-0.99, while the fruit shape of Zivzik variety was found to be between 0.89-0.91.

Soluble solids ratio (%) of Salakhani variety was found to be between 15.1-16.1 % while the soluble solids ratio (%) of Zivzik variety was found to be between 13-17 % (as shown in Table 4). As seen in Table 4 pH of fruit juice of Salakhani cultivars was found to be between 3.05-3.19 %. While, in Zivzik cultivars it was found to be between 3.6-4.0 (Table 4). Titratable acid ratio in the fruit juice of Salakhani variety was ranged between 0.9-1.3 %. Whereas, in Zivzik variety it was found to be between 0.4-0.8 %. The mean values of the pomological characteristics of both varieties are given in Table 5 and Table 6.

3.3. Discussion

Gündoğdu et al. [13], carried out an experiment on pomegranate genotypes planted in Siirt province. They investigated pomological properties of these genotypes such as fruit weights, fruit heights, fruit diameters, fruit volumes, fruit juice amounts, fruit densities, seed weights, calyx heights, calyx half-diameters, total soluble solid (TSS), pH, fruit shape index, total acidity. Valus of these properties ranged between the given numbers respectively 161.45-302.35 g; 60.79-78.67 mm; 67.27-86.92 mm; 177.5-305.0 mL; 69 -121 mL; 0.84-1.17 g cm-3; 80.00-162.35 g; 16.58-34.64 mm; 9.32-14.27 mm; 12-16 brix; 3.63-5.87; 0.84-1.03; and 0.47-1.08 %. In addition to that, in this study the other pomological features of pomegranate genotypes were observed such as fruit taste, seed hardness, seed colour, easiness in separating arils, sub-skin colour, upper skin colour, compartment appearance, upper fruit compartment, sub-fruit compartment and fruit pulp weights as well.

Mars and Marrakchi [17], studied thirty pomegranate (*Punica granatum* L.) accessions to determine the overall degree of polymorphism and to detect similarities among the genotypes grown naturally in Tunisia. In the study, fruit weights ranged from 196 g to 673 g; fruit heights ranged from 46 mm to 96 mm; fruit diameters ranged from 57 mm to 114 mm; peel thickness ranged from to 2.4 mm to 6.1 mm; calyx length ranged from 12 mm to 21 mm; fruit juice amounts ranged from 72 mL to 100 mL; the TSS ranged from 13.3 % to 16.9 %; pH ranged from 0.93 to 4.6; the titratable acidity ranged from 0.25 to 3.17.

Again in another experiment Gündoğdu et al. [18] examined certain chemical and pomological features of standard pomegranate varieties cultured in Turkey. These features are such as fruit weights, fruit height, fruit diameter, fruit volume, fruit juice, fruit densities, total soluble solid content (T.S.S), pH, total acidity. Values are in the following ranges 251.01-530.25 g; 60.30-89.97 mm; 75.57-100.68 mm; 230.00-542.50 mL; 106.66-186.00 mL; 0.92-1.19 g cm-3; 11.50-14.62 %; 3.45-4.71 and 0.19-1.17 %. Furthermore, they also investigated fruit taste, aril colour, easiness of aril separating, seed hardness, peel colour, upper peel colour,

upper chamber number, bottom chamber number, calyx number, appearance of calyx in their study.

Table 4. Chemical properties of pomegranate fruit.

Fruit Features	Salakhani variety	Zivik variety
T.S.S (%)	15.1-16.1	13-17
pH	3.05-3.19	3.6-4.0
Titrable acidity (%)	0.9-1. 3	0.4-08

Table 5. Phenology, flowering and tree characteristics of Salakhani variety.

CULTIVAR NAME	Salakhani cultivars	Fruit Features	Value
Name of location	Halabja	Fruit weight (gr)	502.34
Location of orchard	Byawella	Fruit height (mm)	94.01
Local name	Salakhani	Fruit width (mm)	95.48
Altitude (m)	695 m	Fruit volume (mL)	470
The status of irrigation	watered in summer	Calyx diameter (mm)	15.25
Status of wind	middle	Calyx length (mm)	22.7
		Fruit juice volume (mL)	220
		Fruit pulp and peel weight (g)	251
TREE FEATURES		Fruit taste	sweet-sour
Crown height (mm)	231	Peel colour	light pink- pink
Crown width (mm)	460	Seed hardness	hard
Trunk number (No)	5	Aril yield (%)	68.04
Trunk girth (cm)	18	Weight of 100 arils (g)	42.3
Density of the branches	middle	Upper fruit peel colour	pink- light pink
Cold damage	No	Bottom fruit peel colour	pink
		Peel thickness (mm)	2.72
PHENOLOGICAL OBSERVATIONS		Upper chamber number	5.8
Date of first leafing	18th March	Bottom chamber number	3.4
Flowering time	22th April	Appearance of calyx	clear
Harvest date	5th October	Easiness of aril seperating	easy
		Fruit shape index	0.984
		T.S.S (%)	15.6
		pH	3.12
		Titrable acid ratio (%)	1.1

Muradoğlu et al. [19], conducted an experiment to describe the desirable pomological traits of 46 pomegranate genotypes selected from Çukurca district in Hakkari. The data were recorded as follows: fruit weights ranged from 131 g to 337 g, fruit height ranged from 60.0 mm to 81.0 mm, fruit width ranged from 30.8 mm to 88.9 mm, calyx length ranged from 11.0 mm to 26.1 mm and calyx diameter ranged from 11.2 mm to 18.1 mm. In addition, the total soluble solid content was between 12.2 % and 17.6 %. The values of pH ranged from 2.6 to 3.8. The acidity was between 1.5 % and 2.9 %. Genotypes had green or yellow coloured bottom skins, red or pink coloured aril, soft, semi-hard and hard seeds. Their aril percentages changed between 49.5 % and 71.5 %.

From Turkey some researchers, Usanmaz et al. [20], observed that the yield and pomological properties of three pomegranate cultivars: Wonderful, Acco and Herskovitz cultivated in Cyprus. According to findings of this experiment, the highest fruit weight and second highest yield belongs to Wonderful cultivar (481.12 g/fruit and 14.17 kg/tree). The second highest fruit weight and highest yield belongs to Herskovitz (431.04 g/fruit and 15.44 kg/tree). The lowest fruit weight and yield belongs to Acco (350.31 g/fruit and 11.43 kg/tree). Acco had the highest juice content followed by Wonderful and Herskovitz (40.22 % > 35.60 % > 29.42 %). Wonderful had the highest juice content (5.05 L/tree). Juice content per tree for Acco and Herskovitz was determined to be 4.58 L and 4.53 L, respectively.

Table 6. Phenology, flowering and tree characteristics of Zivzik variety.

CULTIVAR NAME	Zivzik Cultivars	Fruit FeaturesValue	Value
Name of location	Siirt	Fruit weight (g)	233.8
Orchard location	Şirvan/ Zivzik Vill.	Fruit height (mm)	69.23
Local name	Zivzik	Fruit width (mm)	76.26
Altitude (m) (MSL)	850	Fruit volume (mL)	225
The status of irrigation	Watered	Calyx diameter (mm)	16.4
Status of wind	middle	Calyx length (mm)	19.75
		Fruit juice volume (mL)	75
TREE FEATURES		Fruit pulp and peel weight (g)	119.33
Crown height (cm)	340	Fruit taste	sweet
Crown width (cm)	350	Aril colour	dark pink
Trunk number (No)	2	Seed hardness	semi-hard
Trunk girth (cm)	40-16	Aril yield (%)	54.17
Density of the branches	middle	Weight of 100 arils (g)	45.5
Cold damage	No	Upper fruit peel colour	red
		Bottom fruit peel colour	yellow
PHENOLOGICAL OBSERVATIONS		Peel thickness (mm)	3.25
Date of first leafing	13th April	Upper chamber number	5
Flowering time	13th May	Bottom chamber number	4
Harvest date	18th October	Appearance of calyx	semi-clear
		Easiness of aril separating	semi-easy
		Fruit shape index	0.873
		T.S.S (%)	16.5
		pH	3.54
		Titrable acid ratio (%)	0.6

Polat et al. [21], conducted an experiment in Hatay, during which the following results were obtained: fruit weight ranged from 250 g to 461 g, 100 aril weight ranged from 29 g to 50 g, fruit height ranged from 69 mm to 83 mm, fruit width ranged from 80 mm to 94 mm, peel thickness ranged from 3.7 mm to 4.3 mm, aril yield ranged from 54 to 73 %, TSS rates ranged from 14 to 15 % and acidity ranged between 0.3 and 3.9 %. In another study, Yılmaz et al. [22] was carried out on the regional adaptation of pomegranate in the Mediterranean Region and the following results were obtained fruit width was 92-104 mm, fruit height was 79-91 mm, fruit weight was 411-568 g, (T.S.S) was 13-16 % and acidity % ranged between 0.13-1.63 %.

In her study, Gözlekçi [23] examined the characteristics of the fruit, and a relationship in the same direction was determined between fruit diameter with fruit weight, fruit diameter with fruit volume, the volume of fruit with fruit weight, fruit length with fruit width, fruit length with fruit weight and juice yield with aril yield, while there was an opposite relationship between the amount of the peel with aril yield, aril yield with TSS and acidity with TSS.

Appropriate pomegranate varieties have been developed in the internal and external markets as a result of breeding works. The peel colour should be red; titratable acidity less than 1 % (sweet), titratable acidity ranging from 1 to 2 % (sour-sweet), titratable acid ratio more than 2 % (sour) [24]. Muradoğlu et al. [20], conducted an experiment on 45 types of pomegranate fruit in Çukurca, and the following results were obtained: weight was 131-337 g, pH value was 2.60-8.80, titratable acidity rate ranged between 1.50-2.90 %. Türkmen and Ekşi [25], collected pomegranate varieties (Hicaz, Devedişi, Katırbaşı, Ernar, Fellahyemez, Ekşilik, Aşınar) from different provinces of Turkey (Izmir, Gaziantep, Adana, Mersin, Aydın, Antalya, Muğla, Kilis) with the average fruit weight of 374.9 g, peel ratio of 50 %, aril ratio of 49.9 %, juice efficiency of 34.7 % and fruit juice content of 8.3 % when obtained just from the aril.

Tehranifar et al. [26], searched 20 types of Iranian pomegranate in their experiment to define the physicochemical features of these types. In their experiment, fruit weight ranged between 196.89-315 g, fruit length ranged between 69.49-81.56 mm, fruit diameter ranged between 64.98-86.88 mm.

Yıldız et al. [27], conducted a study in Hizan. In their study, fruit weight was 192-388 g, fruit length was 62-78 mm, fruit diameter was 68-90 mm, the number of sepals was 5-8, fruit juice rate was 28-55 %, peel thickness was 1.3-2.8 mm, TSS was 10-17 % and acidity ranged between 0.37-4.3 %. Tibet and Onur [17], selected 35 pomegranate types from the Aegean Region and South East Anatolia and examined their phenological and pomological characteristics. Fruit weight ranged between 223-493 g, fruit width ranged between 78-102 mm, fruit length ranged between 67-88 mm, T.S.S ranged between 12-16 %, aril yield ranged between 41-64 % and the total acidity ranged between 0.19-2.38 %.

Numerous studies have been conducted by various researchers so far to investigate the pomological properties and selection of pomegranate. The above-mentioned studies show that pomegranate genotypes grown in nearby geographies have similar and different characteristics as in Salakhani and Zivzik varieties (Figure 4). Detection of these features is extremely important in terms of increasing genetic diversity.

Figure 4. Longitudinal section of pomegranate varieties.

4. CONCLUSION

Pomegranate fruit specifications of Salakhani and Zivzik varieties can be summarised as follows: Fruit of Salakhani cultivars were of middle to large size, with the weight of 389.43-

578.51 g their peel thickness ranged between 2.11-2.99 mm, peel colour was pink into light pink, aril colour was pink, they had a good flavour and sour-sweet taste, aril separating is easy, appearance of the calyx is clear, fruit juice volume is ranged between 180-250 mL, fruits can be used for fresh consumption or local production of the concentrated pomegranate juice called Ruba Hanar. The harvesting of Salakhani cultivars begins at the beginning of the month of October.

Zivzik variety is characterised by its small-to-middle size, with the weight of 129.8-379.5 g, peel thickness ranging between 2.75-4.00 mm, fruit juice volume ranging between 60-85 mL, peel colour is red-yellow colour, dark pink aril colour, a good flavour and sour-sweet taste, aril separating is semi-easy and appearance of the calyx is semi-clear. The harvesting of Zivzik variety begins in the middle of October, fruits can be used for fresh consumption or making the pomegranate juice. If we compare both varieties in terms of pomological properties such as size, easiness of aril separating, number of chamber, fruit volume, weight, peel thickness and fruit juice volume; could conclude that Salakhani variety is superior to Zivzik variety. Whereas, Zivzik variety was found to be superior to Salakhani variety in terms of T.S.S and pH of fruit juice.

As a result, Salakhani variety was determined to be superior to Zivzik variety; therefore, we recommend more studies on pomegranate fruit (Salakhani) to investigate the components of pomegranate fruit such as vitamins and other elements. Because of the large number of pomegranate (*Punica granatum* L.) variety in the North of Iraq, we recommend further study on Iraq pomegranate varieties in terms of pomological properties. Both varieties have distinguished features in terms of pomological properties. The fruits of the Salakhani variety are larger than the fruits of the Zivzik variety. On the other hand, the taste and aroma of Zivzik variety is very good and fruit juice quality is higher. For that reason, the superior aspects of the varieties identified in our study should be used in breeding studies for the development of new pomegranate genotypes. Breeding works towards the development of new pomegranate varieties, both Iraq and Turkey is important in terms of orcharding.

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ORCID

Khabbat H. Al-Jabbarı https://orcid.org/0000-0002-5638-5304
Mine Pakyürek https://orcid.org/0000-0002-3753-2532
Adnan Yaviç https://orcid.org/0000-0003-2609-2815

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Sequential Green Extraction of Caffeine and Catechins from Green Tea

Gönül Serdar [*1], **Ezgi Demir** [2], **Münevver Sökmen** [3]

[1]Department of Central Research Laboratory, Karadeniz Technical University, Trabzon, Turkey
[2]Department of Plant Sciences, Faculty of Agriculture and Natural Sciences, Konya Food and Agriculture University, Konya, Turkey
[3]Department of Bioengineering, Faculty of Engineering and Arhitecture, Konya Food and Agriculture University, Konya, Turkey

Abstract: Separation of caffeine and catechins from tea extracts usually requires conventional liquid-liquid extraction employing chloroform. This work was planned to improve a green extraction technique to distunguish these important chemicals from green tea. Extraction of caffeine and catechins from Turkish green tea firstly employing MAE, and then to separate these compounds from each other using a SFE method were purpose of this study. Microwave assisted extraction was applied to extract tea components from green tea then *i)* conventional liquid-liquid extraction or *ii)* supercritical carbon dioxide fluid extraction (SFE) method was charged with the effective separation of caffeine and catechins. Initially, an ethanol: water mixture was used in a close microwave system under the particuler extraction situations of green tea samples (*fresh, frozen or dried*) picked up in three collection periods (*first, second and third collection periods*). MAE of tea samples was exerted under a controlled 600 W microwave power for 4 min irradiation time at 80 °C temperature. Then MAE crude aqueous extract was divided in to two portions. The first portion was fractionated first with chloroform to distunguish caffeine then ethyl acetate for catechins. Caffeine and catechins were successfully separated. Second portion was freeze-dried and obtained lyophilized solid was used for SFE. Caffeine (3.68% extract yield) was successfully separated from catechins with SFE at 250 bar and 60°C for 180 min. Constituents of the extracts were determined (caffeine and four catechins namely EGC, EC, C, EGCG) by HPLC to evaluate the effectiveness of the separation.

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1. INTRODUCTION

Green tea has been popular beverage in Asian countries and is consumed all over the World [1]. Green tea is made ready through processing of tea leaves without fermentation [1]. The components of green tea include 10-30% (w/w) polyphenols, 2-4% (w/w) caffeine [2], aroma chemical, fats, vitamins, amino acids, chlorophyll, others [3]. The strain of tea tree, manufacturing process employed, harvest time varies according to ingredient of green tea [4,5]. Catechines, flavanols, flavanonens, phenolic acids, plant pigments and glycosides are which

CONTACT: Gönül SERDAR ✉ gonulserdar@ktu.edu.tr Department of Pharmacy, Faculty of Science, Karadeniz Technical University, Trabzon, TURKEY

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polyphenols of tea [6]. This group of compounds is known to preserve good bioactive effects incluiding anticancer, antioxidation, antimutagenic properties of tea [7,8]. Investigators embarked on enterprise selectively extraction of caffeine and catechins from green tea by various methods.

Chloroform, isopropanol, ethyl acetate and methylene chloride have been effectively used to remove caffeine and to produce decaffeinated green tea [9]. Vuong and Roach [9] have been investigated alternative studies because the use of organic solvents increases health problems in food processing. Chloroform and ethyl acetate are used but not approved resulting from their toxicities in spite of they are effective [10]. Green extraction techniques has important advantages than traditional methods such as greater energy efficiency, shorter extraction time, less solvent and reduction of environmental pollution [11]. Microwave-assisted extraction, carbon dioxide supercritical fluid extraction, ultrasonic-assisted extraction have progressed in recent years owing to its inherent advantages such as solvent volume, reduction in extraction time over more conventional extraction techniques. MAE is novel technique that utilizes nontoxic solvents, microwave energy to extract objective compounds from different matrices and also provides an important reduction in the depletion of organic solvents for extraction. And then MAE is extracting soluble products into a fluid from a wide variety of materials utilizing microwave energy. Microwaves increase speed of heating by directly heating solvent or solvent mixture [12, 13]. Pressure, extraction time, co-solvent, moisture content of tea leaf and temperature affect to SFE selectivity [14-16]. SFE utilising CO_2 as a solvent can be option to toxic organic solvents. Generally, carbon dioxide is utilised as a supercritical fluid owing to it has low critical temperature and pressure [17]. CO_2 is a non-toxic, nonflammable, environmentally-friendly, quite inexpensive, easily separated from the system and do not leave waste. SFE is a fast, leaves no toxic residue and provides less degradation of catechins [9]. Vuong and Roach [9] have been planned in line with the increasing needs for the development of safe, sustainable and environmentally friendly methods to separate caffeine and catechin from green tea in their study. Bimakr [18] and Park [14] found and the optimum condition of SFE was achieved at 200 bar, 60 ◦C and 60min (60.566mg/g extraction yield). Maran [19] advanced SFE method appropriate for extraction of phenolic compounds from tea leaves at differant extraction conditions. And so, they asserted extraction pressure (100–200 bar), co-solvent flow rate (1–3 g/min) have significant effect. Some reports are available on extraction of catechins from both black tea and green tea in supercritical carbon dioxide with the addition of cosolvents such as ethanol and water [14, 16, 20, 21].

MAE is an effective extraction process for green tea which was previously utilised for extraction of various tea samples (*dried*, *fresh or frozen*) collected in first, second and third collection periods [22,23]. MAE provides a mixture of both caffeine and catechins and another separation step should be employed for effective separation of caffeine from catechins. The purpose of this work is to extract caffeine and catechins from green tea firstly employing MAE, then to separate these compounds from each other using a SFE method. We have employed a conventional liquid-liquid extraction method to separate caffeine and catechins to compare the extraction effectiveness of the developed SFE method.

2. MATERIAL AND METHODS

2.1. Standards and Chemicals

Chloroform, ethanol and ethyl acetate were analytical grade from Merck. Caffeine, gallic acid, (−) epicatechin, (−) epigallocatechin, (−) epicatechin gallate and (−) epigallocatechin gallate of standard chemicals were bought from Sigma (St Louis, MO, USA). Green tea samples used in the experiments were supplied by a local tea producer (Sürçay San. Co. Ltd., Trabzon, Turkey) in 2016. Green tea samples were collected at three collection periods (*first, second* and *third collection*) of the same year and location. After collection tea samples were either

extracted directly (*fresh*), extracted after freez drying (*freezed*) or extracted after drying the sample (*dried*).

2.2. Microwave Assisted Extraction

Initially, tea ingredients were extracted utilizing a close microwave assisted extraction system (MILESTONE, START S Microwave, USA). Extraction time and microwave power from MAE parameters can influence the extraction performance [24]. Extraction conditions were previously reported by our group [22,23]. 10 g of tea sample and 200 ml ethanol-water (1:1 v/v) solution were placed in extraction vessel. Extraction was performed under a controlled at 80 ^{o}C temperature, 600 W microwave power for 4 min irradiation time. Aqueous tea infusion was divided into two portions. The first portion was used for liquid-liquid separation and second portion was freeze dried. After drying process the remaining solid vas used for SFE experiments. The same procedure was repeated three times and data was calculated from the results of replicates.

2.2. Conventional Liquid-liquid Extraction

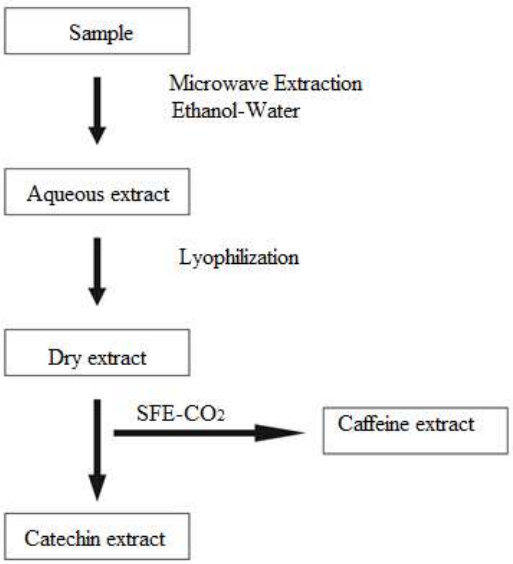
After completion of the MAE obtained aqueous tea infusion was filtrated and the filtrates were initially partitioned with chloroform to remove caffeine. A 50 mL portion of chloroform was used to extract caffeine and this was repeated three times. Then separation of caffeine the aqueous phase was re-extracted with ethyl acetate following the same procedure. The filtrate was concentrated by a rotary evaporator under reduced pressure at 55 °C after the ethyl acetate and chloroform phases were filtered. Extract yield was calculated according to this formula;

$$\text{Extract Yield \%} = (\text{mass of extract/mass of tea sample}) \times 100$$

The yields of caffeine and catechins were calculated after process and purity/combination of the extracts were determined by HPLC.

2.4. Supercritical Carbondioxide Extraction

The freeze dried extracts obtained from MAE were subjected to SFE. Aqueous tea infusion was completely dried by freeze drying. Caffeine extraction was carried out with SFE at 60 °C, 10-30 MPa for 3 hours [25, 26]. Then separation of caffeine the remaining solid mainly contains catechins. The extract yield of caffeine was calculated and purity/composition of the extracts were stated by HPLC analyses. Extraction steps are given in Schema 1.



Schema 1. Extraction and separation steps of caffeine and catechins.

2.5. Quantification of Caffeine and Catechins

HPLC analyses were performed to decide the amount of each catechins in the mixture [27] and the purity of distinguished phases. The instruments used in the work was a HPLC system (Hitachi Elite Lachrom) equipped with a Shim-pack VP-ODS C18 column (5 mm, 4.6 x 250 mm, 35 °C) at 278 nm. Solvents A (water) and B (DMF-methanol-acetic acid mixture, 20:1:0.5) were run with 86% A for 13 minutes. After that, its volume was decreased to 64% within next 15 minutes and back to initial concentration for another 6 min. Concentration of each catechin was measured by their peak areas against those of standards made from original compounds.

3. RESULTS and DISCUSSION

3.1. Liquid-liquid Extraction After MAE

The average caffeine and catechin yields from the first (in May, I. Collection), second (in June, II. Collection) and third collection periods (in August-September, III. Collection) are given in the Figure 1 and Figure 2. Data is the average of three replicates.

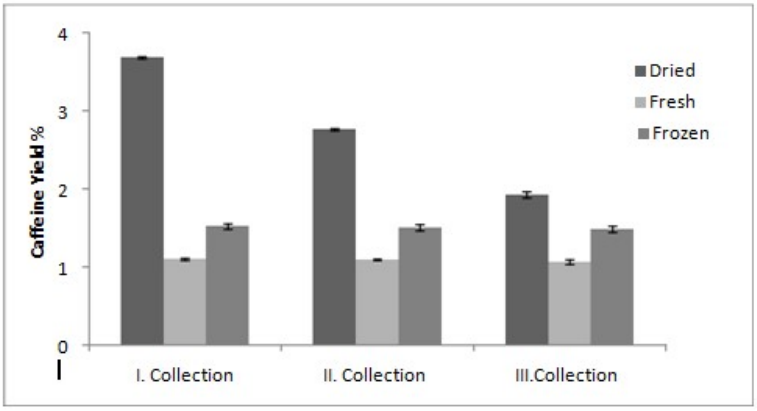


Figure 1. Caffeine content of the dried, fresh and frozen green tea leaves in first, second and third collection periods

Caffeine yields varied between 0.51% and 3.68%. The highest caffeine extract yields were obtained from the dried sample of the first collection. It is expected since dried samples contain higher amount of biomolecules. Almost 60-70 percent of water content of the tea leaves are lost during drying process. First collection gave higher amount of caffeine.

Catechin extract yields are given in Figure 2. Similarly dried samples of first collection period gave highest extract yield. It is gradually decreased at second and third collection periods.

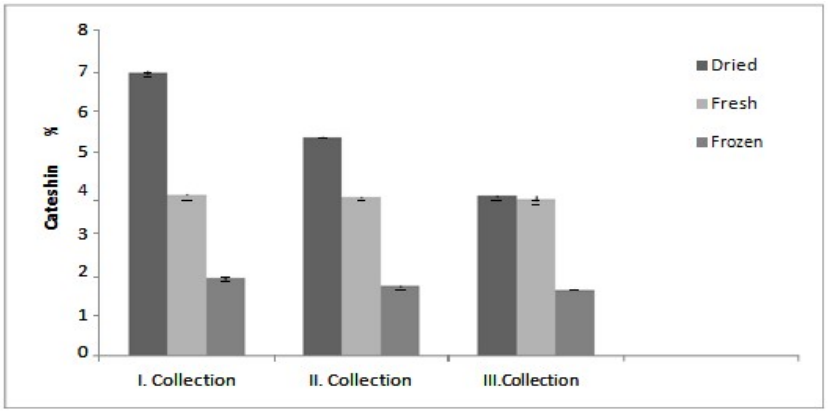


Figure 2. Total catechin content of the dried, fresh and frozen green tea leaves in first, second and third collection periods.

3.2. Supercritical Carbondioxide Extraction After MAE

SFE method was tested only for dried tea samples. Caffeine and catechin extraction from tea samples were already optimized by our group [25]. We reported direct supercritical fluid extraction of these compounds from dried green tea. It was an effective method extracting caffeine first running the instrument for 3 hours employing SFE supercritical fluid (25 MPa and 60 ºC). Additionally, another 3 hours was necessary to extract catechins under the identical situations however, using ethanol as modifier at 0.5 mL/min flow rate. So, at least 6 hours extraction period is needed for a successful extraction of these important tea components. However, MAE is achieved in only 4 minutes and separation of caffeine from catechins is fulfilled within 3 hours. Caffeine extract yields obtained with SFE at different pressures are given in Figure 3.

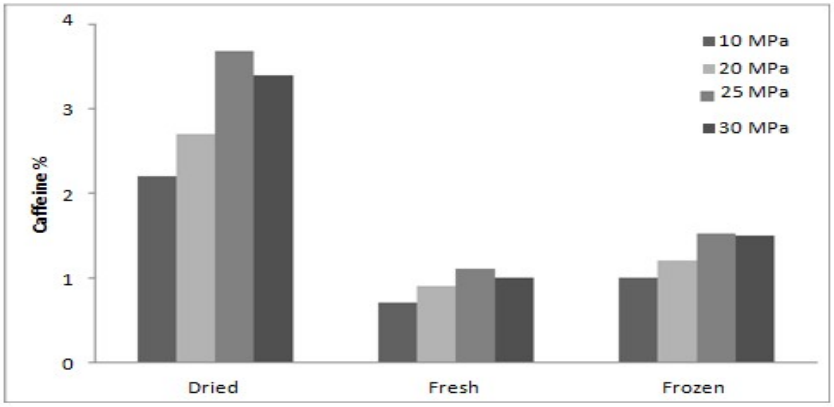


Figure 3. Caffeine yields of the dry, fresh and frozen green tea leaves after MAE (I. Collection period, 60 ºC and 3 h).

It is clear that high pressure is required to separate caffeine from the mixture and 25 MPa provide the highest caffeine yield (3.68%) which is equal to the amount of caffeine separated with liquid-liquid extraction. This is quite strong evidence that SFE is able to separate all caffeine content from the mixture. MAE can be used for total extraction of tea components then following this step SFE might be used as effective green extraction methods for effective separation of caffeine from catechins.

3.3. HPLC Analysis

Caffeine and catechin concentrations of the extracts were identified by reversed-phase HPLC. Catechin standards were catechine (C), epicatechin (EC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG). The chromatogram of the standard mixture is given in Figure 4.

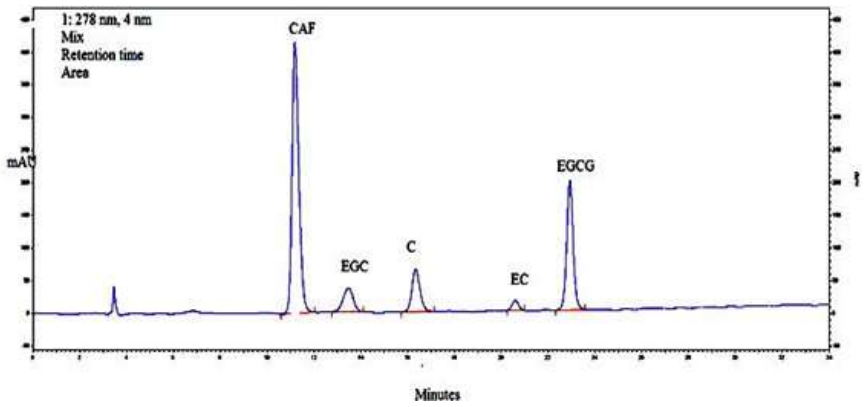


Figure 4. The chromatogram of caffeine and catechin standarts. Retention times are as below; caffeine 11.18; EGC 13.34; C 16.44; EC 20.60; EGCG 22.03 min. Injection volume is 20 µL.

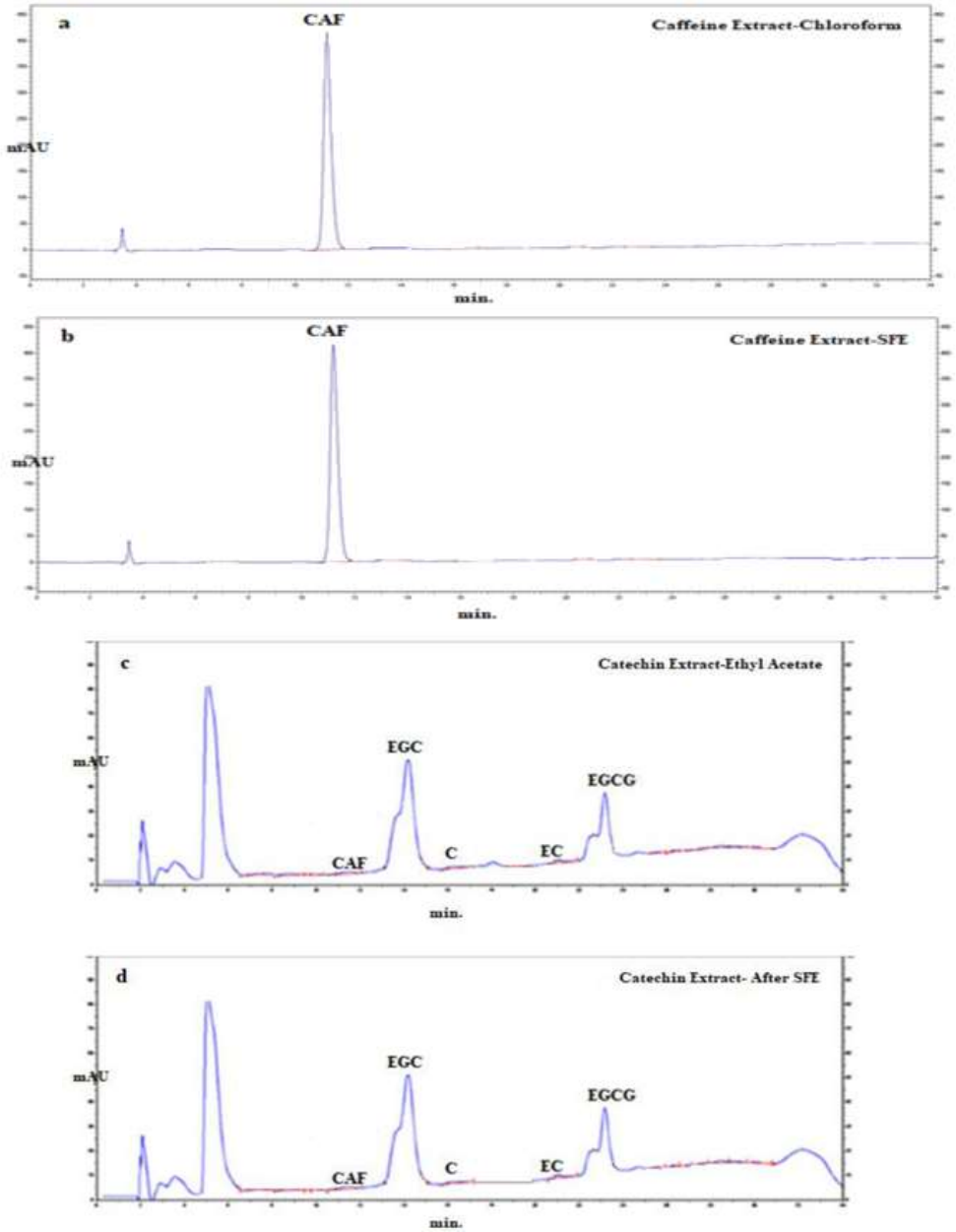


Figure 5. HPLC chromatogram of caffeine and catechin extracts obtained by liquid-liquid extraction (a and c) and SFE (b and d, 25 MPa, 60 °C, 3 h) from dried green tea samples initially extracted with MAE.

The identification of caffeine and catechins were realized by matching their retention times to concerning standards and the amount of individual components was calculated from these chromatograms. Developed SFE method was compared with liquid-liquid extraction method. The purities of caffeine extracts obtained from both methods are given in Figure 5 (a and b). It is clear that caffeine was successfully removed from the mixture and caffeine extract is pure as much as obtained with liquid-liquid extraction. The composition of catechin extract obtained from the extracting tea infusion with ethyl acetat is given in Figure 5c. Similarly, the composition of catechin extract (a residue after separation of caffeine with SFE) was also determined and given in Figure 5d. Purity and combination of both extract was completely same. The quantitative analysis of each cateshin constituent was determined from HPLC chromatograms. The amount of catechin constituents is given in Figure 6.

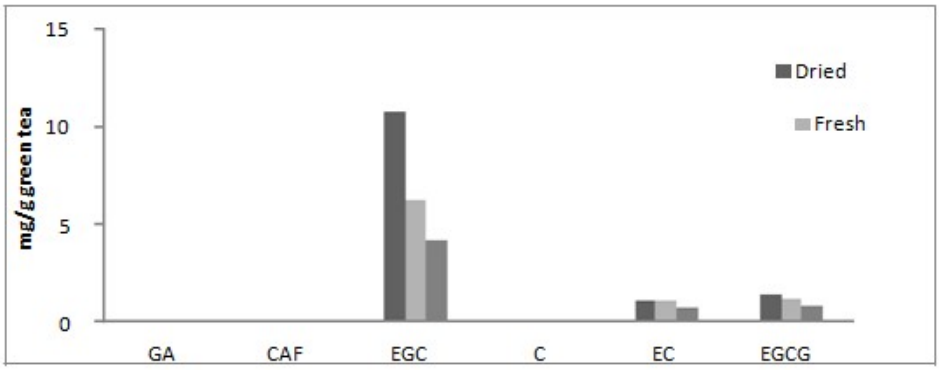


Figure 6. The amount of each cateshin from dried, fresh and frozen using SFE after MAE.

4. CONCLUSION

This study provides a practical approach for extraction of green tea bioactive compounds. A lot of methods have been worked for extraction and separation of catechins such as microwave-assisted extraction, maceration, supercritical carbon dioxide extraction. MAE is economical, fast and green extraction method that effective separation of caffeine and catechin from green tea. Both caffeine and catechin extraction was quite successful with microwave assisted extraction following effective separation with SFE. To overcome use of harmful organic solvents, SFE can be a potential method for isolating catechins from the tea leaves. Further search is useful to evolve new methods or the present methods to exceed the limitations of processes. Tea catechins especially EGC and EGCG can be separated from dried, fresh or frozen green tea using green solvents and green technologies in remarkable process periods.

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ORCID

Gönül SERDAR https://orcid.org/0000-0002-3589-2323
Münevver SÖKMEN https://orcid.org/0000-0002-7671-4160

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