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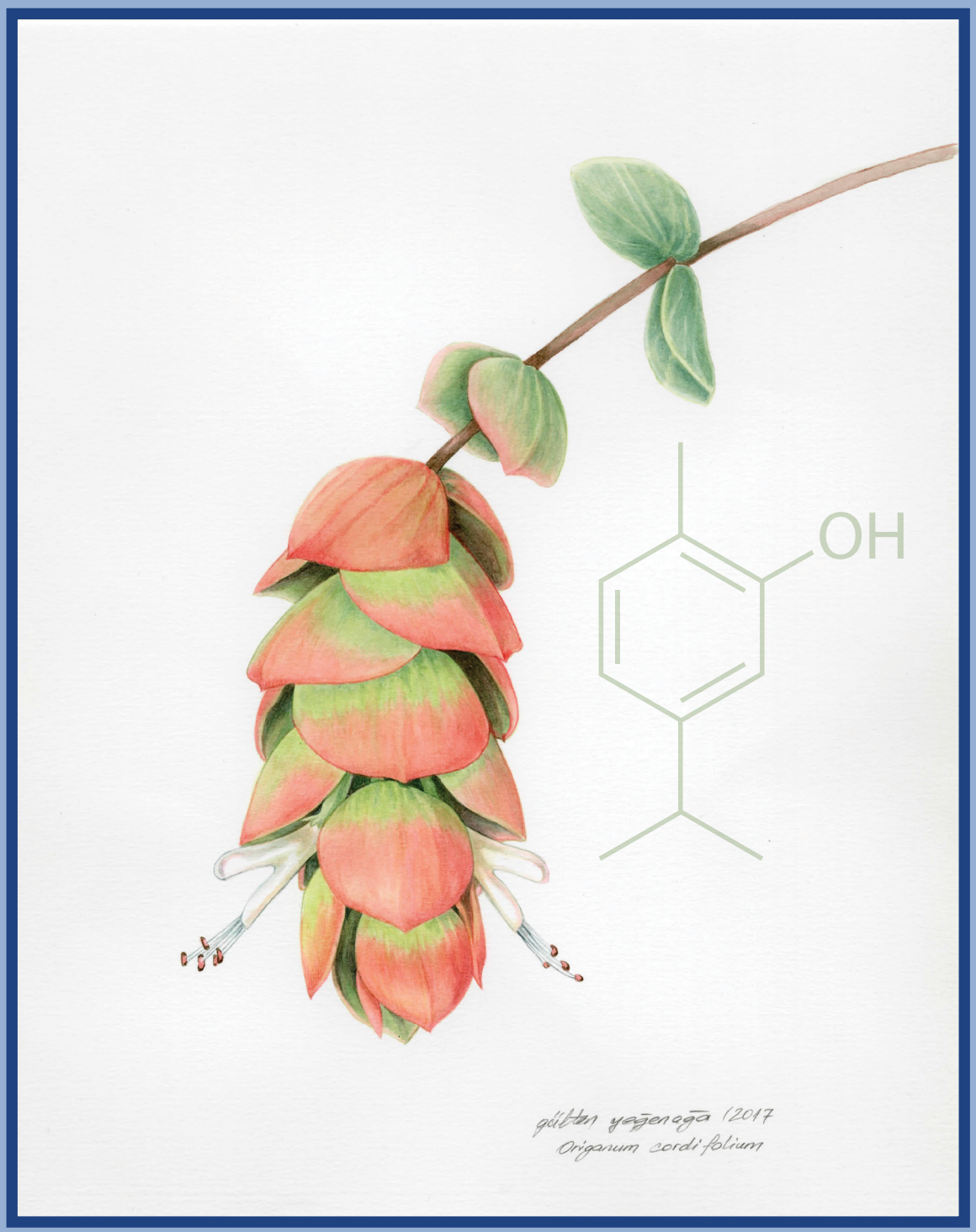

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Origanum cordifolium* **in cover picture was illustrated by Gülten Yeğenağa

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a) Give a brief and clear description of the materials and methods used. Subtitles can be given as appropriate.
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Reviews

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Direct SPME/GC-MS analyzes of small citrus fruits cultivated in Turkey

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Abstract

A headspace-solid phase microextraction (HS-SPME) procedure for isolation and investigation of volatile compounds in hybrid "limequat" and parents "Mexican lime and kumquat" fruits using Gas Chromatography-Mass Spectrometry (GC-MS) for the separation and identification of the volatiles was employed in the present study. Each of mature, fresh and whole fruits was freeze-dried, and each sample was powdered. In all samples, limonene (29.9-46.6%) was the most abundant monoterpene hydrocarbon among the identified monoterpenes. The common characteristic compounds are critically discussed.

Keywords

GC-MS, HS-SPME, kumquat, limequat, limonene, Mexican lime, volatile compounds.

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INTRODUCTION

Essential oils are aromatic oily liquids characterized by strong odors and produced by different methods such as distillation and expression in the case of citrus fruits (Baser and Demirci, 2011). Essential oils are low volume-high value products used in different industrial applications such as food, food ingredients, cosmetic and pharmaceutical preparations. Essential oils, their fractions and natural and/or synthetic aromachemicals are primarily used in the perfumery, flavor and fragrance industries (Palazzolo *et al.,* 2013; Gonzalez-Mas *et al.,* 2019).

Distillation (steam, water and water/steam distillation), cold-pressing (for essential oils), organic solvent extraction (pentane, diethyl ether, *n*-hexane *etc*), simultaneous distillation and extraction (Likens-Nickerson), microwave-assisted distillation/extraction, ultrasonic extraction and supercritical fluid extraction can be employed to isolate essential oils and/or aromatic extracts from plant matrices. European Pharmacopoeia 9.0 defines essential oils as products of distillation or cold-pressing (EP, 2016). Headspace trapping techniques are also used to extract volatile compounds from live plants. One of the most frequently used techniques is headspace-solid phase microextraction (HS-SPME). This technique is employed to isolate volatiles and semi-volatile compounds from materials. It is a versatile, efficient and frequently-used technique in the last few decades for sampling volatiles without using any solvents. Sample preparation is based on sorption of analytes from a sample onto a coated fused silica fiber that is mounted in a specially designed GC syringe. After introducing the coated fiber into a sample, the compounds to be analyzed are enriched according to their distribution coefficients and can be subsequently desorbed thermally from the coating after introducing the fiber into the hot injector port of a gas chromatograph (Kubeczka, 2010). In summary, it integrates sampling, extraction, concentration and sample introduction into a single solvent free-step. Combinations with gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS) are successfully applied to a wide variety of compounds from environmental, biological and food samples (Kusch, 2017). In addition to its applications (HS-SPME), may enhance the detection limits in GC-MS and is favorable for comparative studies of samples that have similar matrices (Spietelun *et al.,* 2013). In the literature, SPME is the subject of numerous studies and is widely applied for sampling a broad spectrum of analytes from gaseous, liquid,

and solid media with diverse matrix compositions (Pini *et al.,* 2004; Stashenko *et al.,* 2006; Perestrelo *et al.,* 2009; Xu *et al.,* 2016; Raza *et al.,* 2019; Song *et al.,* 2019).

Hybridization plays an important role in the evolution of many lineages. In natural populations, hybridization may act in opposition to divergence, introduce adaptive variation into a population, drive the evolution of stronger reproductive barriers, or generate new lineages (Goulet *et al.,* 2017). The study of hybridization in plants has a long history. Among the plant kingdom, *Citrus* products have become one of the world's most economically important fruit crops through a largely obscure history of evolution and domestication. Diversity among *Citru*s and related genera seems to offer opportunities to create new and improved types of *Citrus*. The genus originated in a wide area spanning North-Eastern India to South China and South East Asia is thought to have been cultivated for thousands of years (Butelli *et al.,* 2019). Most of the current cultivars must have been originated from natural or induced mutations rather than from sexual breeding that is considered difficult for most *Citrus* species (Bona *et al.,* 2011). Hybridization of *Citrus* affects secondary metabolites' numbers, diversities and quantities (Kamiya *et al.,* 1979; Shaw *et al.,* 2001; Borges and Pio, 2003; Barboni *et al.,* 2009; Bassene *et al.,* 2009). Essential oils are specific to varieties and taxonomic groups. These oils are often used in comparative studies to assess the genetic diversity of a species, quantify the relationships between varieties or species, and classify the unknown on the basis of discriminating compounds (*e.g.,* in mandarin, kumquat, grapefruit, lemon, lime and citron) (Lota *et al.,* 2002; Luro *et al.,* 2012; Liu *et al.,* 2013; Guney *et al.,* 2015; Sutour *et al.,* 2016). Moreover, essential oils are used to characterize hybrid fruits from their parents' fruits. A few studies found in the literature concluded that the essential oil composition of a hybrid differed from those of the parents. The hybrid fruits were found to have characteristic volatile compounds (Shaw *et al.,* 2001; Barboni *et al.,* 2009; Bassene *et al.,* 2009).

Because of the worldwide economical and nutritional importance of *Citrus* fruits, farmers in Turkey started to cultivate many citrus varieties. As a result of hybridization studies, new citrus varieties are increasingly cultivated. Most of the commonly available and consumed citrus fruits, among others like lemon, orange, mandarin and grapefruits are hybrid fruits. Recently, Mexican lime (*Citrus aurantifolia* (Christm.) Swingle) and kumquat (*Fortunella japonica* Swingle) fruits have started to be cultivated in Turkey. Kumquats (*Fortunella* spp.) are the

smallest species compared to the other citrus species and grow naturally in China. Kumquat essential oils have flavor characters of typical citrus (Guney *et al.,* 2015). *Citrus aurantifolia* is a polyembriyonic species cultivated across the globe, mostly in hot subtropical or tropical regions, such as southern Florida, India, Mexico, Egypt, Iran and the West Indies. The juice and essential oil are the major commercial products (Spadaro *et al.,* 2012). In Cukurova Region, Turkey limequat has been produced as a bigeneric hybrid of *C. aurantifolia* and *F. japonica*. Limequat is similar in size and shape to kumquat but it is closer to Mexican lime in smell and taste. It can be consumed whole or its juice and rind can be used to flavor drinks and foods. It can be made into jams, jellies and preserves (Lim, 2012).

Evaluation of the composition of citrus hybrids may be more challenging because of the contribution of the traits of both parents. In the present study, headspace-SPME coupled with gas chromatography/mass spectrometry was used to characterize the volatile compound of the hybrid fruit "limequat" as well as its parent fruits "Mexican lime" and "kumquat" that were cultivated in Turkey.

MATERIALS AND METHODS

Plant material

Mature fruits were harvested from Subtropical Fruits Research and Experimental Center at Cukurova University, Adana, Turkey, in December 2017.

Fruit powder preparation

The sliced fresh and mature fruits were directly freeze dried for 48 hours. The obtained powders were passed through a 100 mesh sieve.

Isolation of the fruit volatiles

To trap volatile compounds in the fruit powders, Headspace-SPME (Headspace-Solid Phase MicroExtraction) technique was employed. The manual SPME device (Supelco, Bellafonte, PA, USA) with a fiber precoated with a 65 μm thick layer of polydimethylsiloxane/divinylbenzene (PDMS/DVB-blue) was used for extraction of the volatiles. The vial containing each fruit powder was sealed with parafilm. The fiber was pushed through the film layer for exposure to the headspace of the powder for 15 minutes at 40°C. The fiber was then inserted immediately into the injection port of the GC-MS for desorption of the adsorbed volatile compounds for analysis.

Analyzes of the fruit volatiles by GC-MS

The GC-MS analyses were carried out using an Agilent 5975 GC-MSD system. An Innowax fused silica capillary (FSC)

column (60 m × 0.25 mm, 0.25 µm film thickness) was used with helium as the carrier gas (0.8 mL/min). Oven temperature was kept at 60°C for 10 minutes, then programmed to 220°C at a rate of 4°C/min, and maintained constant at 220°C for 10 minutes. Finally, oven was programmed to 240°C at a rate of 1°C/min. Injector temperature was set at 250°C. Split flow was adjusted at 50:1. Mass spectra were recorded at 70 eV with the mass range *m/z* 35–450.

Identification of volatile compounds

Individual components were identified by computer matching with commercial mass spectral libraries (Wiley GC-MS Library, MassFinder 3 Library) and in-house "Baser Library of Essential Oil Constituents", which includes over 3200 authentic compounds with Mass Spectra. Retention data of pure standard compounds and components of known oils as well as MS literature data were also used for the identification (McLafferty and Stauffer, 1989; Joulain and Koenig, 1998; ESO 2000; Koenig *et al.,* 2004). These identifications were accomplished by comparing the retention times with authentic samples or by comparison of their relative retention indices (RRI) to a series of *n*-alkanes (Curvers *et al.,* 1985).

RESULTS AND DISCUSSION

Totally 16 monoterpenes, 42 sesquiterpenes, 23 oxygenated monoterpenes, 10 oxygenated sesquiterpenes and 10 miscellaneous compounds were characterized by GC-MS analysis. Table 1 shows the volatile compounds that were characterized in all of the fruits. As expected, all of the fruits were found to be rich in monoterpenes. In general, the groups in the fruits were as follows: Monoterpenes (48.3-72.0%) > sesquiterpenes (11.6-32.1%) > oxygenated monoterpenes (5.6-13.0%) > oxygenated sesquiterpenes (<1.0). Terpenoids are important due to their wide range of chemical and biological properties. Sesquiterpenes and their oxygenated analogues act as flavors and fragrances, pheromones, plant defense chemicals, or antibiotics (Schmidt *et al.,* 1999).

The genus *Citrus* has important crops like orange, lemons, grapefruits, limes and pummelos. *Citrus* essential oils that have been used widely in many applications over the years, include some major terpenes with biological activities like limonene, linalool, α-/β-pinene, sabinene, β-myrcene, humulene and α-terpineol. *Citrus* fruits are accepted as a major source of limonene (Mahato *et al.,* 2019; Simeone *et al.,* 2020).

The present study agreed with the literature as limonene was the most abundant monoterpene hydrocarbon among the identified monoterpenes. D-limonene is one of the most common monoterpenes in nature and a major constituent in several citrus oils. Limonene is a colorless liquid and exists as two enantiomers (D- or L-limonene) or as a racemic mixture. It possesses a pleasant lemon-like odor. Therefore, it is used widely as a flavor and fragrance additive in common food products. Besides its safety, it is an inexpensive fragrance for use in many products. Limonene was found to exert anti-inflammatory, anti-diabetic, anti-oxidant, anti-cancer, anti-allergic and anti-stress activities (Vieira *et al.,* 2018). Limonene containing essential oils and extracts may be promising dietary supplements. The present study suggested that kumquat, limequat and Mexican lime whole fruit powders may be used for different industrial purposes due to their rich limonene contents.

Other major compounds identified in fruits were germacrene D (6.5% of kumquat), linalool (4.9% of kumquat and 6.1% of limequat), β-bisabolene (4.6% of limequat), γ-terpinene (9.1% of Mexican lime) and *p*-cymene (10.4% of Mexican lime). Germacrene D is predicted as a precursor of many sesquiterpene hydrocarbons. It was shown that germacrene D undergoes acid-catalyzed cyclization yield cadinane, muurolane and amorphane sesquiterpenes (Bülow and König, 2000). Germacrene D's biological activity is on insects and other organisms (Silva *et al*., 2010). β-bisabolene, a sesquiterpene compound, has shown cytotoxic effects in recent studies. Yeo et al. (2015) found that β-bisabolene possessed tumour-specific pro-apoptotic properties in mouse and human breast cells both *in vitro* and *in vivo*. As a result, the researchers suggested further investigation of the use of β-bisabolene in the treatment of breast cancers (Yeo *et al.,* 2015).

When compared with conventional isolation techniques, HS-SPME is considered effective as it saves time and energy. HS-SPME is an eco-friendly technique because of its solvent free nature. For isolation and comparison of the volatiles of *Citrus* species and cultivars and/or detection of changes in volatile compounds during fruit growth, HS-SPME is a preferred technique. In addition, it is used to identify potential markers to that will help to distinguish geographical origin of fruits. The fingerprint analyzes of Pummelo cultivars in China (Zhang *et al.,* 2017), characterization of lemon wax volatiles in Italy (Costa *et al.,* 2019), characterization of orange cultivars

including 137 samples from three different geographical origins, such as Italy, South Africa, and Spain (Centonze *et al.,* 2019), volatiles of Persian lime (*C. latifolia*) from Mexico (Ledesma-Escobar *et al*., 2019), stability of orange essential oil/β-cyclodextrin inclusion complex (Kringel *et al.,* 2017) and characterization of chemical markers in satsuma mandarin (*C. unshiu* Marc.) honey (Jerkovic *et al.,* 2016) include the examples of which HS-SPME was used for the isolation of volatiles.

Table 1: The volatile compounds of kumquat, limequat and Mexican lime.

RRI	Compound	Kumquat	Limequat	Mexican lime	IM
1032	α-Pinene	tr	0.9	4.1	RRI, MS
1076	Camphene	nd	nd	1.6	RRI, MS
1118	β-Pinene	nd	0.6	5.2	RRI, MS
1174	Myrcene	2.1	1.2	2.0	RRI, MS
1188	α-Terpinene	nd	0.4	1.6	RRI, MS
1203	**Limonene**	46.6	37.0	29.9	RRI, MS
1218	β-Phellandrene	0.3	0.5	nd	RRI, MS
1224	*o*-Mentha-1(7)-5,8-triene	nd	nd	3.8	MS
1246	*(Z)*-β-Ocimene	tr	nd	0.2	MS
1255	γ-Terpinene	tr	3.3	9.1	RRI, MS
1266	*(E)*-β-Ocimene	0.6	nd	nd	MS
1280	*p*-Cymene	0.3	3.4	10.4	RRI, MS
1290	Terpinolene	tr	0.4	1.0	RRI, MS
1327	3-Methyl-2-butenol	tr	0.2	nd	MS
1348	6-Methyl-5-hepten-2-one	0.7	0.3	1.0	MS
1382	Alloocimene	0.1	tr	0.3	MS
1413	Rosefurane	nd	nd	0.2	MS
1450	*trans*-Linalool oxide (furanoid)	nd	0.4	0.1	MS
1452	α-*p*-Dimethylstyrene	0.1	0.6	2.6	MS
1466	α-Cubebene	2.0	0.8	nd	MS
1478	Furfural	nd	tr	0.1	MS
1478	*cis*-Linalool oxide (furanoid)	nd	0.4	nd	MS
1479	δ-Elemene	0.9	0.5	nd	MS
1483	Octyl acetate	1.0	nd	nd	RRI, MS
1493	α-Ylangene	0.6	1.3	0.2	MS
1495	Bicycloelemene	0.8	nd	nd	MS
1497	α-Copaene	1.5	0.5	0.1	MS
1506	Decanal	tr	tr	0.1	RRI, MS
1535	β-Bourbonene	0.1	nd	nd	MS
1544	α-Gurjunene	0.1	nd	nd	MS
1549	β-Cubebene	1.0	tr	nd	MS
1553	Linalool	4.9	6.1	1.4	RRI, MS
1562	Isopinocamphone	nd	nd	0.1	MS
1565	Linalyl acetate	1.9	0.4	0.2	RRI, MS
1568	1-Methyl-4-acetylcyclohex-1-ene	tr	0.1	nd	MS
1572	α-Bergamotene	tr	0.4	0.2	MS
1589	β-Ylangene	3.6	0.8	nd	MS
1594	*trans*-β-Bergamotene	tr	3.8	2.2	MS
1600	β-Elemene	0.5	0.4	nd	MS
1610	β-Copaene	2.7	0.7	nd	MS
1611	Terpinen-4-ol	tr	1.5	0.9	RRI, MS
1612	β-Caryophyllene	0.8	3.2	2.4	RRI, MS
1616	Hotrienol	nd	0.2	nd	MS
1617	6,9-Guaiadiene	tr	nd	nd	MS
1628	Aromadendrene	0.3	nd	nd	MS
1645	Cadina-3,5-diene	0.2	nd	0.2	MS
1650	γ-Elemene	0.9	1.6	nd	MS

1659	γ-Gurjunene	1.0	nd	nd	MS
1661	Alloaromadendrene	tr	nd	nd	MS
1661	*trans*-Pinocarvyl acetate	nd	nd	tr	MS
1664	*(Z)*-β-Santalene	nd	0.3	0.2	MS
1668	Citronellyl acetate	0.2	0.1	nd	RRI, MS
1668	*(Z)*-β-Farnesene	nd	0.3	tr	MS
1670	*trans*-Pinocarveol	nd	nd	tr	MS
1687	α-Humulene	0.3	0.6	0.3	RRI, MS
1688	Selina-4,11-diene	nd	nd	0.1	MS
1692	Bicyclosesquiphellandrene	0.8	0.1	nd	MS
1695	*(E)*-β-Farnesene	nd	0.2	0.1	MS
1704	γ-Muurolene	2.1	1.6	nd	MS
1706	α-Terpineol	0.5	3.3	1.5	RRI, MS
1707	δ-Selinene	0.3	0.9	0.6	MS
1719	Borneol	nd	tr	0.2	RRI, MS
1720	4,6-Guaiadiene	0.1	0.2	0.1	MS
1726	Germacrene D	6.5	0.6	nd	MS
1737	Neryl acetate	0.3	tr	nd	RRI, MS
1740	α-Muurolene	0.8	nd	nd	MS
1741	β-Bisabolene	nd	4.6	2.4	MS
1741	Geranial	nd	nd	0.5	RRI, MS
1742	β-Selinene	0.8	0.4	0.4	MS
1744	α-Selinene	0.6	0.6	0.6	MS
1751	Carvone	0.3	0.2	nd	RRI, MS
1757	Dihydrocarveol	0.1	nd	nd	MS
1758	*(E,E)*-α-Farnesene	nd	nd	1.0	MS
1765	Geranyl acetate	2.6	0.2	0.7	RRI, MS
1773	δ-Cadinene	1.3	0.7	0.1	MS
1776	γ-Cadinene	0.9	0.5	tr	MS
1784	*(E)*-α-Bisabolene	nd	0.1	tr	MS
1796	Selina-3,7(11)-diene	0.1	1.2	0.3	MS
1799	Cadina-1.4-diene	0.1	nd	nd	MS
1807	Perilla aldehyde	0.6	0.1	tr	MS
1811	α-Cadinene	0.2	0.1	nd	MS
1837	Eudesma-5,7(11)-diene	tr	0.2	0.1	MS
1853	Calamenene	0.2	0.1	nd	MS
1857	Geraniol	tr	tr	tr	RRI, MS
1864	*p*-Cymen-8-ol	nd	nd	tr	MS
1871	*p*-Mentha-1,8-dien-10-yl acetate	0.2	nd	nd	MS
1900	*epi*-Cubebol	tr	nd	nd	MS
1916	Perilla acetate	0.1	nd	nd	MS
1941	α-Calacorene	tr	0.1	nd	MS
1957	Cubebol	tr	nd	nd	MS
2037	Salvial-4(14)-en-1-one	0.1	tr	nd	MS
2050	*(E)*-Nerolidol	tr	0.1	nd	MS
2096	Elemol	tr	0.1	nd	MS
2144	Spathulenol	0.2	nd	nd	MS
2185	γ-Eudesmol	nd	tr	nd	MS
2232	α-Bisabolol	nd	tr	nd	MS
2250	α-Eudesmol	tr	0.1	nd	MS
2257	β-Eudesmol	tr	0.2	nd	MS
2300	Tricosane	tr	0.1	tr	RRI, MS
2500	Pentacosane	tr	0.3	tr	RRI, MS
2600	Hexacosane	tr	0.3	tr	RRI, MS
2700	Heptacosane	tr	0.4	tr	RRI, MS
2800	Octacosane	tr	0.4	tr	RRI, MS
2900	Nonacosane	tr	0.4	tr	RRI, MS
	TOTAL	95.9	91.6	90.4	

RRI: Relative retention indices calculated against *n*-alkanes; %: calculated from TIC data; tr: Trace (< 0.1 %)
nd: not detected. IM: Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

Kumquat fruits and volatile compounds

In kumquat fruits, 80 volatile compounds were identified representing 95.9% of the sample. The percentages of monoterpenes, sesquiterpenes, oxygenated mono- and sesquiterpenes were calculated as 50.1%, 32.1%, 11.6% and 0.3%, respectively. Limonene (46.6%), germacrene D (6.5%) and linalool (4.9%) were the main terpenic constituents followed by β-ylangene (3.6%), β-copaene (2.7%), geranyl acetate (2.6%), γ-muurolene (2.1%), α-cubebene (2.0%), linalyl acetate (1.9%), α-copaene (1.5%) and δ-cadinene (1.3%). Other characteristic compounds included octyl acetate (1.0%), γ-gurjunene (1.0%), α-muurolene (0.8%), bicycloelemene (0.8%), *(E)*-β-ocimene (0.6%), aromadendrene (0.3%), *p*-mentha-1,8-dien-10-yl acetate (0.2%), spathulenol (0.2%), β-bourbonene (0.1%), α-gurjunene (0.1%), dihydrocarveol (0.1%), cadina-1,4-diene (0.1%), and trace compounds (cubebol, alloaromadendrene, 6,9-guaiadiene).

The essential oil compositions of peel and fruit were reported by previous studies in different countries. The essential oil was characterized with very high limonene content.

Kumquat fruits cultivated in Italy were hydrodistilled by a Clevenger-type apparatus. The essential oil contained 31 compounds. Among them, limonene (91.51%) was the most abundant. Also, myrcene (2.9%), *cis*-muurola-4(14,5)-diene (1.6%), α-thujene (0.8%) and linalool (0.7%) were the other major compounds (Schirra *et al.,* 2008).

Nouri and Shafaghatlonbar (2016) reported that the peel essential oil comprised 51% of limonene and 12.1% of germacrene D.

Limequat fruits and volatile compounds

In limequat fruits, 75 volatiles were detected comprising 91.6% of the sample. General groups were classified as monoterpenes (48.3%), sesquiterpenes (27.4%), oxygenated monoterpenes (13.0%) and oxygenated sesquiterpenes (0.5%). Limonene (37.0%), linalool (6.1%) and β-bisabolene (4.6%) were found to be the major constituents. The other volatiles more than 1% were *trans*-β-bergamotene (3.8%), *p*-cymene (3.4%), γ-terpinene (3.3%), α-terpineol (3.3%), β-caryophyllene (3.2%), γ-elemene (1.6%), terpinen-4-ol (1.5%) and selina-3,7(11)-diene (1.2%). *cis*-linalool oxide (furanoid) (0.4%), hotrienol (0.2%), γ-eudesmol (<0.1%) and α-bisabolol (<0.1%) were only found in limequats. Bigeneric hybrid limequat has common volatile compounds with its parents. Ten compounds were common in limequat and Mexican lime. Among these compounds, β-pinene (0.6%, 5.2%), α-terpinene (0.4%, 1.6%), as β-bisabolene (4.6%, 2.4%) were major

compounds. Twenty-four common volatile compounds were detected in limequat coming from kumquat. Germacrene D (6.5%, 0.6%), γ-muurolene (2.1%, 1.6%), γ-elemene (0.9%, 1.6%), β-copaene (2.7%, 0.7%), β-ylangene (3.6%, 0.8%) and α-cubebene (2.0, 0.8%) were the common major constituents.

In a study carried by Guney *et al.* (2015) investigated on five kumquat species (*F. hindsii, F. crassifolia, F. obovata, F. margarita*, *C. aurantifolia* x *F. japonica*) cultivated in Turkey, each fruit was homogenized with a blender and an automatic headspace technique was used to trap volatile compounds. Limequat was characterized with its high limonene (88.7%) content and β-myrcene (2.5%). Interestingly, when compared to the present study, linalool was not detected and the amount of β-bisabolene (0.8%) was very low. The present study agreed with Guney *et al.* that limequat fruits were rich in terpenic compounds.

Eun-Jin *et al.* (2010) analyzed the peel essential oil of *F. japonica* var. *margarita* native to Island of Jeju, Korea. Limonene (61.6%) and carvone (6.4%) were found to be major compounds.

Mexican lime and volatile compounds

In Mexican lime fruits, 58 volatile compounds were detected representing 90.4% of the sample. Monoterpene content (72.0%) was high in the fruits followed by sesquiterpenes (11.6%) and oxygenated monoterpenes (5.6%) while no oxygenated sesquiterpenes were detected. Monoterpenes like limonene (29.9%), *p*-cymene (10.4%), γ-terpinene (9.1%) and β-pinene (5.2%) were the main constituents. α-pinene (4.1%), *o*-mentha-1(7)-5,8-triene (3.8%), α-*p*-dimethylstyrene (2.6%), β-bisabolene (2.4%), β-caryophyllene (2.4%), *trans*-β-bergamotene (2.2%), α-terpineol (1.5%) and linalool (1.4%) were among the major constituents. *o*-Mentha-1(7)-5,8-triene (3.8%), camphene (1.6%), *(E,E)*-α-farnesene (1.0%), geranial (0.5%), rosefurane (0.2%), isopinocamphone (0.1%), selina-4,11-diene (0.1%), *p*-cymen-8-ol (<0.1%), *trans*-pinocarveol (<0.1%) and *trans*-pinocarvyl acetate (<0.1%) were only detected in Mexican lime fruits.

CONCLUSION

In conclusion, the volatile compounds of kumquat, limequat and Mexican lime fruit powders isolated by HS-SPME coupled with GC-MS were identified. Totally, a hundred and four volatile compounds comprising mono-/sesquiterpenes, oxygenated mono-/sesquiterpenes were identified in the fruits. Monoterpenes were

dominant groups in the fruits with different percentages. Limonene was the abundant monoterpene with 46.6%, 37.0% and 29.9% of the kumquat, limequat and Mexican lime fruits, respectively.

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The effects of some organic solvents on the modified Ellman procedure for the assay of cholinesterases

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Abstract

Ellman's method is the most acknowledged procedure in order to determine the cholinesterase inhibitory potential of substances. It is a quick and high-throughput screening method. Therefore, it is widely used to measure the activities of original molecules and mixtures in aqueous solvents through a UV method. Regarding that many original or known substances as well as mixtures require some organic solvent to guarantee dissolution within this methodology, it becomes critical to be aware of the inhibitory potential of organic solvents. From this perspective, within the present study, it was aimed to screen the inhibitory potential of some organic solvents on human acetylcholinesterase and human butyrylcholinesterase enzymes. The results displayed the potent inhibitory function of DMSO. On the other hand, alcohols also pointed out varying degrees of deactivation of the catalytic function of cholinesterases.

Keywords

Acetylcholinesterase, butyrylcholinesterase, Ellman's method, organic solvents.

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INTRODUCTION

For more than 85 years, scientists have been working on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes from various scientific perspectives. Cholinesterases are the enzymes that cleave acetylcholine to choline and acetate. These enzymes are the primary targets of drugs used in Alzheimer's disease. Moreover, they are employed as pesticides, nerve agents and drugs which are used in the treatment of myasthenia gravis and Parkinson's disease (Dingova *et al.,* 2014). Cholinesterases are also related with the etiopathogenesis of some diseases like cancer, cardiovascular diseases, and obesity (Dingova *et al.,* 2014). From this point of view, the determination of cholinesterase activity or its inhibition is crucial.

Both qualitative and quantitative assays are employed to determine the activity or inhibition of cholinesterases. These assays are applicable in many fields as well as in pharmaceutical sciences (Miao *et al.,* 2009). Cholinesterase activity and its inhibition are determined employing numerous methods (Miao *et al.,* 2009). These methods include, spectrometric (Uv-Vis (McOsker and Daniel, 1959), fluorometric (Guilbault and Kramer, 1965), diffractometric (Walker and Asher, 2005), mass spectrometric (De Jong et al., 2006) assays), thin layer chromatography (TLC) (Marston et al., 2002), radiometric (Wininteringham and Disney, 1962) and calorimetric assays (O'Farrell et al., 1977), biosensor tests (Cesarino *et al.,* 2012), colorimetric sticks or strip based assays (Augustinsson, 1957), histochemical localization of acetylcholinesterase (Koelle and Friedenwald, 1949) and chip techniques (Hadd *et al.,* 1999). Spectrometric assay is still the most extensively used technique among all of these methods (Miao *et al.,* 2009).

Ellman's method is categorized as UV-Vis spectrometric assay and it is the most prevalent type of assays which is used to determine cholinesterase activity. It provides continuous monitoring of acetylthiocholine (an alternative substrate to acetylcholine) hydrolysis by AChE under in vitro conditions (Ellman *et al.,* 1961). This assay consists of two reactions. First one is the hydrolysis of thioesters (acetylthiocholine/ATch or butyrylthiocholine/BTch) to thiocholine by cholinesterases (AChE or BChE). The second reaction involves the interaction of thiocholine with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) which yields out a yellow product, referred to as 2- nitro-5-thiobenzoic acid (TNB) (Dingova *et al.,* 2014). Color intensity of the product that is

measured at 412 nm is proportional to the enzymatic activity (Miao *et al.,* 2010).

Ellman's method is cheap, simple, fast and accurate which makes it indispensable for the measurements and monitoring of cholinesterase activity. The activities of many cholinesterase inhibitors are measured using Ellman's method. In order to conduct these experiments, organic solvents are generally required to aid dissolution, since many compounds are not purely soluble in aqueous buffers employed.

The present study aimed to screen the effects of the widely employed organic solvents on the activity of cholinesterases. Although similar studies on the topic were conducted previously, this study provides sufficient information, since the effects of commonly used organic solvents are investigated and the human enzymes are employed.

MATERIALS AND METHODS

Organic solvents were obtained from Sigma Aldrich (CA, USA). Their purities were more than 96 % as stated on their labels. The effects of organic solvents on human AChE and human BChE were determined by modified spectrophotometric method of Ellman (1961). Human recombinant AChE (HuAChE) (Sigma) and human BChE (Sigma) were used as enzymes for cholinesterase activity studies. Acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as the substrates of the reaction. 5,5′-Dithio-bis(2-nitrobenzoic) acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of the cholinesterase activity. 50 mM Tris HCl buffer (pH 8.0), 6.8 mM DTNB, 2 µl of sample solutions and 10 µl of AChE/BChE solution were added. The reactions were initiated with the addition of 10 µl of acetylthiocholine iodide or butyrylthiocholine chloride. Following the incubation for 15 minutes at 27°C, the hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride was monitored by the formation of the 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines. The measurements and calculations were assessed by using SkanIt Software 2.4.5 RE for Varioskan Flash software. Percentage of inhibition of AChE and BChE was determined by the comparison of rates of reaction of the samples relative to blank sample (buffer) using the formula;

$$(E-S)/E \times 100$$

where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicates.

RESULTS AND DISCUSSION

Percent inhibitions of organic solvents, respectively at 1, 2.5, and 5% v/v concentrations are shown in Table 1 and 2.

Table 1: Effects of different organic solvents on ACHE inhibition.

% Inhibition			
Solvents	**1%˙**	**2.5%˙**	**5%˙**
Methanol	1.0 ± 0.02	3.4 ± 0.08	5.2 ± 0.11
Ethanol	1.4 ± 0.06	2.9 ± 0.17	4.8 ± 0.21
n-propanol	4.9 ± 0.13	6.8 ± 0.05	9.3 ± 0.27
iso-propanol	9.0 ± 0.07	12.7 ± 0.16	27.4 ± 0.24
n-butanol	3.8 ± 0.03	7.9 ± 0.11	9.9 ± 0.10
Ethylene glycol	0.8 ± 0.09	2.9 ± 0.11	4.4 ± 0.17
Dimethyl sulfoxide	18.2 ± 0.21	30.8 ± 0.09	41.0 ± 0.51
Acetone	9.7 ± 0.21	19.8 ± 0.24	28.9 ± 1.21
Acetonitrile	1.4 ± 0.05	2.4 ± 0.21	3.1 ± 0.25
1,4-Dioxane	17.0 ± 0.09	28.4 ± 0.17	34.5 ± 0.47
Donepezil	NT	NT	89.5± 0.68*

NT: Not tested, * 5µM concentration was employed, ˙v/v

Table 2: Effects of different organic solvents on BCHE inhibition.

% Inhibition			
Solvents	**1%˙**	**2.5%˙**	**5%˙**
Methanol	0.9 ± 0.03	1.8 ± 0.11	2.2 ± 0.07
Ethanol	0.8 ± 0.04	1.3 ± 0.08	1.5 ± 0.33
n-propanol	2.5 ± 0.08	5.2 ± 0.18	11.8 ± 0.33
iso-propanol	6.7 ± 0.10	10.9 ± 0.53	19.9 ± 0.08
n-butanol	7.5 ± 0.09	8.4 ± 0.27	10.7 ± 0.25
Ethylene glycol	1.3 ± 0.18	3.5 ± 0.08	4.9 ± 0.07
Dimethyl sulfoxide	16.8 ± 0.87	25.9 ± 1.28	44.8 ± 0.88
Acetone	6.9 ± 0.47	17.9 ± 0.88	31.4 ± 1.04
Acetonitrile	2.8 ± 0.17	5.9 ± 0.77	9.9 ± 1.21
1,4-Dioxane	10.6 ± 0.05	17.5 ± 0.22	26.9 ± 0.55
Donepezil	NT	NT	68.8± 0.14*

NT: Not tested, * 5µM concentration was employed, ˙v/v.

According to the results, varying effects of organic solvents were observed in terms of inhibition of cholinesterase enzymes. In particular, dimethyl sulfoxide (DMSO) has been found as the most potent inhibitor among the organic solvents employed. On the other hand, 1,4-Dioxane was also assessed as a potent inhibitor. Alcohols display varying degrees of inhibition. Mainly, methanol and ethanol, two of the most frequently used solvents, have shown to display weak inhibition even at 5% v/v concentrations.

In general, enzyme inhibition based assays limit the employment of organic solvents at 5%. From this perspective, DMSO, isopropanol, acetone, and 1,4-Dioxane were shown to exhibit equal or higher than 20% inhibition in AChE and BuChE catalyzed reactions. Obviously, this outcome makes the employment of these organic solvents questionable.

Indeed, organic solvents are indispensable parts of enzyme inhibition assays. Many drugs, original drug candidates and mixtures obtained through herbal extracts are not purely soluble in water. In majority of the Ellman's test, buffers are employed at the pH of 7 or 8. Although ionization depending on the functional groups at this pH might aid in aqueous dissolution, it becomes inadequate for many chemicals. Organic solvent application up to certain concentration, therefore, aids in dissolution of these compounds.

The results obviously pointed out the fact that the generalization of the limitation of organic solvent application up to 5% v/v is not suitable, since organic solvents display varying degrees of inhibition at this concentration. Ethanol, methanol, and acetonitrile appeared to be the safest organic solvents under the experimental conditions. In other words, the positive controls wherein the full activity is obtained without a specific inhibitor should include the organic solvent at the specified concentration at least in order to prevent high inhibitory potential measurements. This is particularly valid for DMSO and acetone, as they are water miscible and widely used as solubility enhancer in Ellman's method based cholinesterase inhibition assays.

CONCLUSION

In this study, the effects of some organic solvents, commonly used as solubility enhancers, on the modified Elmman's method were investigated. It was observed that each organic solvent has varying inhibitory effects. Ethanol, methanol, and acetonitrile were found to display the weakest potentials, and therefore they were evaluated as the safest solvents. On the other hand, DMSO and acetone, two of other commonly used solvents were found to have serious inhibitory potential at 5% v/v concentration. This finding definitely requires the employment of these organic solvents in full activity assays where the enzyme catalytic function is determined in the absence of an inhibitor.

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Investigation of CRISPR anti-phage systems of *Lactobacillus plantarum* from pickled olives

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Abstract

Lactobacillus plantarum is the most commonly used microorganism in industrial food fermentations. The multidrug resistance, bacteriocin production, various enzyme activities, probiotic properties and the resistance of the strain against bacteriophages, are important for application in industrial field. Microorganisms have developed various survival strategies in the evolutionary process. Genomic anti-phage role of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas) is one of the defense strategies. This is an RNA-based immune system involved in the collaboration of CRISPR-related (cas) genes with DNA sequences of foreign genetic elements inserted between the repeated sequences.

In this study, a rapid diagnosis of *L. plantarum* was carried out among 59 pickled olives by Real-Time Polymerase Chain Reaction (PCR) with species-specific probes and primers. Twenty-five isolates were identified as *L. plantarum*. The presence of CRISPR-Cas loci of the genomic anti-phage system in these identified species was investigated using DNA based and bioinformatics methods such as PCR, sequencing, and molecular software. Specific primer design for *L. plantarum* CRISPR arrays was performed. CRISPR loci were detected in 14 *L. plantarum* strains via classical PCR method. The results were analyzed using NCBI-Blast, CRISPRFinder databases. In one of the isolates, 6 repeats, and 5 spacers nucleotide arrays were found. CRISPR-related proteins (Dead/Deah box helicases) were detected in remaining isolates.

Keywords

Anti-phage system, CRISPR-cas, CRISPRFinder, *L. plantarum*, pickled olives, TaqMan probe.

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INTRODUCTION

Table olives are of great importance among fermented vegetables worldwide. Turkey has a significant potential in the world's olive production, accounting for 7% of total olive and 17% of total table olive production (Cetin *et al.,* 2004). Izmir province is one of the leading production areas in Turkey. Table olive is very important for local people in Turkey and is fermented by lactic acid bacteria (LAB) and yeasts, which are natural biota of olives. The fermentation process is carried out by competition between these two groups of microorganisms. Yeasts play a minor role in the development flavor and aroma of table olives and in the improvement of LAB.

Lactobacillus spp. is the most frequently isolated bacteria from table olives. The predominant species in most natural and treated fermentation of table olives are *L. plantarum* and *L. pentosus*. In food fermentation, lactobacilli, especially *Lactobacillus* spp., play a major role as starters cultures, bio preservatives, and antimicrobial compounds (de Vries *et al.,* 2006). *L. plantarum* is one of the members of healthy human microbiota and has anti-microbial or anti-tumorigenic activities (Bevilacqua *et al.,* 2010; Chiu *et al.,* 2008; Mathara *et al.,* 2008). *L. plantarum* has an extended environmental niche, including fermented dairies, meat, and vegetables (Kleerebezem *et al.,* 2003).

Microorganisms have developed various strategies to avoid exposure to foreign genetic elements. Although abundant and ubiquitous viruses infect them, microorganisms routinely survive and thrive in the competitive environments. Continuous exposure to exogenous DNA through transduction, conjugation, and transformation has enabled microorganisms to recognize and distinguish "foreign" DNA from their "own" DNA, and develop a set of defense mechanisms that allow them not to be exposed to invasive elements. These systems not only preserve genetic integrity, also allow the uptake of external DNA and conservation of advantageous genetic material for adaptation to the environment. Some strategies, such as adsorption prevention, injection blocking, and abortive infection are effective against viruses. Other defense systems specifically target the invasive nucleic acid, such as the restriction-modification system (R-M) and the use of specific nucleases. Recently, an adapted microbial immune system that provides short palindromic repeats (CRISPR) at regular intervals has been identified and the system is reported to be an element of acquired immunity against viruses and plasmids (Barrangou and Horvath, 2012). CRISPR system includes DNA repeats from archaeal (~90%) and bacterial (~40%) genomes. CRISPR loci are

typically characterized by spacers (mostly phage and plasmid sequences) and *cas* genes are generally associated with CRISPR. Considering the importance of CRISPR loci in the adaptation and persistence of bacteria against viruses, this system clearly provides information about the evolution of genomes belonging to phages and hosts (Horvath *et al.,* 2008).

In this study, the TaqMan 5' nuclease assay method was utilized as a fast and secure vehicle for the detection, identification, and molecular characterization of *L. plantarum.* The presence of CRISPR-Cas loci of the genomic anti-phage system in *L. plantarum* isolates was investigated by molecular and bioinformatics methods.

MATERIALS AND METHODS

Bacterial isolation and media

In the present study, 59 samples were collected from Aydin-İzmir, Turkey. The samples were cultured by spread plate method on Mann Rogosa Sharp (MRS) agar containing 40% cycloheximide. The media were incubated at 30 °C for 48-72 hours under atmosphere containing 10% CO_2. Single colonies were subcultured in MRS broth supplemented with 10% ethanol (EMRS). Colonies were primarily identified based on Gram characteristics and cell morphologies. The strains were further identified by *L. plantarum* specific real-time PCR (RT-PCR). Purified cultures were stored in 30% (v/v) glycerol at -20 °C until further processing.

DNA isolation

QIAamp DNA mini kit was used for extracting DNA of the isolates. Isolated DNAs were used for the identification of the strains by species-specific qualitative PCR. PCR analyzes were carried out by Roche Light Cycler PCR. Amplification products were controlled by gel electrophoresis where the agarose is stained with GelRed.

Species-specific quantitative real-time PCR (TaqMan5' Nuclease Assay)

The optimized probe and primers of *Lactobacillus* spp. and *L. plantarum* specific to 16S-23S intergenic spacing regions that were designed based on EMBL and GenBank DDBJ databases were used (Haarman and Knol, 2006). 6.8 µl of nuclease-free ultra-pure water; 0.5 µL forward primer (20 µM); 0.5 µL reverse primer (20 µM); 0.2 µL of TaqMan probe (10 mM); 10 µL of enzyme and dNTP mixture were used for each reaction. The total volume of PCR reaction was 20 µL with 2 µL of template DNA. Sequences of primers and probes are listed in Table 1. PCR reaction conditions are listed in Table 2.

Table 1: Probe and primers for TaqMan5' Nuclease assay.

Forward primer	TGG ATC ACC TCC TTT CTA AGG AAT
Reverse primer	TGT TCT CGG TTT CAT TAT GAA AAA ATA
Probe	FAM-ACA TTC TTC GAA ACT TTG T NFQ-MGB

Table 2: RT-PCR reaction conditions for identification.

Program	**Denaturation**	**Amplification**			**Cooling**
Analysis mode	None	Quantification mode			None
Cycle	1	45			1
Target [°C]	95°C	95°C	57°C	72°C	40°C
Time[hh:mm:ss]	00:10:00	00:00:10	00:00:30	00:00:10	00:00:30
Rate[°C/s]	**20**	**20**	**20**	**20**	**20**

CRISPR loci analysis in *L. plantarum*

CRISPR sequences belonging to *L. plantarum* were scanned using the CRISPRFinder program and CRISPR site specific primers were designed using the Primer3web database. PCR application was performed using primers specific to CRISPR gene regions of *L. plantarum* (Table 3). Reaction mixture was as follows: 25 µl PCR Master-Mix (2X); 1 µl forward primer, 1 µl reverse primer; 5 µl of template DNA; 18 µl of nuclease-free ultrapure water. PCR reaction conditions are shown in Table 4. PCR products were visualized by agarose gel electrophoresis. PCR products were sent to Altigen-Bio Biotechnology Company (Izmir-Bornova, Turkey) for sequence analysis. Arrangement and analysis of DNA sequences were analyzed by DNA Baser program. The results of the PCR products were analyzed on the CRISPRFinder and NCBI-Blast database.

Table 3. CRISPR loci primers specific to *L. plantarum.*

Forward Primer	5'-TGG CGT GAT ATG AAT TAA TGA GT-3'
Reverse Primer	5'-GGG AAA AGA TGG CGT GAT TG-3'
Degenerate Forward Primer	5'-TCG AAT GGA AAA GTT MAA AA-3'
Degenerate Reverse Primer	5'-AAC CTR TWT TRG TTG GTG AG-3'

Table 4. PCR reaction conditions for CRISPR array.

Stages	**Temperatures**	**Times**	**Cycles**
Initial denaturation	95 °C	2 min	
Denaturation	95 °C	0:30 sec	
Annealing	48 °C	0:45 sec	35
Elongation	72 °C	1:30 min	
Final elongation	72 °C	5 min	

RESULTS AND DISCUSSION

As a result of microscopical examination of isolates, it was determined that 40 isolates from 59 saline samples were Gram positive and rod-shaped. The remaining isolates were cocci or large yeast cells. In previous studies, it was reported that LAB developed spontaneously in processed olives. Also the presence of yeast populations was demonstrated in natural olives. (Hurtado *et al.,* 2008). The isolation of *Lactobacillus* spp. from table olive brine samples and the differentiation of yeasts from these strains are quite difficult. To address this problem, 20 mL/L 1% (w/v) cycloheximide was added to the MRS agar medium for isolation (Sharp, 1962). However, the amount of cycloheximide was not enough to inhibit the yeasts. To overcome this problem, higher concentrations of cycloheximide were tested and the concentration of 40 mL/L 1% (w/v) was detected to have optimal potential to inhibit the yeast population. However, additional procedures were required to separate bacilli and yeasts. EMRS broth was utilized to inhibit yeasts and stimulate the growth of bacilli in a misture of yeasts and lactobacilli. After 3 days of incubation with shaking at 30 °C, high ethanol concentration in EMRS liquid medium was detected. The growth of yeasts was limited, and pure lactobacilli colonies were observed on MRS agar plates. According to the results of the study, the use of EMRS liquid medium in the isolation of *Lactobacillus* spp. is a critical step, and the medium can be used as an effective selective medium for the isolation of *Lactobacillus* spp. Similar to the yeast, *Lactobacillus* spp. has white mucoid colonies in MRS agar. The microscopic appearance of lactobacilli is Gram-positive, rods with straight form. However, they can be in a spiral or coccobacillary form under certain conditions. They are often found in pairs or chains of varying lengths (Altermann *et al.,* 2005). On the other hand, yeasts can have circular, elliptical, triangular, bottle-shaped appearance (de Becze, 1956). Therefore, the phenotypic identification is often unreliable, and molecular identification of *Lactobacillus* spp. is required.

In recent years, PCR is utilized as a rapid and powerful technique for *in vitro* amplification of DNA (especially 16S rDNA genes) to identify bacterial genus and species (Goldstein *et al.,* 2015). Moreover, in the last few years, the quantification of 16srRNA gene by quantitative RT-PCR, has become one of the most useful methods (Schefe *et al.,* 2006). However, in addition to the operational advantages, RT-PCR (TaqMan5' Nuclease) is more sensitive and reproducible. Therefore it has recently

replaced traditional PCR in diagnostic studies (Gibson *et al.,* 1996). In a study conducted by Haarman and Knol (2006), all *Lactobacillus* spp. from the samples were correctly detected by RT-PCR, while closely related *Enterococcus* spp. and *Propionibacterium* spp. strains did not yield any amplification.

The Minor Groove Binder (MGB) probe was used in this study, which consisted of two sections: 5' reporter dye and 3' Non-Fluorescent Quencher (NFQ). The advantage of NFQ is to provide a lower background signal, which results in better sensitivity in the quantification. On the other hand, the MGB fraction balances the hybridization of the probe with single-stranded DNA targets by increasing the melting temperature and thereby reducing the length requirements of the oligodeoxynucleotides. In practice, the specificity of an MGB probe on a traditional TaqMan probe should be increased (Yao *et al.,* 2006). Due to the stability of the generated DNA duplexes, it is possible to use shorter probes by MGB probes, with higher sensitivity to single base mismatches, providing additional sequence specificity when the mismatch is below the MGB site. To benefit from these properties, fluorogenic 3′-MGB probes were prepared and studied in 5'-nuclease PCR analysis. MGB probes are shorter length probes with better sequence specificity and lower fluorescence background than none-MGB probes (Kutyavin *et al.,* 2000). The sensitivity of the TaqMan probe technique and the specificity of the probes and primers are found to be 1,000 to 10,000 times more sensitive than conventional PCR analysis.

In this research, DNA was extracted from 40 samples of LAB with similar morphology after incubation in MRS broth media and then, visualized by nano-spectroscopy and agarose gel. All of the DNA extracts were detected to be between 55-200 ng/µL at 260-280 nm wavelength. Twenty-five of 40 isolates obtained by culture method were identified as *L. plantarum* by RT- PCR assay using a gene-specific TaqMan probe and primer pairs specific to the 16S-23S intergenic region (Figure 1).

PCR analysis performed by CRISPR primers showed amplification in 14 of 25 *L. plantarum* strains. A typical CRISPR sequence (Repeat-Spacer) was found in only one of the 14 isolates (Figure 2). In the other 13 isolates, no locus was found on the CRISPRFinder database. However, each of the 14 isolates was identified to have CRISPR-associated proteins in the NCBI database. The spacer sequences, which were detected in an isolate (designated as the L3), were also examined. The length of the CRISPR region was found to be 440 base pairs (bp) and the repeat region was 36 bp (Table 5).

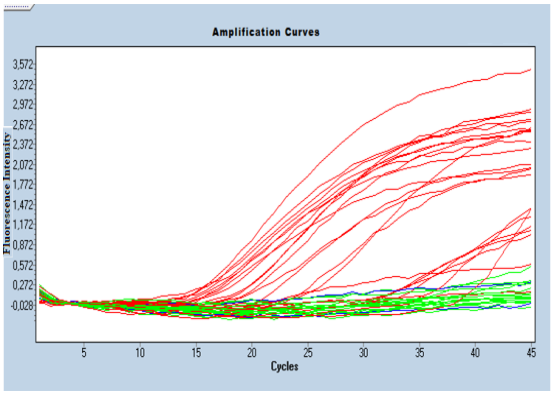

Figure 1: RT-PCR amplification curve.

The red lines running exponentially in the amplification curve shown in the figure indicate the species identified as *L. plantarum*.

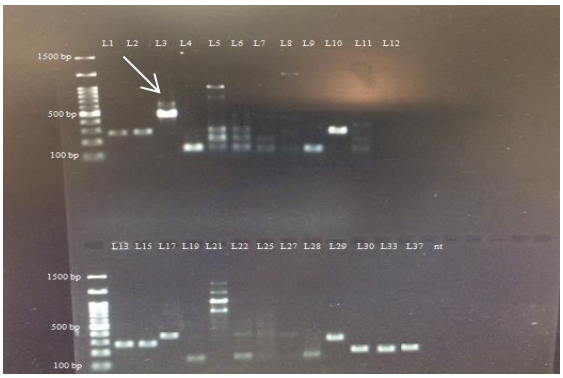

Figure 2: Agarose gel electrophoresis of CRISPR gene regions in *L. plantarum* isolates.
A complete CRISPR sequence approximately of 500 bp long was detected in L3.

According to studies on LAB, eight CRISPR families are generally represented. Eight CRISPR families were identified by a comparative analysis of repeat and Cas 1 gene sequences. Members of CRISPR families called Sthe3, Sthe1, Efam1, Lsal1, Blon1, Lhel1, Sthe2 and Ldbu1 consist of various LAB genera and species. It was found that the repeat length which was determined to be 36 bp was preserved in the first 5 families (Horvath *et al.,* 2008). As a result of this study, the repeat sequences detected in the isolate of L3 were also found to be 36 bp.

Table 5. CRISPR array results of L3 in CRISPRFinder database.

Repeat sequences	Spacer sequences
ATTCTACAACTGCTTAAAATGACCACTGTCC GAGAC	CCGGATGACAGGTCGAAACGATCCT
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	CGATTTGGATGGCTCTTTTTTTTGAATT
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	GACCTAGCTAAAACGTCTGAGAACGTCTTA
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	TAGCTACGCAAACTCATGGCAAAATGACTA
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	CGGCATTCTCACATAATCCACTCATTAATTCA AATCACGCCATNTN
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	GCCCACGATTCAAGGATTACACGGGCGGCG
GTTCTAAACCTGTTTGGTATGACTACTATTC AAGAC	

The samples, in which CRISPR sequences could not be detected in CRISPRFinder database, were also analyzed using NCBI-Blast. Analyzes were first performed based on nucleotide similarities. Later, nucleotides were used "blastx" to examine their homology with proteins. The sequences of the isolates encoded as L29, L30, L10, L17, L5, L1 and L2 showed homology with the DEAD / DEAH helicase family whereas the sequences of those designated as L9, L13, L15, L21, L37 and L3 showed homology with ATP-dependent helicase. In a study conducted by Crawley

et al., (2018), CRISPR typing and activities of *cas* genes were studied in *Lactobacillus* spp. CRISPR type I, II, and III vary in *Lactobacillus* spp. Type II CRISPR loci is detected mainly in *L. plantarum* strains and type I is rare. Type III CRISPR locus was not found in *L. plantarum* strains (Crawley *et al.,* 2018). The results of this study are similar to the type I CRISPR loci. Because the DEAD / DEAH helicase family, one of the domains of the key Cas3 protein in type I CRISPR systems, shows high homology with the sequences of the samples analyzed in this study. Sinkunas *et al.,* (2011), revealed the structural analysis of Cas3 protein in *Streptococcus thermophilus* DGCC7710 strain, which is a LAB species. The Cas3 protein consists of three domains in the strain. One of these domains is defined as DExD helicase. Cas 3 shows a nuclease and helicase function that cleaves single-stranded DNA. While nuclease activity is carried out by the HD domain, helicase activity is due to the function of the DEAD / DEAH domain (Sinkunas *et al.,* 2011). Moreover, in Type I-F systems, fusion of Cas3 and DEAD / DEAH family exonuclease with a different Type IE system version in several genomes, and various conserved Cas2 fusions in Firmicutes, was detected (Makarova and Koonin, 2013). In our study, predominantly detection of the DEAD / DEAH helicase family in *L. plantarum* strains indicates the presence of CRISPR loci in these species. However, CRISPR-Cas systems are not always naturally active in the microorganisms that have *cas* protein domains. For example, *L. paracollinoides* strains always contain the *cas* gene but do not contain repeat sequences (Crawley *et al.,* 2018).

In this study, strains without repeat-spacer sequences were determined. To determine whether the sequences scanned in fasta format in the analyses are present in databases such as CRISPRFinder, the sequence must have at least 2 repeat and 1 spacer regions. Therefore, it is not possible to detect CRISPR sequences with the CRISPRFinder database in strains that have not been exposed to phage or in strains that have lost their function over time and are inactive.

One of the important findings of the present study is the ability of the TaqMan5 'Nuclease test to detect bacteria with the same morphology as well as bacteria that have close genetic similarity with high sensitivity at a species-specific level.

L. plantarum isolates, analyzed in this study, were isolated from home-type pickled olive waters collected from Aydın and Izmir. The formation of CRISPR sequences usually occurs by exposure of the microorganism to phage and plasmids or by horizontal gene transfer. Of the 14 strains of *L. plantarum* isolated from domestic fresh

brine, a typical CRISPR sequence was found only in one isolate (L3). For the isolates in which CRISPR sequences were not encountered but the presence of CRISPR-related proteins was detected, further studies have to be performed using transcriptomic approaches in detail to determine whether these proteins are active or not. However, CRISPR loci may show polymorphism in microorganisms. Therefore, the primers used in CRISPR locus analysis may not always give results since spacer contents may change and new sequences can be formed in the locus with horizontal gene transfers. Whole-genome analysis with the approach of next-generation sequencing will contribute to the field for the analysis of gene regions that are constantly dynamic such as CRISPR.

From recent studies on LAB phages, it is clear that a two-pronged approach is required to fully understand phage biology. It is necessary to understand the genetics and biology of both phage and host to define the phage requirements for infection and to determine how phage and host evolved together to adapt to the threat posed by the other. It does not seem possible to eliminate the problem of phage contamination in LAB used in various food fermentations. However, the primary approach to gain insights into phage and host interactions should be the development of tools and strategies to control and prevent phage infection during food fermentations.

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Morphological and leaf anatomical structure of *Pimpinella cypria* Boiss.

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Abstract

Pimpinella cypria Boiss (Apiaceae) is an endemic species of Turkish Republic of North Cyprus. It is locally known as 'Cyprus-rocky anise'. Three species of the genus *Pimpinella* that are *P. cretica* Poir., *P. peregrina* L., and an endemic *P. cypria* grow in Cyprus. Specimens of *P. cypria* were collected from their nature habitats; St. Hilarion castle, and specimens were dried according to the standard procedures, as herbarium specimens and kept in the Eastern Mediterranean University, Faculty of Pharmacy Herbarium (EMUH). Anatomical structure of leaves were examined. Leaves were bifacial, multicellular and long hairs were observed especially on the midrib. Leaves are amphistomatic where stomata are less on upper epidermis than those of lower epidermis. Stomata guard cells have a characteristic shaped resembling kidney.

Keywords

Apiaceae, anatomy, leaf, *Pimpinella cypria*, North Cyprus

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INTRODUCTION

Cyprus is the third largest island in the Eastern Mediterranean region after the Sicilia and Sardinia islands that is a crossing point of Asia, Africa and Europe. It measures 240 kilometres long and 100 kilometres wide. Cyprus is 75 kilometres away from Turkey. Other neighbouring regions include Syria (105 kilometres away) and Lebanon (108 kilometres to the east), Israel (200 kilometres to the southeast) and Egypt (380 kilometres to the south). The land of North Cyprus is divided into three areas; Besparmak Mountains – lying along the cape of Korucam to cape of Zafer, Mesaria plain- extending from Guzelyurt district to the eastern coastline and the third plain along the shore in the North (Yildirim, 2010). The general view and location of North Cyprus is shown in Figure 1. Apart from limited number of floristic studies (Meikle, 1977; Viney, 1994; Viney, 1996) carried out in North Cyprus, the data related with the anatomical structure of the leaves of *P. cypria* are scarce. Palynological studies belonging to the endemic taxa were published by Yıldız *et al.,* (2009) and Gucel *et al.,* (2008). The aim of the present study was to investigate the anatomical structures of the leaves of *Pimpinella cypria*.

According to the Flora of North Cyprus is one of the richest floras in the Mediterranean region (Meikle, 1977). The flora comprises 1649 indigenous taxa (species and subspecies), 254 introduced taxa occurring in the wild, 43 hybrids and 81 species with unclear status (as at March 2019). For the Flora of North Cyprus; it has 1610 species and 1738 taxa (Viney, 1994; Viney, 1996) 19 of species endemic for the Northern Cyprus (Yildiz and Gucel 2008). Many species of North Cyprus especially endemics are guaranteed because of they are smaller and few populations. Many of these species become endangered by the human influence; increase of the danger depends on the changes in agriculture, increasing the tourism activity and urbanization of natural places. The location and distribution of the species shown in Table 1 (Yildiz and Gucel 2008; Gucel *et al.,* 2009).

Figure 1: Location of South and North Cyprus.

Table 1: Location of some endemic species of Northern Cyprus.

Taxa , family	Locality
***Delphinium fissum* subsp. *caseyi* (Burtt) C.Blanché & Molero (Ranunculaceae)**	Kyrenia, St. Hilarion castle, Southwest limestone hill, north slope.
***Brassica hilarionis* Post (Brassicaceae)**	Kyrenia, Girnekaya, scrubs and limestone cliffs.
***Arabis cypria* Holmboe (Brassicaceae)**	Kyrenia, St. Hilarion castle limestone cliffs and rocks.
***Dianthus cyprius* A.K.Jacks. & Turrill (Caryophyllaceae)**	Nicosia, Halevga, rocks and near road, southeast slopes.
***Silene fraduatrix* Meikle (Caryophyllaceae)**	Nicosia, Halevga, under forest.
***Sedum lampusae* Boiss. (Crassulaceae)**	Kyrenia between Halevga- Girnekaya north slope
***Pimpinella cypria* Boiss. (Apiaceae)**	Kyrenia from Halevga to Girnekaya, north slope.
	Kyrenia, St. Hilarion castle, rocky places.
***Ferulago cypria* Post (Apiaceae)**	Kyrenia, from to Nicosia, under St. Hilarion castle.
	Famagusta between Geçitköy-Geçitkale, near road
***Limonium albidum* (Guss.) Pignatti subsp. *cyprium* Meikle (Plumbaginaceae)**	Kyrenia, Tatlısu Village, sea level.
***Onosma caespitosum* Kotscy (Boraginaceae)**	Nicosia, Halevga-Kalavaç road.
	Nicosia, Buffavento castle, south slopes.
***Salvia veneris* Hedge (Lamiaceae)**	Nicosia above Değirmenlik lake, sandstone hills.

Chemical composition and medicinal usage of the family Apiaceae and genus *Pimpinella*

This family plants accumulate in flavonoids, mainly in the form of flavones and flavanols; significant antioxidant, antispasmodic and anti-inflammatory effects, due to the effects they are often used for muscle pain, stomach cramps, irritable bowel syndrome and nausea (Gebhardt *et al.,* 2005). Common flavonoids are; apigenin, quercetin, lutein, bisabolene, rutin. Vegetables in the family Apiaceae e.g fennel, carrot, parsley, celery are high in polyacetylene especially falcarinol, some research shows to be cytotoxic to five different cancer cell line to against acute lymphoblastic leukoma (Zidorn *et al.,* 2005). Another important constituent is coumarin. It is common in all plants and expansively distributed, but for the carrot families (Apiaceaeor Umbelliferae) are high in coumarin. Coumarins has vascular effect, hepatoprotective effect, anticancer effect, antispasmodic effect, hormonal effect and immune enhancing effect (Stansburg 2016). Common coumarins are osthol, umbelliferon, scopoletin, bergapten, angelicin, impertorin, avicennin, avicenol, xanthotoxin etc., in the terms of plants which are rich in coumarin especially 'osthol'; shows anti-aggregating effects, antiproliferative effects on vascular smooth

muscle, protective effect on liver and antihypertension effects (Ramesh and Pugalendi 2005; Lee *et al.,* 2003; Guh *et al.,* 1996; Chiou *et al.,* 2001). 'Umbelliferone' constituent shows an anti-hyperlipidaemic, improve the glycaemic action in diabetic patient and most important activity is anticancer effect include the inhibition of cell proliferation and the induction of apoptosis. This constituent also shows as an antiviral effect as well as direct effect on skin disorders, it's photosensitizing (Ramesh and Pugalendi 2005; Okamoto *et al.,* 2005). Approximately 70-80% of world population treated with the traditional medicine practices by using the plants. People uses plant extract for medicinal purposes by making the syrup, tablets and oral spray to treated the anxiety, central nervous system disorder, some spasmodic disorders and decreases some pain about headache, teeth or throat (Arceusz *et al.,* 2010). Also, the essential oils are obtained from the plants; they have special odour and aroma therapeutic properties. They have antimicrobial, antioxidant and anticancer effects (Hammer *et al.,* 1999; Jayaprakasha *et al.,* 2002; Lee and Shibamoto 2002; Vardar-Unlu *et al.,* 2003). For the Apiaceae family especially Anise and Caraway seeds are very important effects on traditional medicine. Anise (*Pimpinella anisum)* is an important plant and spice; used in pharmacy, perfume and food industry. It has antispasmodic, antioxidant, antimicrobial, insecticidal and antifungal activity (Tunc and Sahinkaya 1998; Tirapelli *et al.,* 2007; Tepe *et al.,* 2006; Ozcan and Chalchat 2006; Gulcin *et al.,* 2003). Also, it can be used in throat pain, flu and expectorant (Blumenthal 1998). Some hormonal effect has been noted in this family especially in Fennel (*Foeniculum vulgare*) and Anise *(Pimpinella anisum)* seeds tea promote lactation in nursing women and some gonadotropin activity in central nervous system (Stansburg 2016; Tabanca *et al.,* 2004). Lastly this family plants can be used in allergy treatment; *Angelica* species has been found to have an antihistaminic and antiserotonin effect, so used in asthma or bronchitis (Matsuda *et al.,* 2002). It has cognitive function to improve the enhance memory especially the essential oil forms (Stansburg 2016). For this family plants mostly, contraindications are limited to hypersensitivity to active substance. Many of the oils in this family especially green anise (*Pimpinella anisum*) are neurotoxic because of the presence of particular ketones or phenonic ethers (Price and Price 2011). Some species produce phototoxic substances mainly furanocoumarin constituent species may cause photodermatitis also some members (carrot, celery, fennel, parsley, parsnip) exhibit a cytotoxic effects

(Stansburg 2016). Apart from some chemical analysis; *P. cypria* contains essential oils which is 81.7% of the total composition. The essential oils are; oxygenetaned sesquisterpenes (33.9%), sesquiterpenes (22.0%), monoterpenes (11.4%), oxygenated monoterpenes (2.6%) and phenylpropanoids (7.5%).

The main components of the oil were (*Z*)-β-farnesene (6.0%), spathulenol (5.9%), *ar*-curcumene (4.3%), and 1,5-epoxy-salvial(4)14-ene (3.8%).

P. cypria essential oil demonstrated moderate antimicrobial activity against Gram-negative and Gram-positive bacteria except for *Candida albicans.* Additionally, it has insecticidal activity against yellow fever mosquito's *(Aedes aegypti)* and it shows a cytotoxic effect (Tabanca *et al.,* 2016).

The genus *Pimpinella* in North Cyprus

According to the Flora of North Cyprus published by Viney, 2000, the genus is represented by 4 species; *Pimpinella anisum* L.*, P. cretica* Poir., *P. peregrina* L. and *P. cypria* Boiss**.** *P. cypria* (North Cyprus burnet) locally known as Cyprus-rocky anise (kıbrıs tas anasonu) (Anon 2011). *P. cretica,* slender erect annual plant with 40 cm height, zigzag-branched above, basal leaves are simple, roundish with scalloped edge, the oval segments often 3-lobed uppermost with narrow segments; umbels opposite with leaves, flowers are white or pinkish and frequent in north-central area. *P. peregrina* hairy biennial plant to 1m with a carrot-shaped taproot, basal leaves are simple or ternate but normally withered by flowering time, upper leaves pinnate with 5-7 oval, toothed segments, umbels dropping in bud and flowers are white. Very local in Kyrenia range; shady banks in Lapta (Viney 1994; Viney 1996).

Morphological characteristics of *Pimpinella cypria* Boiss.

Pimpinella cypria is a herbaceous perennial (Apiaceae/ Umbelliferea) which is an endemic species to North Cyprus. Erect with a stout, woody rootstock clothed in the upper part with the remains of withered and indurated leaf-sheats; stems conspiously sulcate, rather densely (rarely sparingly) clothed with soft spreading hairs, not much branched except in the inflorescence; basal leaves oblong, pinnate with 5 (less commonly 3) segments, or rarely undivided and flabelliform, up to 12 cm long and 7 cm wide; segments broadly ovate-cuneate, up to 4 cm long 3 cm wide, thinly puberulous above, more densely pubescent below with prominent reticulate nervation, apex obtuse, margins bluntly toothed and irregularly lobed, the terminal segment often deeply 3-lobed; petioles up to 12 cm long, densely pubescent, canaliculate above, basal sheats conspiouscus, overlapping, thick, distinctly nerved; stem

leaves very sparse, similar to basal or sometimes divided into two narrow, linear or oblanceolate, pinnatisect or laciniate segments, shortly petiolate or subsessile; umbels terminal, in lax spreading panicles, 6-14-rayed; peduncles 2-9 cm long; bracts and bracteoles wanting; rays shortly hispidulous, spreading, subequal up to 3 cm long at anthesis; flowers mostly hermaphrodite and fertile, numerous and rather dense in each umbellule; pedicels hispidulous, up to 5 mm long at anthesis; sepals obsolete; petals dirty white, broadly oblong-obovate, unequally 2-lobulate, flattish, about 1 mm long 0.9 mm wide, conspicuously unequally-2-lobulate, flattish, about 1mm long, 0.9 mm wide, conspiuously pilose dorsally; filaments up to 1.5 mm long, glabrous, inflexed; anthers yellow, oblong, about 0.5mm long, 0.4 mm wide; stylopodium convex or shortly conical, about 0.6 mm diam. with a crenulate margin; styles filiform, spreading, about 2mm long; stigmas. Fruit narrowly ovoid-ellipsoid, about 4 mm long, 2.5 mm wide, laterally compressed, conspicuously white-pilose; carpophore deeply bipartite; mericarps dark brown when ripe, convex dorsally with 5 distinct, pallid, filiform ridges, attenuate towards apex; endosperm flattish along its commissural face.

Figure 2: General habit of *P. cypria* by D.A.Viney.

Distribution of *Pimpinella cypria*

P. cypria is common quite on North facing slopes beside the steps leading up to the St. Hilarion castle, rocky places; 800 m, Girne (Kyrenia) from Alevkayasi – North slope; 820 m, Girne (Kyrenia) near Girnekaya, north slope, rocky places; 750-800 m. *P. cypria* grown in a sunny, sheltered and rocky places.

Figure 3: *P. cypria* in its natural habitat by G Konstantinou.

Figure 4: Inflorescences of *P. cypria.*

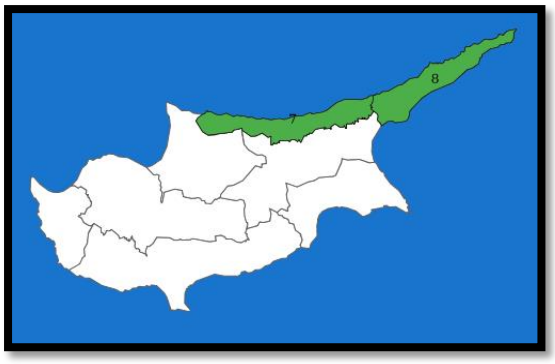

Figure 5: Distribution of *P. cypria* (green parts) (dynamic checklist- Flora of N. Cyprus).

MATERIALS

Field surveys about research material was conducted on April- 14.04.19, and May 24.05.19. Young and mature individual of *P. cypria* are shown in Figure 6.

The plant specimens that was collected on 14.04.19 (young individuals) and 24.05.19 (mature individuals) then dried according to the standard procedures and transformed into the Herbarium of Eastern Mediterranean University, Faculty of Pharmacy (EMUH), the number of the specimen SNM 002.

Study area

The survey area located in Northern Cyprus, at the west of the Besparmak Mountains; St. Hilarion Castle. It has rocky places and above 700 m sea level. The picture and map of the study area are shown in Figure 7.

In literature review, it was seen that *P. cypria* is found in North Slope on Kyrenia mountains; Kyrenia from Halevga to Girnekaya and St. Hilarion castle. This species grows in rocky places, Halevga and St. Hilarion castle rocky places have similar topography (Gucel and Yildiz 2008).

Figure 6: Basal leaves of young *P. cypria* in April (left) and flowering stage of mature *P. cypria* in May (right) (taken by author).

Figure 7: Study area (left; picture taken by author) and map.

METHODS

In this study; the anatomical and morphological characteristics of leaves are examined. Morphological examinations were carried out with the help of literature reviews. The general view as well as the shapes of the leaves drawn by hand due to their significance in the identification of the specimens also taken photographs by mobile phone. For anatomical examination were taken cross and superficial section of leaves that use by hand with the aid of razor blade. In cross sections were taken perpendicular to the long axis (90˚) with the help of razor blade. In superficial sections were taken both upper and lower layer, peeled the layers (upper& lower) separately and carefully pulled out the transparent layer from foliole, after placed the samples into the microscope slides then dropped Sartur Reagent which is 'developed in 1949 by two Turkish scientists, Sarim Celebioglu and Turhan Baytop. It is prepared to identify many elements in one preparation' (Celebioglu and Baytop 1949), to the sample and close with coverslide, heated on hot plate because Sartur reagents must be activated by using heat. Finally slides examined under microscope and photographed by mobile phone.

RESULTS

Morphological examination

The plant is perennial with 60-70 cm height. Leaves are compound and imparipinnate with 7 leaflets, basal leaves are hairy and the stem is erect with soft hairs (Figure 8). Flowers are dirty white showing a characteristic features of an umbel.

Figure 8: Compound basal leaves and leaflet (taken by author).

Anatomical examination

Anatomical investigation based on the superficial and cross section of leaves.

Cross-section

Leaves are exhibit dorsiventral (bifacial) structure. Epidermis is distinctly, has long and multicellular hairs for both upper and lower epidermis; upper epidermis hairs are thinly while lower epidermis hairs are thickly and compactly but on midrib hairs distinctly seen and more than hairs has been observed than upper and lower epidermis. Interior of leaf between upper and lower epidermis contain veins and parenchymatous cells, differentiated into two region; palisade parenchyma below the upper epidermis which is vertically elongated, parallel and cylindrical cells and rich in chloroplast therefore they are the main set of photosynthesis and spongy parenchyma between the lower epidermis and the palisade parenchyma which is oval, rounded and branched also they have chloroplast and cell walls are thin. Both palisade and spongy parenchyma has crystals while on palisade parenchyma has more than the spongy parenchyma. Vascular bundles not seen distinctly so some literature review shows; It is made up of a number of vascular bundles of varying size, found at the boundary between the palisade and spongy parenchyma, they are almost rounded, possess both phloem and xylem which lie on the same radius. Xylem lies towards the upper side of the leaf while phloem is found towards the lower surface, xylem consists of vessels, tracheids, xylem parenchyma and a few xylem fibres, xylem parenchyma stores food and allows lateral movement of water and mineral salts, phloem parenchyma cells store food and help in the lateral conduction of food'

(Fritsch and Salisbury 1943). However, on result photographs have not been viewable because of the sartur reagent; when drop sartur reagent on to leaves; parenchymatous cells has been become dark purple because of the starch content of plant (Figure 9).

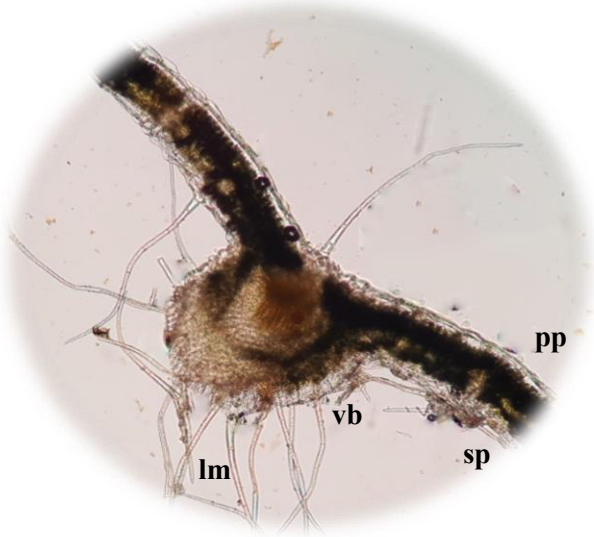

Figure 9: Cross section of the leaves (midvein).
pp: palisade parenchyma, sp: spongy parenchyma, vb: vascular bundles (xylem & pholem), lm: long and multicellular hairs.

Superfacial sections- abaxial and adaxial surface

Shown as an amphistomatic characteristic feature which means both upper (adaxial) and lower(abaxial) sides have stomata on their surfaces; tiny pores in tissue to allow gas exchange and each stomata has a narrow pore bounded and controlled by small specialised kidney-shaped epidermal cells called guard cells. However *P. cypria* stomata on upper (adaxial) side lesser than lower (abaxial) side (Figure 10 and 11).

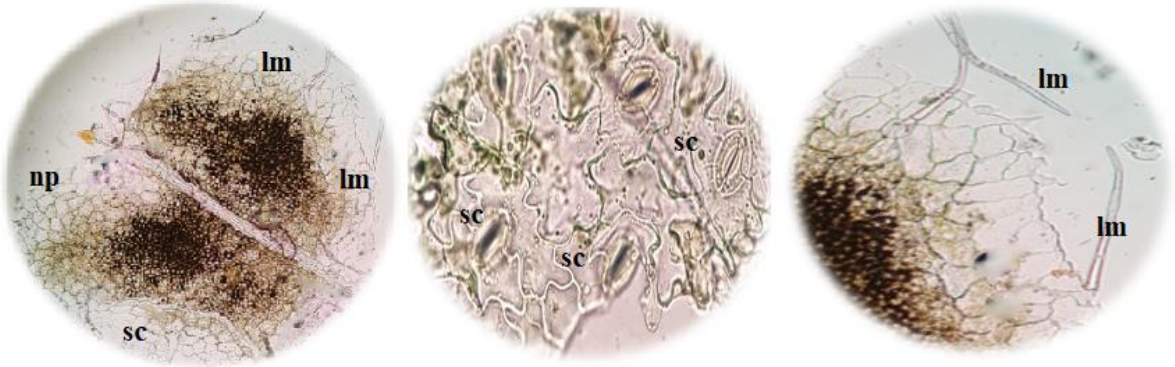

Figure 10: Upper epidermis (adaxial epidermis).
lm: long and multicellular hairs, np: normal polyhedral cells, sc: stomata cell.

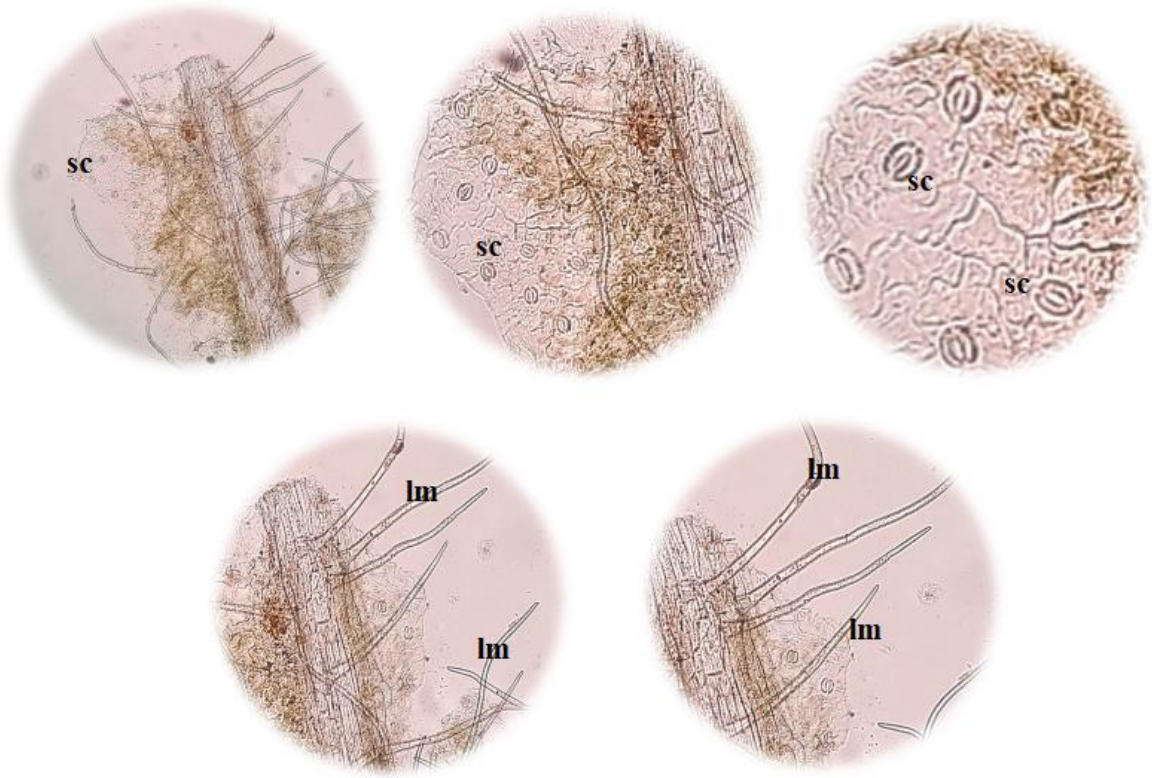

Figure 11: Lower epidermis (abaxial epidermis).
lm: long and multicellular hairs, sc: stomata cell.

CONCLUSION

In conclusion, the main objective of this study is to investigate the anatomical and morphological characteristics of the leaves which are presented for the first time. Young and mature individuals collected from nature habitat with the different times after that cross-section and superfacial section were taken from the leaves and examined under microscope. During the observations; leaves are exhibit dorsiventral structure, upper epidermis rich in palisade parenchyma and lower epidermis rich in spongy parenchyma, long and multicellular hairs are observed for both sides, on the upper epidermis hairs were thinly while on the lower epidermis hairs were thick and compactly. This plant shows amphistomatic structure however on the upper epidermis has lower stomata than lower epidermis.

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The ethnobotany, systematics and morphological studies of the genus *Ornithogalum* that naturally grows in Kahramanmaras province of Southern Turkey

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Abstract

The specimens of the genus *Ornithogalum* L. (Asparagaceae) were collected from Kahramanmaras province in Southern Turkey between 2012 and 2013 during the PhD study. As a result of detailed examination and definitions of the collected specimens, it was determined that these specimens belonged to 19 species of two subgenera: "Subgenus *Ornithogalum* and Subgenus *Beryllis*'. The identification key of the species was conducted, distributions in Kahramanmaras have been shown on the maps and photographs of each species from natural habitats have been presented. During the study, information was obtained from the local people about their local names and uses.

Keywords

Kahramanmaras, local uses, *Ornithogalum*, Southern Turkey, vernacular names.

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INTRODUCTION

The genus *Ornithogalum* L. (Asparagaceae) was revised by Cullen (1984) in the Flora of Turkey and the East Aegean Islands, in which 22 species were recognized. It was divided into four subgenera: *Beryllis* (Salisb.) Baker*, Ornithogalum*, *Myogalum* (Link) Baker and *Caruelia* (Parlatore) Baker. After publication of the Flora, 42 new taxa have been added to the Turkish flora by various authors (Davis *et al*., 1988, Speta 2000a, 2000b, Ozhatay 2000, Dusen & Sumbul 2002, 2003, Dusen & Deniz 2005, Uysal *et al*., 2005, Ozhatay & Kultur 2006, Dalgıc *et al*., 2006, Varol 2008, Bagcı *et al*. 2009, Yildirimli 2009, Koca & Yildirimli 2010, Ozhatay *et al*., 2011, Bagcı *et al*., 2011, Mutlu & Karakus 2012*,* Demirci & E.Kaya 2014, Demirelma, 2020). Thus, the total number of species has increased to 64. *Ornithogalum* species are known as vernacular names such as "köpek soğanı, tükrük otu, akyıldız soğanı, itdirseği, kurtsoğanı, akbaldır, sabunotu, çiğdem çiçeği, karga sarımsağı, eşek susamı, it keseri, sakarca" (Guner *et al*., 2012). *Ornithogalum* species are named as "akyıldız" in Kahramanmaras (Demirci & Ozhatay, 2012). Bulbs of *Ornithogalum* have been used as an emetic since Dioscorides. *Ornithogalum* species have been used as medicine and are thought to be poisonous (Baytop 1999). However, it has been determined that the leaves of *Ornithogalum* species are cooked and consumed as food in some regions of Turkey (Demirci & Eroglu Ozkan, 2017). The herb of *O. narbonense* was used as food in Mediterranean area (Rivara *et al.*, 2006). The bulbs of *O. umbellatum* was used as food in Italia and Balkans ((Tizio *et al.,* 2012; Novella *et al.,* 2013). It was determined that bulbs are poisonous in Jordan (Al'Quarayn, 2005). In Southern Italy, bulbs are eaten (e.g. *Ornithogalum pyrenaicum* (Scherrer *et al*., 2005, Salerno *et al*., 2006). Kahramanmaras is situated at the south part of Taurus Mountains in the Southern Turkey. Its plant diversity is very rich because its localization lies in meeting point of three phytogeographic regions. While Irano-Turan elements are found in the east, Mediterranean elements are common in the south. Some formations of the Euro-Siberian geographical region can also be seen in humid areas (Ozhatay *et al*., 2008; 2009; Yildiz, 2006; Demirci, 2014; Demirci and Eroglu Ozkan, 2017). Bulbs of *Ornithogalum* have been used as medicine and food in Kahramanmaras (Demirci and Ozhatay, 2012). The ethnobotanical studies of Turkey were scanned and uses and vernacular names of *Ornithogalum* species are given in Table 1.

Table 1: The uses and vernacular names of *Ornithogalum* species in Turkey.

Scientific name	Used parts	Vernacular name	Uses	Province	References
O. armeniacum Baker	Leaves, flower	Soryaz	Food, eaten cooked	Antalya	Bulut 2006; Guner *et al.,* 2012
O. lanceolatum Labill.	Whole plant	Bulumbışık	Eaten as vegetable	Mersin	Baytop 1999; Guner *et al.,* 2012
O. narbonense L.	Bulb, leaves	Akbaldır	Eaten as vegetable	Igdır	Altundag 2009; Guner *et al.,* 2012
	Bulb	Akbaldır	Eaten cooked	Erzurum	Aksakal and Kaya 2008; Guner *et al.,* 2012
	Leaves	Akbaldır	Eaten cooked	Sakarya	Koyuncu 2005; Guner *et al.,* 2012
	Leave, bud	Akbaldır	Acnedisease, emetic,diuretic, cardioactive	Adana	Ozer *et al.,* 2001; Guner *et al.,* 2012
	Leave, bud	Akbaldır	Eaten cooked	Adana	Ozer *et al.,* 2001; Guner *et al.,* 2012
	Leave	Akbaldır	Eaten as vegetable	Siirt	Yapici *et al.*, 2009; Guner *et al.*, 2012
	Herb	Akbaldır	Food	Inner Anatolia	Dogan et al., 2004; Guner et al., 2012
O. oligophyllum E.D.Clarke	Bulb, leave	Kurtsoğanı	Eaten cooked	Igdır	Altundag 2009; Guner *et al.,* 2012
	Leave, shoot	Kurtsoğanı	-	Amasya	Cansaran and Kaya, 2010; Guner *et al.,* 2012
	Scape, leave	Kurtsoğanı	Eaten cooked	Blacksea region	Ozbucak *et al.,* 2006; Guner *et al.,* 2012
O. platyphyllum Boiss.	Bulb, leave	Dağ akyıldızı	Eaten cooked	İzmit	Kizilarslan and Ozhatay 2012; Guner *et al.,* 2012
	Scape, leave	Dağ akyıldızı	Eaten cooked	Blacksea region	Ozbucak *et al.,* 2006; Guner *et al.,* 2012
O. pyrenaicum L.	Whole plant	Eşek susamı	Animal food	Aksaray	Ertug 2000; Guner *et al.,* 2012
O. sigmoideum Freyn. & Sint.	Whole plant	Sakarca	Eaten cooked	İzmit	Kızılarslan and Ozhatay 2012; Guner *et al.,* 2012
	Scape, leave	Sakarca	Eaten cooked	Blacksea region	Ozbucak *et al.,* 2006; Guner *et al.,* 2012
Ornithogalum sp.	Whole plant	Akyıldız	Eaten as vegetable	Ordu	Turkan *et al.,* 2006; Guner *et al.,* 2012
O. sphaerocarpum A.Kern	Leave, bud	Salkım sakarca	-	Amasya	Cansaran and Kaya 2010; Guner *et al.,* 2012
O. umbellatum L.	Whole plant	Sunbala	-	Aksaray	Ertug 2000, Guner *et al.,* 2012
	Bulb	Sunbala	Boil and acne disease	Usak	Deniz *et al.,* 2010; Guner *et al.,* 2012

MATERIALS AND METHODS

The *Ornithogalum* specimens were collected from Kahramanmaras province (Figure 1) between 2012 and 2014. Approximately 200 *Ornithogalum* specimens were collected as a result of field studies in the research area (Demirci, 2014). Characteristics such as distribution areas, habitats, leaf widths, perigon shapes and color, tepals shape, filaments length, capsule shapes were recorded in the natural habitat. Photos of the general appearance, leaves, flowers and capsules of the specimens were taken. The flowering and fruiting specimens that were required for laboratory studies were collected from the field, pressed and dried in accordance with herbarium techniques. Herbarium specimens were kept in the Herbarium of the Faculty of Pharmacy of Istanbul University (ISTE).

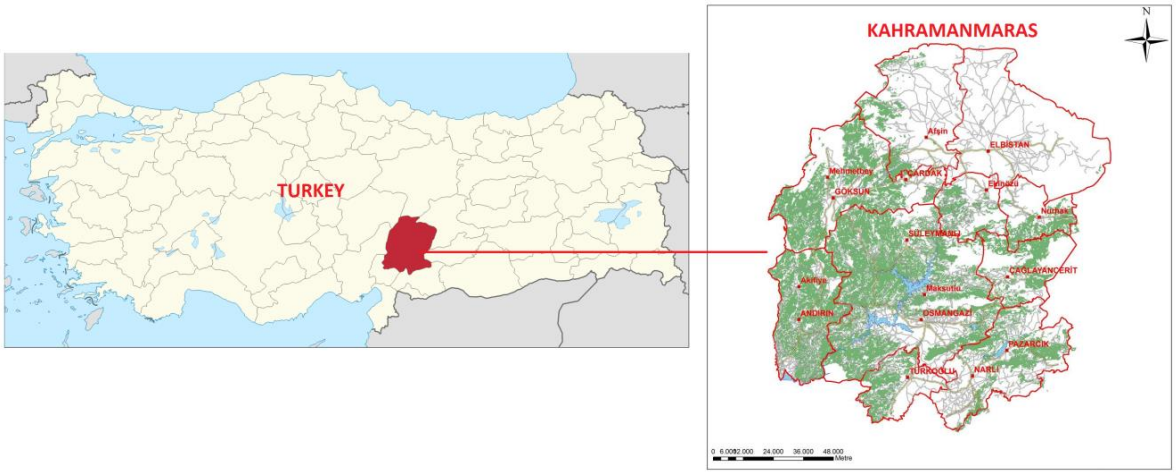

Figure 1: The map of Kahramanmaras province in Turkey.

RESULTS AND DISCUSSION

As a result of our studies, it was determined that 18 *Ornithogalum* species have been found to grow naturally in Kahramanmaras province. The identification key of these species and distribution maps have been given with the photographs of each species taken from natural habitats. *Ornithogalum* species determined in previous floristic studies from the research areas consist of 11 species. These are: *O. alpigenum* Stapf., *O. comosum* L., *O. lanceolatum* Labill., *O. montanum* Cirillo, *O. narbonense* L., *O. orthophylum* Ten., *O. platyphyllum* Boiss., *O. sigmoideum* Freyn & Sint., *O. sorgerae* Wittmann, *O. sphaerocarpum* A.Kern *and* *O. umbellatum* L. In this study, it was determined that 8 more *Ornithogalum* species were distributed in

Kahramanmaras. Additional floristic new records are as follows:

One new record for Turkey: *Ornithogalum pedicellare* Boiss. & Kotschy, the species is recorded as endemic to North Cyprus (Hand *et al*, 2011). Five new records for the province Kahramanmaras: *O. vasakii* Speta (endemic), *O. oligophyllum* E.D.Clarke, *O. neurostegium* Boiss. & Blanche, *O. balansae* Boiss. (endemic), *O. hajastanum* Agapova.

In our study, the genus *Ornithogalum* was grouped under 2 subgenera: *O.* subgen. *Ornithogalum* and *O*. subgen. *Beryllis*. The identification keys of the *Ornithogalum* species are given below. The distribution of the species is shown on the map of Kahramanmaras (Figure 2).

The identification key for subgenus *Beryllis* and subgenus *Ornithogalum*

1. Long racemose and multiflowered inflorescences, tepals usually with a green band visible on the abaxial side……..……. Subgenus: *Beryllis*

1. wide and short corymbose or pseudocorymbose raceme;tepals white on the adaxial face bearing a central green band on the abaxial face…….. Subgenus: *Ornithogalum*

A. Subgenus: *Beryllis* (Salisb.) Baker Syn: *Loncomelos* Speta

Ornithogalum subgen. *Beryllis* (Salisb.) Baker includes about 160 species (cf. Wittmann 1985) distributed in the Mediterranean basin and Western Asia and is characterized by the long racemose and multiflowered inflorescences, tepals usually with a green band visible on the abaxial side –at least partially–, capsules subrounded in transversal section and seeds irregularly compressed with rugose testa. This subgenus has been recently treated as the genus Loncomelos Rafinesque by Speta (2000, 2001, 2006. 2010, 2011) and Martínez-Azorín *et al.* (2011), which differs from *Ornithogalum* sensu stricto by clear differences regarding inflorescence, fruit and seed morphology. Five taxa belonging to this subgenus have been distributed in Kahramanmaras.

The identification key of *Ornithogalum* species (Subgenus *Beryllis*) distributed in Kahramanmaras

1. Perianth segments 6-11 mm, racem 17-55 flowered, and flowers rotate or not…… **2**

2. Perianth segments 6-9 mm, racem 30-55 flowered, flowers not rotate. ***O. sphaerocarpum***

2. Perianth segments 6-11mm, racem 17-25 flowered, flowers rotate……***O. sorgerae***

1. Perianth segments 9-16 mm, racem more than 75, flowers not rotate…….. **3.**

3. Pedicels patent in fruit, arcuate in upper part ……..... ***O. magnum***

3. Pedicels are not erect in fruit, parallel of scape or not…….**4.**

4. Tepals with green stripe, racemes 25-75 flowered, style 2.7- 4mm…. ***O. narbonense***

4. Tepals with wide green stripe, racemes 25-50 flowered, styles 2.5mm ***O. hajastanum***

O. hajastanum, *O. magnum*, *O. narbonense* species are shown in Figure 3. Figure 4 shows *O. sorgerae*, *O. sphaerocarpum* species.

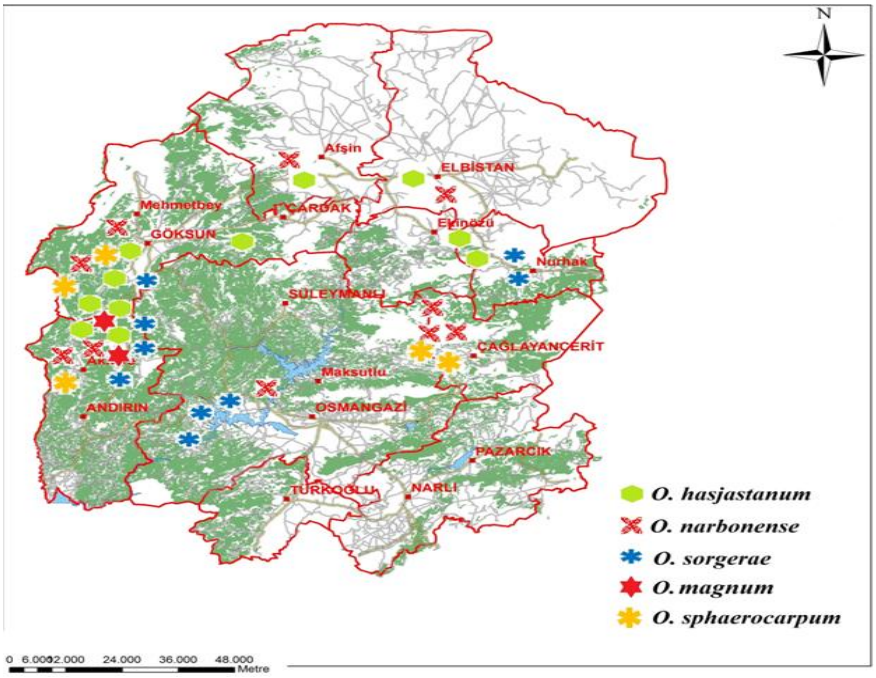

Figure 2: *Ornithogalum* species of the subgen. *Beryllis* distributed in Kahramanmaras.

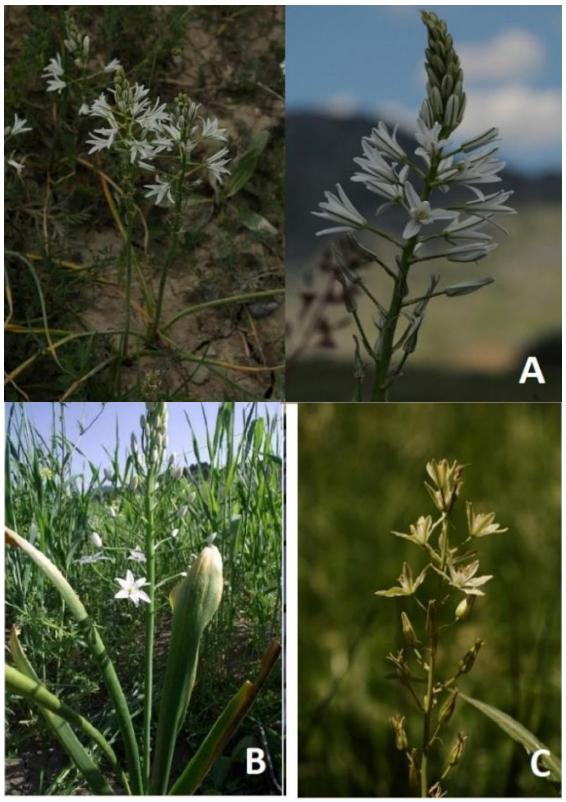

Figure 3: *Ornithogalum* species of the subgenus: *Beryllis* in Kahramanmaras;
A: *O. hajastanum*, B: *O. magnum*, C: *O. narbonense*.

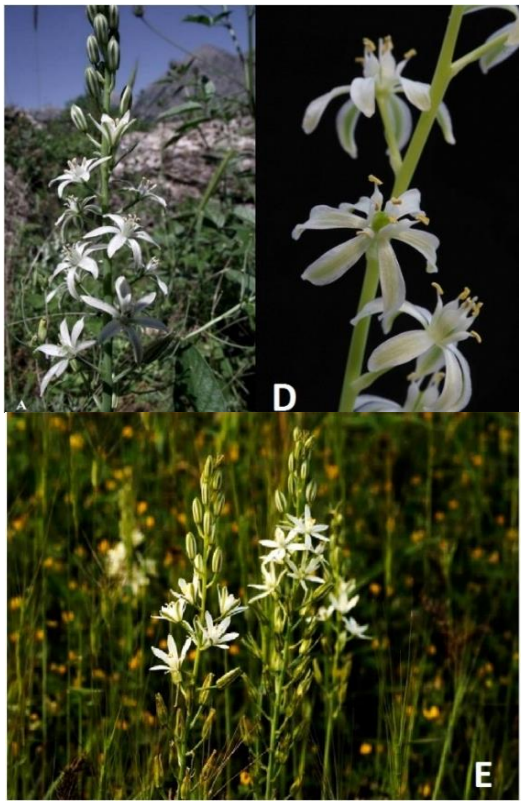

Figure 4: *Ornithogalum* species of the subgenus: *Beryllis* in Kahramanmaras;
D: *O. sorgerae*, E: *O. sphaerocarpum*

B. Subgenus: *Ornithogalum* Baker

Subgen. *Ornithogalum* is characterized by wide and short corymbose or pseudocorymbose raceme; pedicels usually erect-patent to reflexed at anthesis and in fruit; tepals white on the adaxial face bearing a central green band on the abaxial face; filaments linear or tapering contracted abruptly at their apexes; oblong, ovoid, or obovoid ovary; style long and filiform and stigma small trigonous; and seeds globose with reticulate testa (Moret *et al.* 1990). Thirteen taxa belonging to this subgenus have spread naturally in Kahramanmaras (Figure 5). These taxa are: *O. alpigenum* Stapf.; *O. balansae* Boiss.; *O. lanceolatum* Labill.; *O. montanum* Cirillo; *O. neurostegium* Boiss. et Blanche; *O. oligophyllum* E.D. Clarke; *O. orthophylum* Ten; *O. pedicellare* Boiss. & Kotschy; *O. platyphyllum* Boiss.; *O. sigmoideum* Freyn & Sint; *O. umbellatum* L.; *O. vasakii* Speta; *O. wiedemannii* Boiss. var. wiedemannii.

The Identification key of *Ornithogalum* species of the Subgenus *Ornithogalum* distributed in Kahramanmaraş

1. Leaves margine ciliate, lower surface pilose..... ***O. neurostegium***

1. Leaves margine and surfaces glabrous ……. 2

2. Leaves broadening above ground level; acute apex……. .3

3. Scape absent or to 2 cm longer; raceme± sessile between leaves; leaves (10-)15-20 mm broad at ground level.....***O. lanceolatum***

3. Scape evident; racem longer than leaves; leaves less than 15 mm…… 4

4. Leaves born stiffly erect and racem erect ……. ***O. platyphyllum***

4 Leaves horizontal or rarely arching.....***O. montanum***

2. Leaves broadening above ground level or equaly broad to upper part; tapering abruptly to subacute or blunt……5

5. Pedicels patent at fruit, thickening at base..... ***O. sigmoideum***

5. Pedicels erect or patent at fruit, rare patent, not , thickening……...6

6. Leaves 2-3, without a white line on upper surface; pedicels shorter than to as long as flowers at anthesis…… ***O. oligophyllum***

6. Leaves 4 or more, parallel-sided for most of their length, with a white line on upper surface; pedicels usually longer than flowers at anthesis……. . 7

7. Perianth segments 10(-12) mm.....…. ***O. alpigenum***

7. Perianth segments 12-21 mm……. 8

8. Capsule prominently winged……. 9

9. Raceme dense; leaves 6-10..... ***.O. vasakii***

9. Raceme lax, rarely dense; leaves 2-4 …… 10

10. Scape 5-10 cm, 2-9 flowered, tepals oblong-linear, 5-14 mm, styles 2.5-3 mm..... ***O. balansae***

10. Scape 0.5-16 cm, 1-10 flowered…..…… ***O. wiedemannii***

8. Capsule not winged……11

11. Bulbs with bulbils or not; pedicels stout at fruit, 0.5-9 cm…… ***O. umbellatum***

11. Bulbs without bulbils; pedicels thin at fruit, 0,7-3.5 cm……..12

12. Ovary 3-5 mm, ovoid, styles 2-3 mm..... ***O. orthophyllum***

12. Ovary 3 – 3.1 mm, obovoid, styles 2 – 2.1 mm…… ***O. pedicellare***

O. alpigenum, O. balansae, O. lanceolatum, O.montanum, O. neurostegium, O. oligophyllum, O. platyphyllum, O. pedicellare, O. orthophylum species are shown in Figure 6. Figure 7 shows *O. umbellatum, O. vasakii, O. wiedemannii., O. sigmoideum* species.

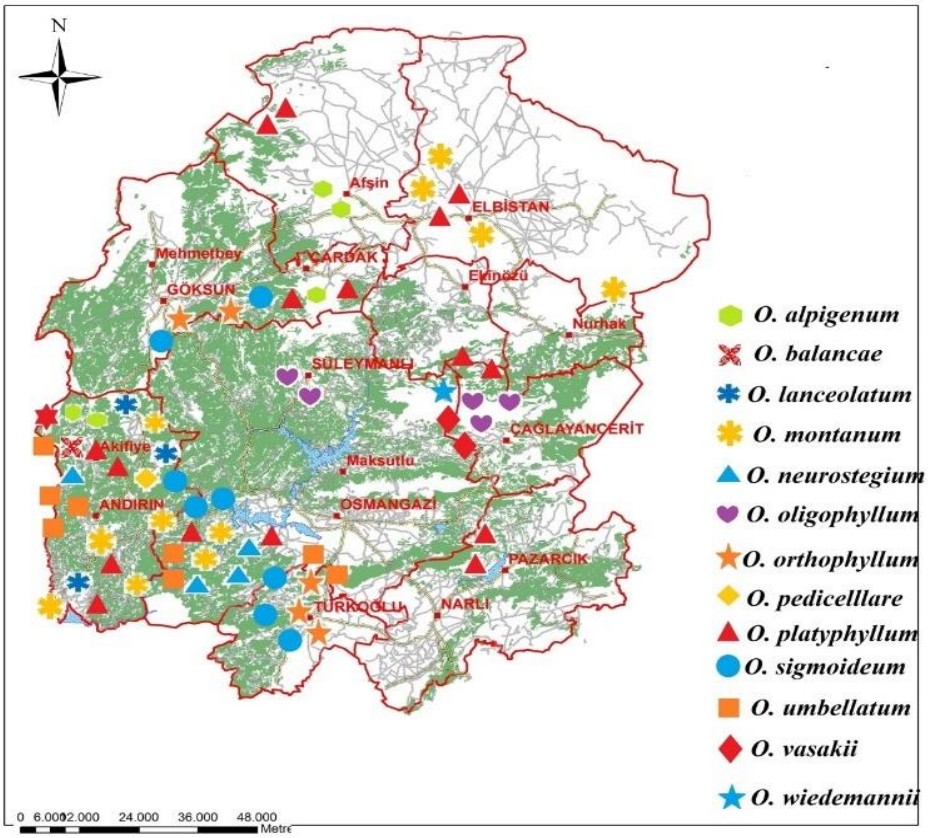

Figure 5: *Ornithogalum* species of the subgen. *Ornithogalum* distributed in Kahramanmaras.

Figure 6: Photos of *Ornithogalum* species of subgen. *Ornithogalum* in Kahramanmaras.
A: *O. alpigenum*, B: *O. balansae*, C: *O. lanceolatum*, D: *O.montanum,* E: *O. neurostegium*, F: *O. oligophyllum*, G: *O. platyphyllum,* H*: O. pedicellare,* I: *O. orthophylum.*

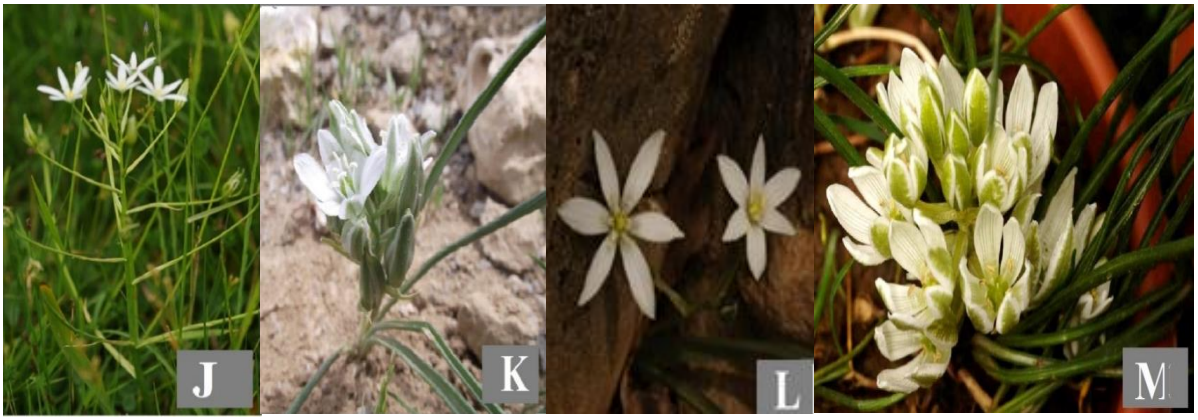

Figure 7: *Ornithogalum* species of subgen. *Ornithogalum* in Kahramanmaras*,*
J: *O. umbellatum,* K: *O. vasakii,* L: *O. wiedemannii.,* M: *O. sigmoideum*

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Formulation and chracterization of meloxicam loaded niosome-based hydrogel formulations for topical applications

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Abstract

Analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) have gastrointestinal side effects and, particularly for local pain, topical dosage forms of these drugs are mainly preferred. The aim of this study was to develop meloxicam loaded niosomal hydrogel for enhanced transdermal and controlled drug delivery. Niosomal formulations were prepared by thin film hydration method using different types of non-ionic surfactant in the presence of cholesterol. Niosomal vesicles were characterized in terms of droplet size, zeta potential, surface morphology and entrapment efficiency. For enhanced residence time, niosomes were further loaded into the carbopol gel. The niosomal formulation containing Span 60, Tween 80 and cholesterol at a molar ratio of 6:1:0.6 had an optimally high percentage of drug entrapment with a mean vesicular diameter of 236.80 nm. Within 24 hours, a maximum of 46.83% drug release was achieved showing faster releasing profile than commercial meloxicam gel. Dermal and transdermal delivery of meloxicam using niosomal-gel formulations may offer promising alternative to traditional delivery systems of non-steroidal anti-inflammatory drugs with improved local and systemic but decreased adverse effects.

Keywords

Hydrogels, meloxicam, niosomes, topical drug delivery.

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INTRODUCTION

Meloxicam (MX) is a nonsteroidal anti-inflammatory drug (NSAID) that is structurally related to the 4-hydroxy-1,2-benzothiazine carboxamide enolic acid class. It was first approved by the United State Food and Drug Administration (US-FDA) in 2000 as a 7.5 mg tablet (Mobic- Boehringer Ingelheim). It was later approved and sold in capsule and suspension forms. These dosage formulations are used clinically to relieve acute and chronic pain and inflammation, as well as to reduce swelling, stiffness and discomfort due to arthritis. MX has also been studied as a possible treatment for Alzheimer's disease and as a potential adjuvant therapeutic chemotherapy agent for various tumors, including breast, colorectal, prostate and urinary bladder cancers, and has been found to have comparable but less toxicity than other NSAIDs to alleviate pain and inflammatory symptoms. In addition, MX is a drug with a low risk allergic reactions associated with NSAID intolerance (Ah *et al.,* 2010). For patients who are intolerant to other NSAID medications, MX is an efficient alternative drug. However, adverse reactions, such as gastrointestinal toxicity/bleeding, headaches, rash, increased risk of cardiovascular events are commonly reported when high-dose and long-term treatment of this medication is administered. Topical drug delivery is an alternative to oral administration, often with comparable effectiveness but theoretically with a more suitable tolerability profile. A variety of benefits over oral NSAIDs are offered by topical administration: These benefits are the ability to deliver the active ingredient more selectively to a given area with both local and systemic effects, the avoidance of first-pass effects, the elimination of gastrointestinal side effects and the improvement in patient compliance (Engelhardt *et al.,* 1995; Graeme, 2005; Noble and Balfour, 1996). Advancing technologies to promote the delivery of drugs to the skin site was a primary subject of study as the barrier function of the skin impairs the penetration and absorption of drugs by the skin barrier of the stratum corneum. The most advanced and less invasive methods for improved delivery of drugs through the skin barrier include a number of formulation strategies, such as micelles, liposomes, niosomes, and nanoparticles (Lengert *et al.,* 2020).

Niosomes provides comparable benefits to phospholipid vesicles (liposomes) and are capable of combining both water-soluble and lipid-soluble drugs as efficient drug delivery mechanisms for a wide variety of

applications and control releases. In addition, niosomes can be used as alternatives to liposomes that are both chemically and mechanically stable (Patel *et al.,* 2012). Niosomes may be formulated using basic methods and surfactants widely used in pharmaceutical technology. In case niosomes are introduced into vehicles such as hydrogels, the residence time for topical drugs will also be expanded (El-nabarawi *et al.,* 2015; Peppas *et al.,* 2000).

In this study, the goal was to investigate the formulations of niosomal hydrogel as potential carriers for the dermal delivery of MX. MX-loaded and gel-dispersed noisomes were subjected to structural tests and applied to *in vitro* release experiments.

MATERIALS AND METHODS

Materials

MX Span 60, Tween 20, Tween 80, Span 20, Carbopol 934P and cholesterol have been purchased from Sigma. Ethanol, ammonia, chloroform and regular saline have been purchased from Merck. Dialysis membrane filters have been obtained from Ashke Shishe, Tehran-Iran.

Preparation of niosomal vesicles

Using safe and non-toxic surfactants such as Tween 20, Tween 80 and Span 20, MX niosomes were prepared utilizing a thin-film hydration process followed by a sonification process (Figure 1). Cholesterol was used as a niosomal membrane rigidity enhancer (El-nabarawi *et al.,* 2015; Manconi *et al.,* 2002; Tavano *et al.,* 2013). For this study briefly, the ratios of non-ionic surfactants, cholesterol and MX that are listed in Table 1, were dissolved in 10 mL of chloroform: methanol: ammonia (3:1:1) mixture in a 100 mL round bottom flask. In a rotary flash evaporator at 45°C under reduced pressure (435 mbar), the flask was allowed to rotate for 15 minutes at 140 rpm to obtain a dry film.

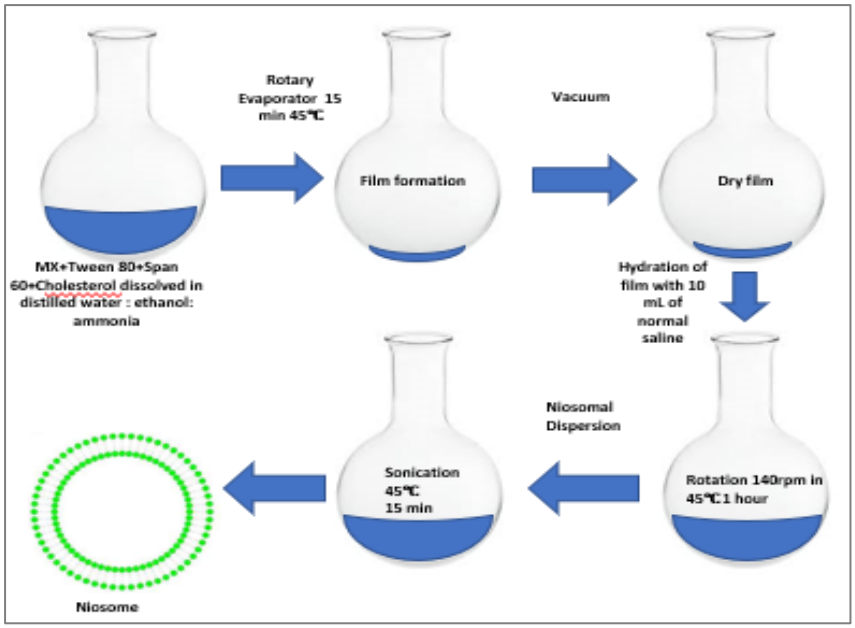

Figure 1: Procedures used for the formulation of MX niosomes using the thin-film hydration process.

This film was hydrated with 10 mL of saline solution and allowed under similar conditions to rotate further for 15 minutes. In a sonicator, bath the niosome dispersion was subsequently sonicated for 15 minutes at 45 °C (Ultrasons-HD 5 Selecta, Spain). The prepared niosomal dispersions were filtered through a 0.45 µm membrane filter to obtain transparent dispersions. The formulated formulations of vesicles (supernatant) were stored in airtight containers at 4°C prior to use.

Table 1: Meloxicam, non-ionic surfactant and cholesterol ratio used for the formulations.

	F1	F2	F3	F4	F5
Meloxicam	0.03	0.03	0.03	0.03	0.03
Tween 20	-	0.18	-	-	-
Tween 80	0.07	-	0.07	-	-
Span 20	-	-	-	-	0.02
Span 60	0.23	-	-	0.22	-
Cholesterol	0.05	0.06	0.02	0.1	0.02

Entrapment efficiency (EE%)

Standard stock solution of 1mg/mL MX was prepared for the calibration curve and validation of the assay method. Six working standard solutions with concentration of 1, 2, 5, 10, 20 and 30 µg/mL were prepared from stock solution and diluted with distilled water: Ethanol: Ammonia; 50 mL: 48 mL: 2 mL, respectively. The absorbances of resulting solutions were measured at λmax (362 nm) and plotted a calibration curve to get the linearity and regression equation.

Encapsulated MX was determined by ultra-centrifugation at 1500 rpm for 45 minutes (Hitachi/Cp100NX, Japan). Briefly, the supernatant was separated with a glass pipette and the precipitated vesicles were washed three times with distilled water to remove residual MX and surfactants in the environment. The incorporation efficiency was calculated from the collected niosomes by UV spectrophotometry (UV1800 Shimadzu Spectrophotometer, Japan) measured at λmax 362 nm, expressed as a percentage of the total amount of MX used initially (EE %).

EE% = amount of MX entrapped/total amount of MX *100

Vesicle characterization

The particle size and polydispersity index (PDI) of the niosomes were determined by a complex light-scattering method using the Malvern Zetasizer (Nano ZS, England). Vesicle formation and morphology were examined with optical microscopy by a camera attached to the optical microscope (Nikon HFX-DX, Japan) at 10×40 and 10× 100 magnifications.

Preparation of MX loaded niosomal-hydrogels

Carbopol-934 P (2.0% w/v) hydrogels containing MX-loaded niosomes equivalent to 1 % w/w of the drug were prepared by technique adopted by French *et al.* (French *et al.,* 1995). Through gentle mixing, a small portion of carbopol-934P was applied to the water. After the full inclusion of the polymer, hydrogel was naturally created by the addition of a few milliliters of triethanolamine. The formulated niosomes dispersed hydrogel was stored prior to use in airtight containers at 4°C.

Characterization of MX loaded niosomal-hydrogels

The rheological analysis of prepared niosomal-gel was evaluated using the Brookfield Digital Viscometer (DV-II, USA) at 37°C. Measurements at varying shearing speeds were applied for rheogram profile. In addition, pH measurements have been evaluated using an electronic pH meter (Jenway, U.K.). All experiments were done in triplicate.

***In vitro* dissolution studies MX loaded niosomal hydrogels**

In vitro drug release from the selected MX niosomal gel sample (F1) and market gel known as Ocam® (1% MX; Galeno) gel were investigated using semipermeable dialysis membrane filters (from Ashke Shishe, Iran). Membranes were hydrated by ethanol and ammonia over a night. 1% MX loaded niosomal hydrogel was inserted into dialysis membrane. Subsequently, filters were placed

within vessels containing 100 mL of release medium (distilled water: ethanol: ammonia; 50 mL: 48 mL: 2 mL respectively,) and stirred at 100 rpm at 37°C. In order to maintain the sink condition, samples of 1 mL of the receptor medium were replaced with 1 mL of the fresh receptor medium 24 hours (Qumbar *et al.,* 2017). Samples were analyzed utilizing spectrophotometry. In order to compare the dissolution profiles obtained in the release studies, the similarity factor (*f2*) and the difference factor *(f1*) were determined according to the SUPAC (Scale-up and post-approval changes) (FDA, 2017).

RESULTS AND DISCUSSION

Impact of formulation components on the encapsulation efficiency

The assay method was validated, and the analytical validation parameters (accuracy, precision, limit of detection, limit of quantification) were calculated. The linearity range of the method was 1–30 µg/mL with R2 of 0.9998. The limit of detection (LOD) was found to be 0.430 µg/mL and the limit of quantification (LOQ) was 1.302 µg/mL. The relative standard deviation for both intra-day and inter-day precision was less than 2%. Table 2 describes the results of encapsulation efficiency. F1 had the highest trapping efficiency of 56.00 % and F3 had the lowest trapping efficiency of 11.87%.

Table 2: Encapsulation efficiency results.

Formulation	Encapsulation efficiency (±SD)
F1	56.00 % (±0.85)
F2	22.12 % (±0.22)
F3	11.87 % (±0.17)
F4	37.98 % (±0.55)
F5	40.23% (±0.23)

The results shows that, relative to Tween-based formulations, Span 60 based niosomes had a substantially higher trapping performance ($p<0.05$). This may be due to Span surfactants' chemical composition. The increase in alkyl chain length could have resulted in greater efficiency of trapping. Span 60 has the longest alkyl saturated chain, which may be responsible for the highest efficiency of encapsulation (Hao *et al.,* 2002). Therefore F1, formulation was chosen for further characterization and assessment studies.

Impact of formulation components on the vesicle characterization

Optical microscopic images indicate that the resulting vesicles were almost spherical in shape and uniform in scale (Figure 2).

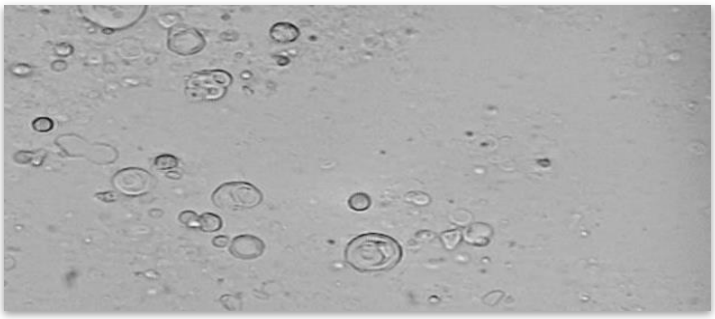

Figure 2: Optical microscopic image of prepared noisome vesicles.

The average size and PDI of the prepared niosomes are shown in Table 3.

Table 3: Average vesicle size and PDI of niosomal formulation.

Formulation	Mean particle size (nm) (±SD)	PDI
F1	236.70 (±2.21)	0.24(±0.06)
F2	350.00 (±0.75)	0.45(±0.03)
F3	384.00(±3.8)	0.52(±0.12)
F4	422.00(±3.2)	0.38(±0.04)
F5	301.00(±0.65)	0.30(±0.13)

The vesicle size distribution ranged between 0.24 and 0.65 with a narrow peak, indicating that this method produced relatively homogeneous vesicles. For all formulations, the particle size spectrum was observed to be between 236–422 nm. Based on the results, the scale of the niosomes showed a steady rise with an increase in HLB surfactant values. This is clearly observed with F1 formulation that consisted of Tween 80 (0.07; HLB: 15) and Span 60 (0.23; HLB: 4.7) (Figure 3). It is predicted that vesicles consisting of a mixture with a lower HLB surfactants values would have a smaller vesicle size than those with higher HLB values. This may be due to surface-free energy, since it decreases with increasing hydrophobicity (Gupta *et al.,* 2011; Nowroozi *et al.,* 2018; Sternberg and Florence, 1994)

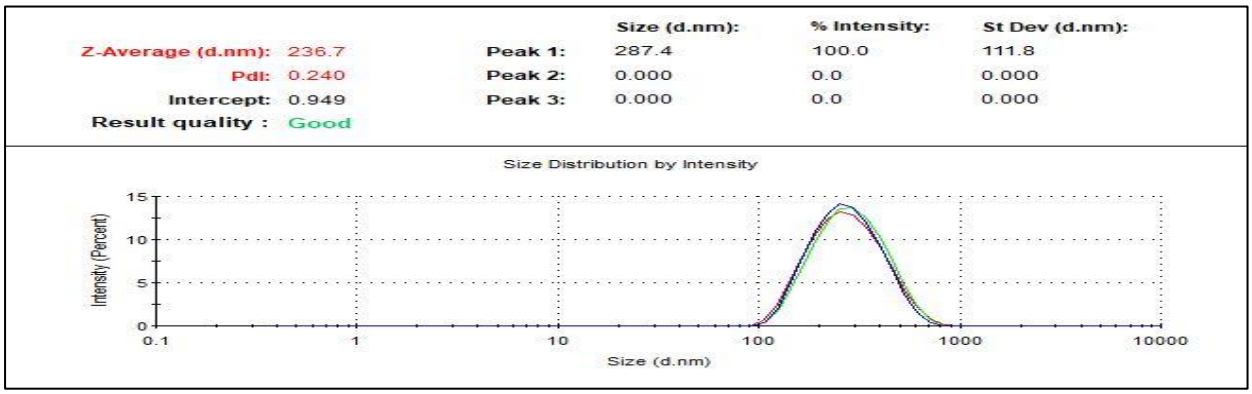

Figure 3: Vesicle size and PDI of F1 formulation niosomes measured with Malvern Zetasizer (Nano ZS, England).

Adequate findings were seen for the drug content, viscosity and pH of gel containing F1 niosomes as 97.4%, 244.66 cP, 7.1 respectively, (Figure 4).

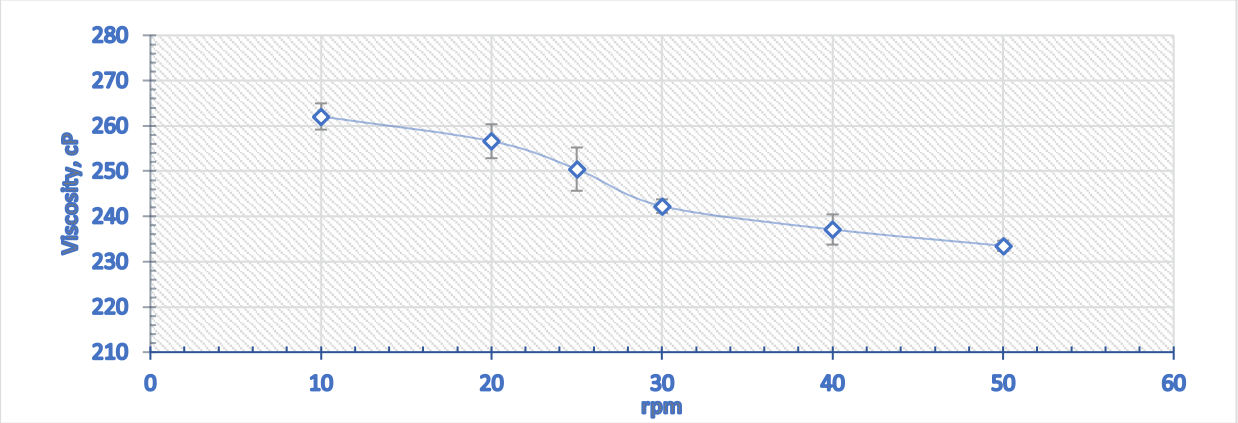

Figure 4: Viscosity profile of F1 niosome loaded hydrogel.

In vitro release of meloxicam from niosome based hydrogel

From pervious investigation, hydrogel loaded with formulation F1 niosomes was chosen for *in vitro* study because of its suitable size (236.7 nm), uniformity (PDI=0.240) and favorable entrapment efficiency (56.00%). The dialysis system was used to track, the amount of MX released from the vesicles within 24 hours. The release profiles of Ocam® (1 % MX gel) and MX niosome based hydrogel formulation are shown in Figure 5.

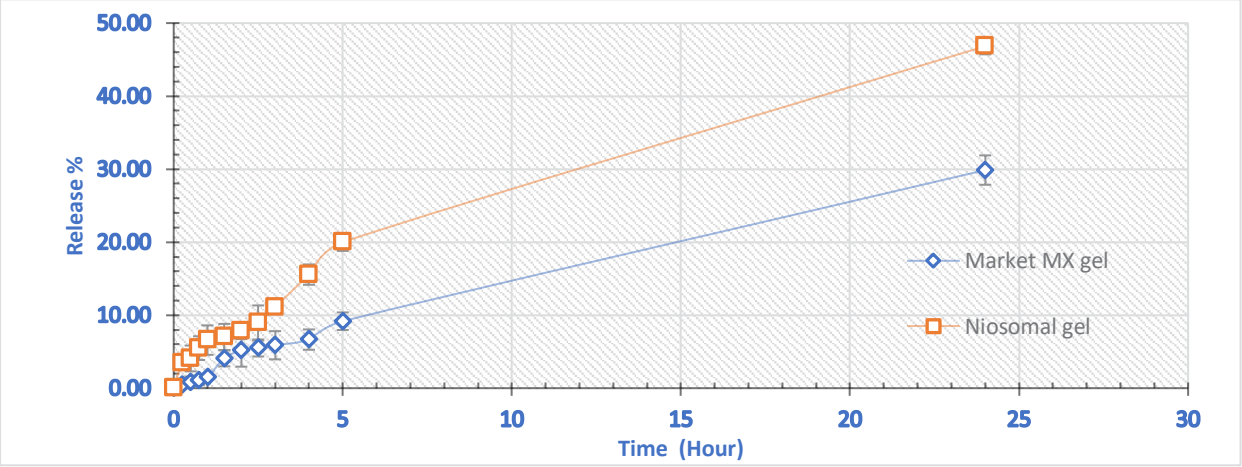

Figure 5: Comparative percentage release of MX from market product (Ocam®) and MX- niosome-based hydrogel.

Different methods have been published in the literature that can be used to compare dissolution profile data (Kassaye and Genete, 2013; Santos Júnior et al., 2014). The most commonly used tools, known as fit factors, have been used in this research. The fit

factors can be represented by two approaches: f1 (the factor of difference) and f2 (the similarity factor). In order for two dissolution profiles to regarded as identical and bioequivalent, f1 should be between 0 and 15, while f2 should be between 50 and 100 (Simionato et al., 2018). In this analysis, the dissolution profiles corresponding to the market product and the MX noisome-based hydrogel are found to be different according to this guideline (Table 4).

Table 4: Fit factors for Market product (Ocam®) and MX Niosomal-hydrogel.

Fit factor	Market product (Ocam®) -Niosomal gel comparation
f1 (the difference factor)	94.80
f2 (the similarity factor)	57.25

To predict the release trend of the drug from the MX niosome-based hydrogel, the *in vitro* release data was fitted to different release kinetics models. The findings indicated that the chosen formulation was best defined by Higuchi release kinetics (displaying the highest linearity and determination coefficient R2=0.976) suggesting that the concentration was independent of drug release.

CONCLUSION

High molecular weight and hydrophobicity of MX may restrict its tissue permeation for topical applications. Different types of non-ionic surfactants were used to prepare meloxicam-containing niosoms that could help MX to overcome this restriction. Hydrophobicity of surfactants has proven to play a role in the size of the niosomes. The devoloped niosome formulation showed a spherical shape, improved entrapment efficiency, and an acceptable polydispersity index and vesicle size. *In vitro* release studies have demonstrated a potential for improved topical delivery of the MX-charged niosomal-hydrogel formulation when compared to its commercial product.The present research has therefore successfully demonstrated the value of niosomal gel as an excellent delivery method for MX. More comprehensive animal and human trials should be undertaken to verify the potential of MX niosomal hydrogel for its anti-inflammatory activities.

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Myrtle (*Myrtus communis* L.) and potential health effects

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Abstract

Myrtus communis L., the common myrtle, is a plant which can be found in the Mediterranean and Middle East regions. The aim of the study is to evaluate the effects of myrtle on the human health. The fruit of myrtle has a unique flavour and can be in two different colours as black or white. Since ancient times, myrtle has been reported to be used in traditional medicine as a food and spice in the treatment of diarrhoea, peptic ulcer, bleeding, headache, palpitations, urethritis, conjunctivitis, pulmonary and skin diseases. In several studies, it has shown that different parts of the myrtle plant contain various bioactive compounds. The leaves of the plant contain quercetin, catechin and myricetin; its fruit contains phenolic compounds and anthocyanin. In the studies investigating the health effects of the myrtle plant, essential fatty acids obtained mostly from various parts of the plant, such as leaves, roots and fruits, were used. Essential fatty acids obtained from the plant are used in scientific and commercial fields such as cosmetics, medicine, food industry, aromatherapy and phytotherapy. It has been thought that positive effects on health due to the bioactive compounds contained in different parts of the myrtle plant. In previous studies, it has been found that the plant has antioxidant, antimicrobial, antidiabetic, anti-inflammatory, anti-ulcerative and antidiarrheal activities. However, it has been observed that most of these studies are animal studies and thus more human studies are needed.

Keywords

Antimicrobial, antioxidant, *Myrtus communis* L., myrtle.

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INTRODUCTION

Myrtus communis L., known as myrtle, is a flowering shrub that grows in the Mediterranean region and in the Middle East (Asgarpanah and Ariamanesh, 2015; Aleksic and Knezevic, 2014). The fruit of the myrtle is covered with a waxy layer, has a unique flavour and can be in two different colours, black or white (Soke and Elmaci, 2015). It has been reported that myrtle has been used in traditional medicine as treatment for diarrhea, peptic ulcer, bleeding, headache, palpitations, urethritis, conjunctivitis, pulmonary and skin diseases in the form of food or spice since the ancient times (Messaoud *et al.,* 2012; Akin *et al.,* 2010; Mahmoudvand *et al.,* 2015; Aksay, 2016). Essential oils obtained from various parts of plants have been used in scientific and commercial fields for many years including, cosmetics, medicine, food industry, aromatherapy and phytotherapy (Donmez and Salman, 2017). Essential oils and ingredients of plants have multiple biological activities (Hsouna *et al.,* 2014). The aim of the study is to evaluate the effects of myrtle plant and the essential oils obtained from the plant on the human health.

Nutritional composition of myrtle

Extracts obtained from various parts of the plant contain the same compounds in different amounts. The leaves of the myrtle contain quercetin, catechin and myricetin (Alipour *et al.,* 2014).

The fruit of myrtle contains various bioactive compounds, but mainly phenolic acids and anthocyanins (Asgarpanah and Ariamanesh, 2015; Sumbul *et al.,* 2011). The dark blue coloured fruit of the myrtle mainly contains polyphenolic compounds and shows high antioxidant activity while white coloured fruit of the myrtle predominantly contains unsaturated fatty acids such as myrtenyl acetate, linoleic acid and oleic acid (Messaoud *et al.,* 2011).

The energy, protein, fibre, fat, sugar, tannin, and essential oil content of the myrtle berries in Turkey were determined as 11.21 kcal/g, 4.17%, 17.41%, 2.37%, 8.64%, 76.11 mg/100 g, and 0.01%, respectively (Aydin and Ozcan, 2007). The berries of myrtle contain 74.1% of unsaturated fatty acids and 25.7% of saturated fatty acids, which are mainly 72.1% oleic acid and 15.7% palmitic acid. The fatty acid pattern is shown in Table 1.

Table 1: Fatty acid pattern of the fruit of myrtle.

Fatty Acids	Amount (%)
Caprylic acid	-
Capric acid	-
Lauric acid	4,3
Myristic acid	3,0
Pentadecanoic acid	0,5
Palmitoleic acid	0,3
Palmitic acid	15,7
Linolenic acid	<0,01
Linoleic acid	1,7
Oleic acid	72,1
Vaccinic acid	-
Stearic acid	2,2
Arachidonic acid	-
Eicosenoic acid	-
Arachidic acid	-
Saturated fatty acids	25,7
Unsaturated fatty acids	74,1

Myrtle contains various polyphenolic compounds. The essential oil obtained from the leaves contains α-pinene (31.8%), 1,8-cineol (24.6%), limonene (14.8%) and linalool (8.3%) (Ghasemi *et al.,* 2011). In its berries polyphenolic content have been found as ellagic acid (54.64%), gallic acid (12.70%), quercetin (3.72%) and quercetin 3-O-rhamnoside (3.71%) (Correddu *et al.,* 2019). It has been thought that the myrtle plant has positive effects on the health due to its phytochemical content (Sumbul *et al.,* 2011). Figure 1 shows the potential positive effects on health of myrtle and its products according to the information obtained from in vitro and in vivo studies.

Antioxidant activity of myrtle

The cell uses oxygen to generate energy, free radicals are formed as a result of the ATP production. These by-products are usually reactive oxygen and nitrogen species (Lobo *et al.,* 2010). The presence of these molecules in large amounts causes oxidative stress, which can cause many chronic diseases such as inflammation, diabetes and atherosclerosis (Percário *et al.,* 2020).

Anthocyanins are the C15 phenolic glycosides that give plants their colours. Anthocyanins have been found to have positive effects on oxidative stress related diseases (Skrovankova *et al.,* 2015). Studies have shown that essential oils obtained from the myrtle plant have high antioxidant activity (Dahmoune *et al.,* 2015). Delphinidin 3-O-glucoside (31.5%), petunidin 3-O-glucoside (25.8%), malvidin 3-O-glucoside represented (24.3%) and minor amounts of anthocyanins such as delphinidin-pentose (4%), delphinidin-pentose (3.8%), cyanidin 3-O-glucoside (6.3%), petunidin-pentose (0.7%), petunidin-pentose (1.6%), and peonidin 3-

O-glucoside (2%) were found in the Italian myrtle berries (Scorrano, 2017).

In the study conducted by Mimica-Dukić *et al.* (2010), it was reported that essential oil of the plant reduces the oxidant effect of DPPH, as well as the effects of t-BOOH mutagen. Xanthine oxidase activities of myricetin-3-o-galactoside and myricetin-3-o-rhamnocide isolated from myrtle leaves inhibit lipid peroxidation and oxidant effects of DPPH, while inhibiting the mutagenic activities of aflatoxin B1, nifuroxazide and H_2O_2. Methanol and ethyl acetate extracts obtained from myrtle plant inhibited the antioxidant effects as well as the genotoxic effects of aflatoxin B1 and nifuroxazide (Hayder *et al.,* 2008). Liquors of white and dark blue coloured myrtle were analysed in a study and it was found that white liquor has higher antioxidant capacity due to its high content of gallic acid and its derivatives (Serreli *et al.,* 2017).

Antimicrobial activity of myrtle

The consumption of contaminated foods with pathogenic bacteria is a major health problem (Cherrat *et al.,* 2014). *Salmonella*, *Clostridium perfringens*, *Campylobacter*, *Staphylococcus aureus*, *Clostridium botulinum*, *Listeria monocytogenes*, *Escherichia coli*, *Vibrio* are common foodborne bacteria that may the most pose health risk (European Food Safety Authority and European Centre for Disease Prevention and Control, 2018)

In a study investigating the antibacterial activity of essential oil obtained from myrtle leaves collected in Northern Cyprus consisted of eucalyptol (50.13%), linalool (12.65%), α-terpineol (9.05%) and limonene (4.26%). The results showed promising antibacterial effect on Staphylococcus aureus, *Listeria monocytogenes, Enterococcus durans*, *Salmonella*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* (Akin *et al.,* 2010). Yadegarinia *et al.* (2006) have found that the myrtle collected from Iran has mainly consisted of α-pinene (29.1%), limonene (21.5%), 1,8-cineol (17.9%) and linalol (10.4%). In addition, it has been determined that the myrtle plant with this content shows high antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* (Yadegarinia *et al.,* 2006). Similarly, in another study, it was found that the ethanolic extract obtained from the leaves of the myrtle has a strong antibacterial activity against *Escherichia coli*. Accordingly, the extracts obtained from the leaves of the myrtle plant could have a potential antibacterial effect on pathogenic bacteria (Douhri *et al.,* 2017).

In recent years, the increase in the frequency and variety of fungal infections has risen the importance of components with antifungal properties (Costa and Alexander, 2009). Essential oil of myrtle is known to cause damage to fungus cell

membranes, cellular material leakage and death of microorganisms (Yangui *et al.,* 2017). It has been reported that essential oil of myrtle have anti-fungal activity of on various pathogenic fungi such as *Rhizoctonia solani*, *Fusarium solani*, *Colletotrichum lindemuthianum*, *Sclerotiniaminor*, *Nigrospora oryzae*, *Cladosporium herbarea* and *Botrytis cinerea* (Kordali *et al.*, 2016).

Antidiabetic and anti-inflammatory activity of myrtle

Diabetes is a metabolic disease characterized by insulin secretion disorder or hyperglycaemia resulting from insulin insufficiency. Chronic hyperglycaemia that occurs with diabetes can cause dysfunction and failure of different organs, especially eyes, kidneys, nerves, heart and vessels (American Diabetes Association, 2014). The antidiabetic and antioxidant activity of the aqueous extract of the Myrtle was reported using diabetic rats. In the same study, serum glucose, aspartate aminotransferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) levels were significantly reduced in diabetic mice consuming 1000 mg/kg of myrtle aqueous extract for 14 days compared to the control group. When compared with the control group, it was determined that aqueous myrtle extract showed significant antioxidant activity in diabetic rats due to its superoxide dismutase activity, increased glutathione levels and decreased malondialdehyde levels (Demir *et al.,* 2016). In a study conducted on mice, it has been reported that myrtle has a potential anti-inflammatory effect in diseases related with inflammation and reduces oedema (Touaibia, 2017).

Antiulcerative and antidiarrheal activity of myrtle

The gastrointestinal system (GIS) is about 10 meters long and is a large system that starts from the mouth, runs through the chest, abdominal and pelvic spaces and ends in the anus. The main task of GIS is to convert nutrients in the diet into forms used by cells in the body for certain tasks (McErlean, 2016). Ulcers that can be found anywhere on the GIS mucosa are a cutaneous bare wound or lesions of mucosal tissue that exhibit gradual tissue breakdown (Kahn and Hall, 2014). It was found that the powder of the Myrtle berries has a significant effect on the healing of oral wounds in an animal study (Hashemipour *et al.,* 2017).

Diarrhea is usually characterized by negative effects on the intestines caused by a bacterial or viral infection, drug reaction, food allergy, or systemic disease (World Health Organization, 2020) Sisay *et al.,* (2017) has found 80% methanol extract from myrtle leaves had an antidiarrheal effect in mice.

Gastroesophageal reflux disease is one of the common chronic gastrointestinal diseases that can cause symptoms such as epigastric pain, indigestion, dysphagia, chronic cough and chest pain (Fock and Poh, 2010). The disease that characterized by spasm or impaired lower oesophageal relaxation, results in impaired flow of food into the stomach and subsequent displacement of stomach contents towards the oesophagus (Boeckxstaens *et al.,* 2011). In a double-blind randomized controlled study, it was reported that myrtle syrup reduced disease-induced symptoms in individuals with gastroesophageal reflux (Salehi *et al.,* 2017).

CONCLUSION

In conclusion, the extracts obtained from myrtle plant has antioxidant, antiulcerative, antimicrobial, antidiabetic and anti-inflammatory effects due to the phytochemical content. Thus, consumption of myrtle has potential positive effects on health. Since the researchers have found results at the cellular level or on animals, its effects on the human body are not fully known. Therefore, experimental human studies are needed to be thoroughly done.

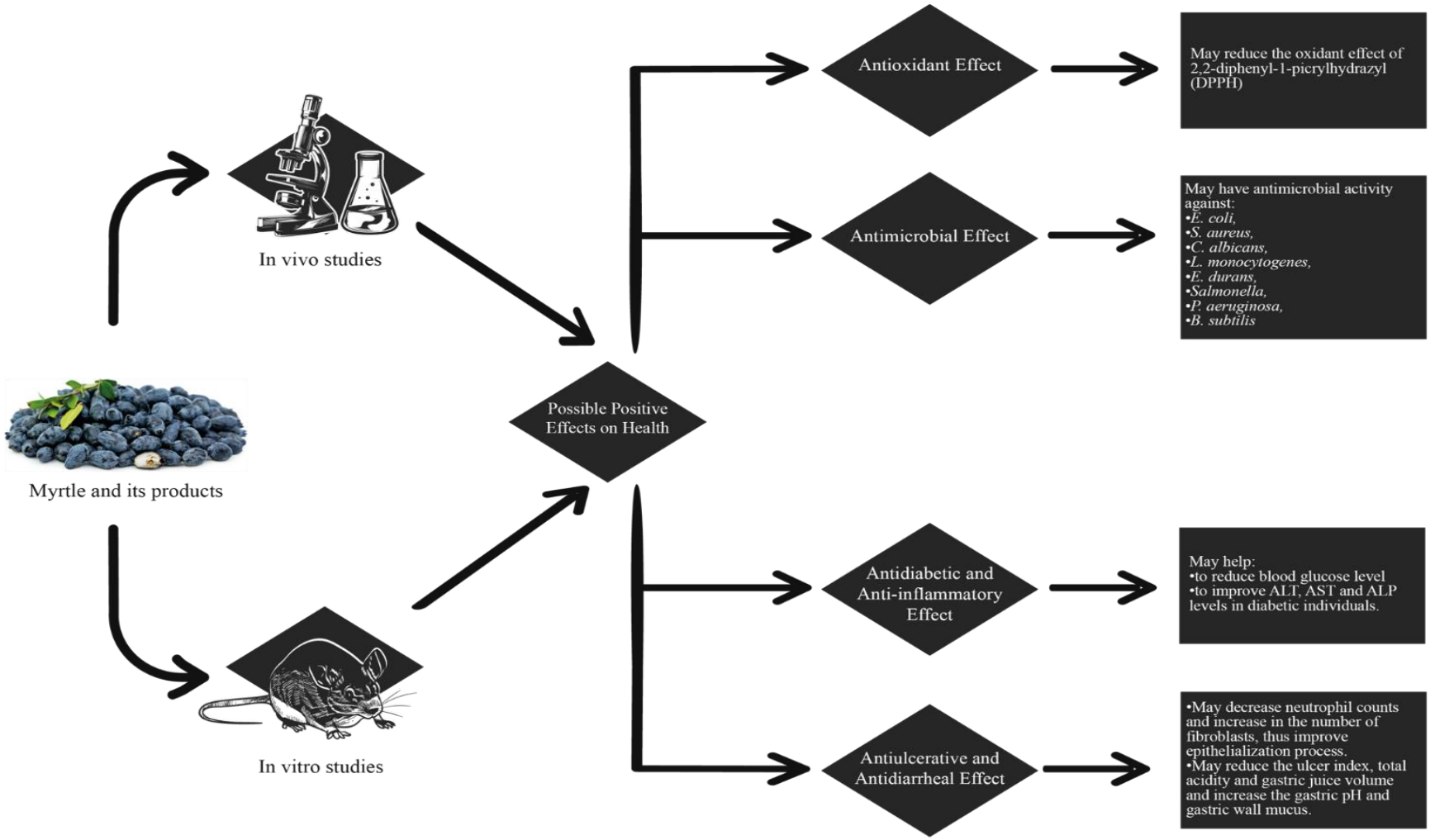

Figure 1: The potential positive effects on health of myrtle and its products according to the information obtained from in vitro and in vivo studies.

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