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Research Article

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Isolation and Identification of Bacteria from Fruit Garden Soils in Aydın Province

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Abstract: In this study, different fruit garden soil samples were collected in Aydın province and bacterial diversity was examined. These isolated bacteria were identified by 16S rRNA sequencing and using BLAST. The Maximum Parsimony method was used to produce a Molecular Phylogenetic analysis and a phylogenetic tree was constructed. As a result of this study the following bacteria *Bacillus thuringiensis* (5), *Bacillus mycoides* (1), *Brevibacterium frigoritolerans* (1), *Bacillus circulans* (1), *Bacillus cereus* (5), *Bacillus weihenstephanensis* (1), *Pseudomonas putida* (1), *Stenotrophomonas maltophilia* (1), *Azotobacter chroococcum* (1), *Paenibacillus xylanilyticus* (1), *Variovorax paradoxus* (1), *Aerococcus viridans* (1) were found. These bacteria are significant because they regulate soil ecosystem. In addition bacteria isolated from soil are used in industrial applications such as agriculture, textile, plastic industry, chemical industry.

Keywords: Bacteria, 16S rRNA, biodiversity, soil,

1. Introduction

Soil is the most precious part of nature due to containing minerals, organic components and microorganisms The physical, chemical and biological properties of the soil profoundly affect life on earth [1]. Soil is an important source of nutrients and food for all living things and it has large variety of different microorganisms. The role of these microorganisms in soil is to stabilize the soil structure for the ecosystem services. Soil microorganisms as bacteria, fungi and archaea contribute to the cycling of all major elements (e.g. C, N, P) in the recycling of wastes, and the detoxification of environmental pollutants [1]. Microbial activity in soil can influence oxygen distribution within soils that anaerobic microbes catalyse a variety of soil processes such as methane production and denitrification [2].

Bacteria in soil ensure a great contribution to organic matter production and soil formation. In addition some bacteria in soil promote plant growth and products synthesized by these bacteria strengthen the structure of the soil [3]. Bacteria are major determinants of the carbon storage capacity of soils because they degrade carbon and nutrients in soil. Denitrifying and methane producing bacteria regulate nitrous oxide (N_2O) and methane (CH_4) emissions from soils [3].

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Although up to now many bacteria have been cultured from soil researches but this subject still continues. Reason for this is these bacteria are used in many industrial fields, especially in agriculture, plastic industry, chemical industry as enzyme, antibiotic, polysaccharide productions and bacterial plastics [4, 5, 6, 7, 8]. Nowadays it has left this place to molecular methods while using the classical taxonomy for bacterial isolation from soil [9]. The diversity of bacteria in the soil is better understood by the use of molecular methods. Although bacteria have been subdivided into more than 100, they have phylum fewer than 10 in soil [10]. While Proteobacteria, Acidobacteria and Actinobacteria are widespread in different soil types Verrucomicrobia, Bacteroidetes, Firmicutes, Chloroflexi, Planctomycetes, and Gemmatimonadetes are usually less dominant. Although the number of the phylum in soil is low it is high compared with other environments [11]. However, bacteria phylum determinated in soil samples is less than 10% as to unknown phylum [10, 12].

In this study, it was investigated biodiversity of bacteria isolated from different fruit garden soils in Aydın province.

2. Experimental

2.1. Collection of soil samples

Various soil samples were taken from six different fruit gardens (mulberry, plum, fig, pomegranate, quince, lemon) in autum and spring from Aydin province. The ground was dug as deep as 10-15 cm for the collection of soil samples. These soil samples were packeted in sterile polythene bags and brought to the laboratory for microbiological analyzes.

2.2. Isolation of microorganisms

Each one gram of the sample was suspended in 99 mL of sterile distilled water and shaken. The samples were heated at 80ºC for 5 min in water bath for *Bacillus* sp. isolation from soil. Mannitol Agar Medium (10g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.2 g $FeCl_3.6\ H_2O$, 0.005 g), *Pseudomonas* Selective Agar (Difco) and Nutrient Agar (Sigma) were used for isolation of *Azotobacter* sp., *Pseudomonas* sp. and *Bacillus* sp. respectively. Later, the liquid media were serially diluted in sterile 0.85 % FTS (NaCl) solution and the dilutions from 10^{-1} to 10^{-6} were plated on Agar Medium. Plates were incubated at 28-37ºC for 24-48 h. After incubation each different colony were isolated and stocked in skim milk [13].

2.3. Identification of microorganisms

Morphological, cultural and biochemical identifications were made according to the Bergey's Manual of Systematic Bacteriology [14]. For molecular identification DNA isolation of the samples were made according to Green and Sambrook [15]. After isolations DNA concentration and purity was measured with nanodrop spectrometer (Thermo Scientific). 16S rRNA PCR reactions were carried out at initial denaturation 95ºC 5 min, denaturation 94ºC 40 sec, annealing 50ºC 40 sec, extension 72ºC 40 sec with 35 cycles and final extension at 72ºC 10 min. Reagents concentrations were 10X Taq Buffer, 0.5M dNTP mix, 10 pM from each primer, 7.5 mM $MgCl_2$ and 1U Taq polymerase with the final volume of 25 µl. PCR products were sent to the sequencing (GATC BioTech, Germany) after electrophoresis at 1.4% agarose jel at 90 V 40 min.

2.4. Phylogenetic analysis of isolates

Phylogenetic tree was constructed by using the Maximum Parsimony method [16]. Reference sequences which were aquired from GenBank, were compared with sequences from our isolates. Sequences were aligned with ClustalW program which is inside MEGA 7.0 software [17].

3. Results and Discussion

20 bacterial species were identified and the results were *Bacillus* sp., (13) *Pseudomonas* sp. (1), *Stenotrophomonas* sp (1), *Azotobacter sp*. (1), *Brevibacterium sp*. (1), *Paenibacillus* sp. (1), *Variovorax paradoxus* (1), *Aerococcus* sp. (1) (Table 1).

Table 1. Characterization and number of bacteria isolated from different soils

Bacteria	**Number of isolates**	**Sample numbers**	**Characterizations**
Bacillus sp.	13	A35,B9,B18,B20,B22,B25,B30, B31,B33,B37,B38,B45,B47,	Gr(+), rod shaped bacteria
Pseudomonas sp.	1	P30	Gr(-), rod shaped bacteria
Stenotrophomonas sp.	1	P4	Gr(-), rod shaped bacteria
Azotobacter sp.	1	A30	Gr(-), rod shaped bacteria
Brevibacterium sp.	1	B12	Gr(+), rod shaped bacteria
Paenibacillus sp.	1	P11	Gr(+), rod shaped bacteria
Variovorax sp.	1	P20	Gr(-), rod shaped bacteria
Aerococcus sp.	1	P14	Gr(+), coc shaped bacteria

Pseudomonas sp., *Stenotrophomonas* sp., *Azotobacter* sp., and *Variovorax paradoxus* are Gr (-) rod shaped bacteria. *Bacillus* sp., *Brevibacterium* sp. and *Paenibacillus s*p. are Gr(+) rod shaped bacteria. *Aerococcus* sp. is Gr(+) coc shaped bacteria. Some biochemical tests were applied to isolated bacteria and results were given in Table 2.

Table 2. Biochemical characteristics of bacteria isolated from different soil samples

	Biochemical Characteristics								
Bacteria	Spore formation	Catalase	Glucose	Lactose	Sucrose	Starch hydrolysis	Nitrate reduction	Citrate utilization	Voges-Proskauer
Bacillus thuringiensis	+	+	+	-	Variable	+	+	+	+
Bacillus mycoides	+	+	+	Variable	Variable	+	+	Variable	+
Bacillus circulans	+	+	+	+	+	+	Variable	-	-
Bacillus cereus	+	+	+	-	Variable	+	+	-	+
Bacillus weihenstephanensis	+	+	+			+	Variable	+	+
Pseudomonas putida	-	+	+			-	+	+	
Stenotrophomonas maltophilia	-	+	+	+		-		Variable	-
Azotobacter chroococcum	-	+	+		+	+	+		
Paenibacillus xylanilyticus	+	+	+			+	-	-	
Aerococcus viridans	-	-	+	+	+	-	-		

PCR results of these samples were send to GATC BioTech, Germany for sequencing. Molecular identification made by comparing sequence results with Genebank using BLASTn software. Species we found are as following *Bacillus thuringiensis* (5), *Bacillus mycoides* (1), *Brevibacterium frigoritolerans (1), Bacillus circulans* (1), *Bacillus cereus* (5), *Azotobacter chroococcum* (1), *Paenibacillus xylanilyticus* (1), *Aerococcus viridans* (1), *Bacillus weihenstephanensis* (1), *Pseudomonas putida* (1), *Stenotrophomonas_maltophilia* (1), *Variovorax paradoxus* (1). The analysis of the soil samples of Aydın provinces showed that there were twenty species with accession number (Table 3).

Table 3. Molecular identification of the species isolated from fruit garden soils (pomegranate, plum, fig, lemon, quince, mulberry, grape) in Aydın province.

Name of The Species	Name of Samples	Number of Strains	Accession No	Similarity (%)
Bacillus thuringiensis	Plum, Mulberry,Fig, Quince	5	KJ784474.1	98
			KJ676099.1	99
			FJ981909.1	98
			KU179338.1	97
			KX832697.1	98
Bacillus cereus	Lemon, Quince, Pomegranate	5	FJ763650.1	99
			KX694390.1	99
			KM349191.1	98
			KT922033.1	99
			KU179332.1	99
Bacillus mycoides	Grape	1	JX122613.1	97
Bacillus circulans	Lemon	1	KT983982.1	98
Bacillus weihenstephanensis	Quince	1	KF836527.1	97
Pseudomonas putida	Plum	1	KU977141.1	99
Stenotrophomonas maltophilia	Lemon	1	KC857484.1	98
Azotobacter chroococcum	Plum	1	KX108861.1	99
Brevibacterium frigoritolerans	Lemon	1	HQ202870.1	99
Paenibacillus xylanilyticus	Grape	1	KM378595.1	98
Aerococcus viridans	Pomegranate	1	KR140225.1	98
Variovorax paradoxus	Grape	1	KX530771.1	99

A neighbour-joining phylogenetic tree was constructed by MEGA 7.0 software from a partial 16S rDNA sequence of the bacterial isolates obtained in this study with selected sequences downloaded from GenBank, shown in Fig. 3. The ClustalW program in MEGA 7.0 was used to align the sequences. These isolates found were *Bacillus thuringiensis* (5), *Bacillus cereus* (5), *Bacillus circulans* (1), *Bacillus mycoides* (1), *Bacillus weihenstephanensis* (1), *Pseudomonas putida* (1), *Stenotrophomonas maltophilia* (1), *Azotobacter chroococcum* (1), *Brevibacterium frigoritolerans* (1), *Paenibacillus xylanilyticus* (1), *Aerococcus viridans* (1), *Variovorax paradoxus* (1) (Fig. 3).

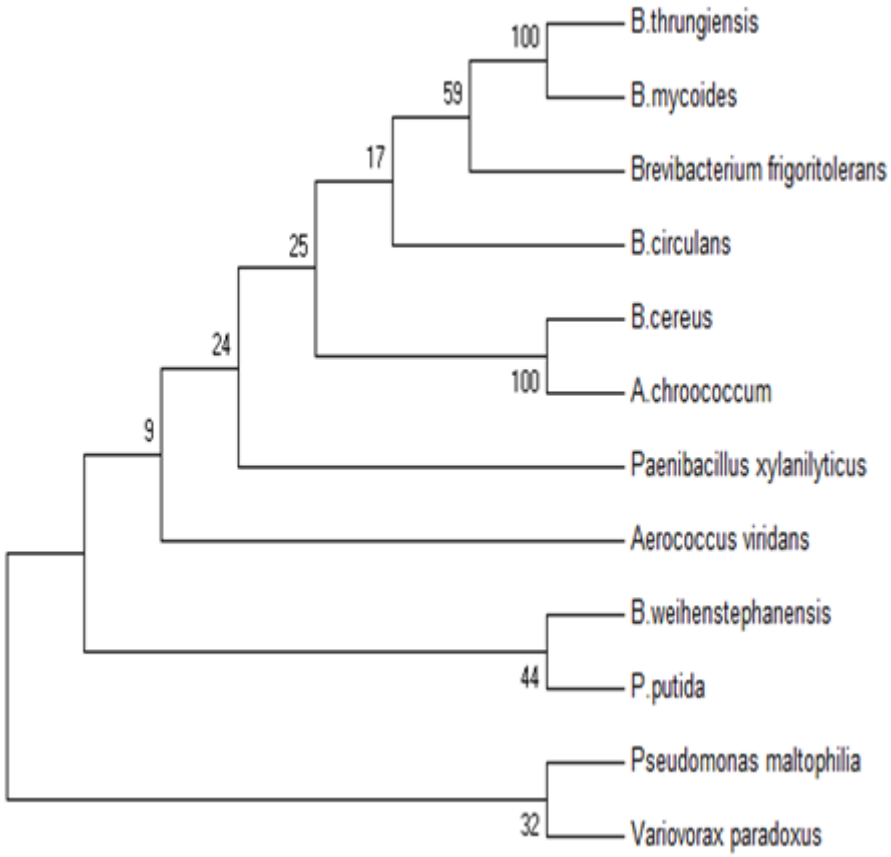

Figure 3. Molecular phylogenetic analysis by Maximum Likelihood method.

Using the Tamura based model of the maximum likelihood method the evolutionary history was found and the as shown in the figure is the highest log likelihood. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths representıng the number of substitutions per site. The analysis involved 12 nucleotide sequences. Codon posıtıons were the usual trıplets wıth some non codıng nucleotıdes. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 166 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Many researchers have isolated and used molecular methods such as 16s rRNA-PCR, ERIC-PCR, REP-PCR in identifying PHB producing bacteria from different soil samples.

Hayat et al. [4] isolated Gram positive *Bacillus* strains from legumes rhizospheric soil and identified using 16S rRNA gene sequencing for plant growth promoting activities in legume. Issar et al. [18] isolated *Pseudomonas* spp. from root nodules of various leguminous plants and identified that these 8 bacterial isolates belonged to genus *Pseudomonas* using 16S rDNA sequencing. Lakshmi et al. [19] carried out molecular identification using 16S rRNA sequencing of bacteria isolated from rhizospheric soils and the organism was identified as *Pseudomonas aeruginosa* KC1. Kasa et al. [20] researched isolation, screening, and molecular characterization of plant growth promoting rhizobacteria isolates. They identified *Azotobacter* as 85.59% polymorphic using randomly amplified polymorphic DNA analysis. Pathania et al.

[21] carried out molecular characterization of diazotrophic bacteria isolated from rhizosphere of wheat cropping system. These bacteria were identified as diverse genera of *Pseudomonas* sp., *Bacillus* sp., *Azotobacter* sp., *Rhizobium* sp., *Azospirillum* sp., *Beijerinckia* sp. and *Derxia* sp. Dadook et al. [22] isolated twelve nitrogen-fixing bacteria from six different soil samples and identified as *Azotobacter chroococcum*. Bhuvaneswari [23] executed molecular characterization using 16S rDNA gene sequences of bacterial strains isolated from the biomass sludge sample. These bacteria were confirmed as *Pseudomonas* sp., *Staphylococcus* sp., *Alcaligenes* sp., *Agromyces* sp., *Stenotrophomonas* sp., *Reichenowia* sp., *Achromobacter* sp., *Brevibacterium* sp. and *Pseudaminobacter* sp. Kumar et al. [24] identified as *Paenibacillus elgii* using 16S rDNA gene sequences of isolated strain from forest soil. It was reported that *P. elgii* indicated wide spectrum effect against all human and plant pathogenic microorganism. Someya et al. [25] researched the bacterial community of a potato phytosphere at the flowering stage and found 82 genera from 8 phylum. These bacteria were identified using 16S rDNA gene sequences and assigned as *Variovorax*, *Pseudomonas*, *Paenibacillus*, *Bacillus* etc. Olukunle [26] isolated bacteria associated with crude oil polluted sites using traditional methods and identified as *Aerococcus viridans*.

The aim of this study was to isolate and identify, using 16S rRNA sequencing methods, soil bacteria from fruit garden soil samples. According to these; it has been showed that morphological methods are not always adequate and confidential for identification of species. Thus, both morphological and molecular methods for identification of bacteria were used. It can be seen that, in recent years, molecular identification gained more importance.

4. Conclusion

In this study isolation of soil bacteria from fruit garden soils (pomegranate, plum, fig, lemon,quince, mulberry, grape) in Aydın province were carried out. In addition, we obtained twenty various bacteria as *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus mycoides*, *Bacillus weihenstephanensi*, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, *Azotobacter chroococcum*, *Brevibacterium frigoritolerans*, *Paenibacillus xylanilyticus*, *Aerococcus viridan*, *Variovorax paradoxus*. These bacteria regulate the circulation of matter in the soil and encourage plant growth.

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Research Article

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Screening some of plants from *Silene* Genus for 20-Hydroxyecdysone

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Abstract: Eight *Silene* species include *S. brachuica*, *S. guntensis*, *S. linicola*, *S. oreina*, *S. praemixta*, *S. pseudotites*, *S. viridiflora* and *S. wallichiana* were screened for the main ecdysteroid 20-hydroxyecdysone by HPLC using the UV spectroscopy. HPLC analyses have shown all *Silene* plants except *S. oreina* contain 20-hydroxyecdysone, but in different concentration. Studies have shown that *S. praemixta* and *S. viridiflora* are rich phytoecdysteroids containing plants and the yields of total ecdysteroids are 2.0% and 1.6%, respectively. The results of investigation species of *Silene*: *S. brachuica*, *S. praemixta*, *S. viridiflora*, *S. guntensis*, *S. linicola*, *S. pseudotites* and *S. wallichiana* showed that the yields of 20E of these plants are 0.03, 0.27, 0.35, 0.082, 0.367, 0.071 and 0.08% respectively. The plants of *S. linicola*, *S. praemixta* and *S. viridiflora* suggested to be used for production of ecdysteroids containing preparations.

***Key words*:** Phytoecdysteroids, Caryophyllaceae, *Silene*, 20-hydroxyecdysone,

1. Introduction

Phytoecdysteroids are contentedly widespread in the plant world. They are isolated from the main types of higher plants - ferns, gymnosperms and angiosperms, but their function in plants are yet studied insufficiently. The application of phytoecdysteroids is a promising alternative to the use of anabolic-androgenic steroids because of the apparent lack of adverse effects. Toxicity is very low, at an LD_{50} for 20-hydroxyecdysone (20E) of 6.4 g/kg (per os) and 9.0 g/kg (pre oral) [1]. 20E may be extended to treatments of pathological conditions where anabolic steroids are routinely applied. 20E does not bind to the cytosolic steroid receptors, but rather is likely to influence signal transduction pathways, just as the anabolic steroids, possibly via membrane bound receptors. One of the most cited effects of phytoecdysteroid application is the increase of muscle size.

The tonic and anabolic preparations are produced from the plants *Rhaponticum carthamoides* (Asteraceae), *Pfaffia irisinodes* (Amaranthaceae), *Ajuga turkestanica*

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(Lamiaceae) and *Serratula coronata* (Asteraceae). The limited availability of the natural resources and relatively low content of 20E in these plants leads to a high cost of these preparations. Regional variations in the concentration levels of 20E range from 2.10-5–3% of dry weight with the average being between 0.001-0.01%.

More than 170 *Silene* species (family Caryophyllaceae) have been analyzed for their phytoecdysteroid content, and 140 of them were found to be positive and 93 different ecdysteroids have been detected from these plants. Some of them contain a high concentration of 20- hydroxyecdysone, such as *Silene otites* (almost 1%) and *Silene multiflora* (1.9%) [2]. It is established that the promising 20E containing species are plants of genus *Silene* L. and it is necessary to find novel plants among this genus. In this study we screened content of 20E from aerial parts 8 *Silene* species plants: *S. brachuica* Boiss., *S. guntensis* B. Fedtsch., *S. linicola* L., *S. oreina* Schischk, *S. praemixta* M. Pop., *S. pseudotites, S. viridiflora* L. and *S. wallichiana* Klotzsch.

2. Materials and Methods

2.1. Plant Material

Aerial parts of the *Silene* species such as *S. brachuica* Boiss., *S. guntensis* B. Fedtsch., *S. linicola* L., *S. oreina* Schischk, *S. praemixta* M. Pop., *S. pseudotites, S. viridiflora* L. and *S. wallichiana* Klotzsch. were collected in the Surkhan-Darya, Samarkand, Tashkent, Namangan regions and Botanical Garden of Uzbekistan in the summer time during 2011-2013. Aerial parts were collected when the plants were at the flowering stage. The plants were identified at the Department of Herbal Plants, Institute of the Chemistry of Plant Substances, Uzbekistan, by Dr. O.A. Nigmatullaev and voucher specimens were deposited at this Department.

2.2. Extraction

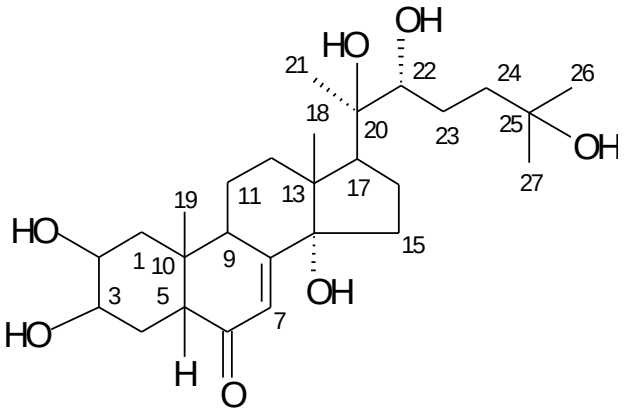
The collected plant material was air-dried away from direct sunlight at room temperature and then ground to a fine powder in a Waring blender. After grinding 100 g unit of the powdered plant material was extracted for 24 h in 500 ml of methanol. The extraction solvent were subsequently filtered to remove plant debris and then evaporated to dryness using a rotary vacuum at 40^{o}C. Solvents methanol, acetonitrile and water were purchased from Sigma (Italy).

2.3. HPLC analysis

The contents and quantity of the 20E in the extracts of *Silene* species were investigated by HPLC. Chromatographic profiles of the extracts were generated using a high performance liquid chromatograph LC-10ATvp connected to a UV-VIS detector SPD-10Avp (Shimadzu Co, Kyoto, Japan). Extracts were diluted to 1 mg/ml, filtered through 0.22 μm and 20 μl were injected. For separation of these extracts, a Nucleosil 100-5 C18 column with a size 250 mm × 4 mm (Macherey-Nagel GmbH & Co, KG) was used. Elution was carried out by a mobile phase consisted of A (water) and solvent B (acetonitrile) and the gradient profile was as follows: from 0% B to 5% B in 8 min, from 5% B to 85% B at 8-30 min, from 95% B to 100% B% at 30-35 min and at 100% B% until 40 min. Flow rate was 1 ml/min and detection was at 242 nm and 200 nm. The quantifications of 20-hydroxyecdysone in the extracts were carried out using a calibration curve of corresponding standards at different concentrations. Authentical phytoecdysteroid 20E was obtained from the Institute of the Chemistry of Plant Substance, Tashkent, Uzbekistan. The purity of the tested compound was >95 %, as determined by HPLC. Standard of 20E was dissolved in MeOH and stored in dark at 4 ^{o}C.

3. Results and Discussion

Plants comprise rich sources of ecdysteroids in high concentration and with broad structural diversity. Zibareva et al. (2000) indicate that some sections and groups of the genus *Silene* probably include only ecdysteroid-containing species (e.g., the sections Siphonomorpha, Dipterospermae, Silene, Otites), whereas others probably comprise only ecdysteroid-negative species (e.g., Auriculatae, Conomorpha, Eudianthe, Heliospermae, Inflatae). The highest content of ecdysteroids both in annual and perennial species was associated with the reproductive organs. Ecdysteroid content was also high in leaves and lowest in stems. In annual species, the highest concentrations were found during budding or flowering stages. In *S. linicola* and *S. viridiflora*, ecdysteroid concentrations followed the same pattern: reproductive organs > leaves > stems> roots and the highest concentrations of 20E were found in flowers [3, 4].



Scheme 1. 20-Hydroxyecdysone (**20E**)

Eight *Silene* species include *S. brachuica*, *S. guntensis*, *S. linicola*, *S. oreina*, *S. praemixta*, *S. pseudotites*, *S. viridiflora* and *S. wallichiana* were screened for the main ecdysteroid 20E by HPLC using the UV spectroscopy. HPLC analyses have shown all *Silene* plants except *S. oreina* contain 20E, but in different concentration. Studies have shown that *S. praemixta* and *S. viridiflora* are rich phytoecdysteroids containing plants and the yields of total ecdysteroids are 2.0% and 1.6% respectively (from weight of air dried aerial parts). The results of investigation species of *Silene*: *S. brachuica*, *S. praemixta*, *S. viridiflora*, *S. guntensis*, *S. linicola*, *S. pseudotites* and *S. wallichiana* showed that the yields of 20E of these plants are 0.03, 0.27, 0.35, 0.082, 0.367, 0.071 and 0.08% respectively. The chemical analysis of the present study confirms already reported [5-7]. Determination of structures and content of isolated 20E was established on basis of data of physical-chemical constants and HPLC. The absorption maximum is due to the presence of a conjugated ketone occurs near 242 nm.

20-Hydroxyecdysterone (**20E**), $C_{27}H_{44}O_7$, mp 241-242°C (acetone), $[a]_D$ +58.9±2° (*c* 0.3, CH_3OH). **IR** spectrum (KBr, n, cm-1): 3435 (OH), 1665 (7-en-6-on). **UV** λ_{max}^{MeOH} nm: 242 (log ε 3.98). **ESI-MS,** m/z (%): 480 $[M]^+$ (0.03), 462 (1), 446 (14), 444 (2), 411 (4), 408 (10), 393 (5), 363 (10), 345 (33), 327 (20), 301 (16), 300 (12), 161 (5), 143 (10), 125 (8), 107 (6), 99 (100), 81 (34), 69 (21). **HR-MS** m/z: for $C_{27}H_{47}O_7$ $[M+H]^+$ calcd. 481.3165, found 481.3233 [8].

4. Conclusion

Determination of content of 20E in Silene species were analyzed by UV–vis and mass spectral characteristics to standard. Based on validation results, the developed method proved useful for 20E analysis under the specified conditions. The conducted screening results confirm the content of 20E in those *Silene* plants of Uzbekistan. The plants of *S. linicola, S. praemixta* and *S. viridiflora* can be used for production of the tonic and anabolic preparation "Ecdisten".

5. References

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Research Article

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Recycling of Tea Waste: Simple and Effective Separation of Caffeine and Catechins by Microwave Assisted Extraction (MAE)

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Abstract: This study was designed to develop a cost effective green extraction method to separate valuable chemicals from black tea waste (BTW) and tea dust called "caffeine dust" (CD). These materials are totally unwanted residue and treated as rubbish. Two extraction methods were used for extraction of waste materials obtained in three collection periods. In conventional extraction method, material was subjected to hot water extraction at 80 ^{o}C for 40 minutes. In microwave assisted extraction (MAE) method, different eco-friendly aqueous solvents (water, citric acid-water or ethanol-water) were used under a controlled 600 W microwave power at 80 ^{o}C for only 4 minutes treatment in a close microwave system. MAE and conventional method gave similar extract yields for both waste materials but MAE was more successful and economical completing the extraction in shorter time. Using citric acid-water solvent in MAE significantly increased the extraction of individual catechins such as epicatechin (EC) and epigallocatechin gallate (EGCG). This study showed that tea waste could be utilized for production of valuable chemicals using nontoxic solvents at extremely short extraction periods.

Keywords: Tea waste, caffeine, catechins, microwave assisted extraction,

1. Introduction

Black tea is one of most favored drink in all societies. Different methods are used for black tea production to obtain its red bright infusion. Basic steps for production line are withering, rolling, fermentation and drying. Among them, fermentation step is the most significant process of making black tea since it defines the quality of the tea.

In Turkey, tea is planted in Eastern Black Sea region and harvested in three collection periods; first collection is in May, second is in June, and the third is in August-September. Almost half of black tea is processed by the government based tea processing plants in seven grades [1]. All tea factories use there main techniques namely orthodox, rotorvan and CTC (curling, tearing, crushing) or combination of these techniques. Çaykur is a government based company and use either orthodox (nonpressing orthodox+sieving+pressing orthodox) or modified Çaykur method (nonpressing orthodox+rotorvan+sieving+conical orthodox). During these processes tea leaves are broken down to small particles and this is not desired by tea producers. Small particles are separated by sieving or air flow from black tea that has a market value. This waste called "caffeine dust" and produced at large quantities. It is totally unwanted

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residue and treated as rubbish or burned for energy production. Another waste material is also produced during production of black tea. This material is different than caffeine dust and mainly contains cellulosic residue of tea stalks. Çaykur sells 200.000 tones waste material in 2014 and guaranties that they will supply 20.000 tons/year for next 10 years. According to new legislation in Turkey damping these waste materials are not allowed and it must be consumed in an appropriate way. Burning is one of the possible option but created massive air pollution.

On the other hand both black tea waste (BTW) and caffeine dust (CD) contain the same compounds as black tea has: tea polyphenol, caffeine, amino acids, saponins, tannins, etc., [2]. Tea polyphenols are catechins, flavanols, phenolic acids, flavanonens, glycosides and plant pigments [3] . The catechins can be categorized into two groups based on their structure: epistructured catechins and non-epistructured catechins. The epistructured catechins are epigallocatechin (EGC), epicatechin (EC), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG), of which EGCG is the major constituent and the most powerful one. Non-epistructured catechins are gallocatechin (GC), catechin (C), gallocatechin gallate (GCG), and catechin gallate (CG) [4]. This group of compounds is known responsible from anticarcinogenic and antimutagenic properties of tea, as well as protective action against cardiovascular diseases. They are good antioxidant agents and have been used to enhance the shelf-life of food products [5]. Therefore, these waste materials can be utilized for production of valuable chemicals such as caffeine and catechins. But an economical and eco-friendly extraction method should be used for separation of these chemicals from tea waste. Extraction time and consumed energy is an important issue to process the waste materials at large quantities.

Conventional solid-liquid extraction is one of the methods that are used for effective extraction of caffeine and catechins from tea samples. Many different treatment steps were used for extraction of caffeine and catechins that mainly consists hot water as extracting solvent [6-12].

Other methods have been proposed for effective extraction of chemicals from tea. A review published by Vuong et al. (2011) contains all important techniques for the extraction of tea samples. Although hot water extraction methods are quite simple and easy they require long extraction periods and energy that are not feasible for large scale production. Separation of valuable chemicals from tea waste at large quantities need sophisticated techniques that: *i*) has relatively lower and economical extraction periods *ii)* provide high extract yields and *iii*) selective to target compounds.

Microwave assisted extraction (MAE) is a new techniques that requires lower extraction period and less energy consumption. Microwave power produces high temperature regions that ease the disruption of the cell wall and facilitates the extraction. As a result of these effects caffeine and catechins are readily extracted into the extracting solvent [2,13]. Recently Nkhili et al. (2009) has reported a study for extraction tea using a 600 W microwave oven. The temperature was controlled between 80 and 100 ^{o}C for 30 min and the solvent to tea ratio was 20:1 (ml/g). Under these conditions the extraction efficiency for the tea catechins was reported to be higher than for the conventional hot water methods. Therefore, MAE of tea waste might be a good approach to meet all requirements.

The objective of this study is to establish MAE methods for cost effective extraction of caffeine and catechins from black tea waste (BTW) and caffeine dust (CD). Two extraction methods, conventional hot water and microwave assisted extraction (MAE), were employed and different extracting solvents were tested for MAE. Waste material was collected from the same tea factory in different collection periods and subjected to extraction. After successive separation of caffeine and catechins from extraction medium these constituents were quantified by chromatographic analyses to determine the amount of individual catechin. The results of this study can be used as reference for recycling of these waste materials for cost effective

production of valuable chemicals. This is the first report employing MAE for extraction, isolation and quantification of industrial important chemicals from waste materials such as caffeine dust.

2. Material and Methods

2.1. Standards and chemicals

Methanol, ethanol, ethyl acetate and chloroform were analytical grade from Merck. The standard chemicals of (−)epigallocatechin (EGC), (−)epicatechin (EC), (−)epigallocatechin gallate (EGCG), (−)epicatechin gallate (ECG) and caffeine were purchased from Sigma (St Louis, MO, USA). Black tea waste and caffeine dust used in the experiments were collected from Sürçay San. Co. Ltd. (Sürmene, Trabzon, Turkey). All experiments works were repeated three times.

2.1.2. Conventional hot water extraction

Briefly, 10 g of waste material was extracted with 200 ml of pure water at 80 °C for 40 min. Residue was removed by filtration using a filter paper (pore size: 5 μm) and filtrates were initially partitioned with chloroform to remove caffeine. Then aqueous phase was extracted with ethyl acetate three times using 150 ml ethyl acetate in each extraction to separate catechins. The chloroform and ethyl acetate phases were filtered and the filtrate was concentrated by a rotary evaporator under reduced pressure at 55 °C.

2.1.3. Microwave assisted extraction (MAE)

Tea catechins and other components were extracted using a close microwave assisted extraction system (MILESTONE, START S Microwave, USA). MAE parameters such as microwave power, extraction time and extracting solvent can affect the efficiency [14]. 10 g of sample and 200 ml extracting solvent were placed in vessel and soaked for 90 minutes at room temperature. Presence of citric acid or ethanol facilitates the selective extraction of catechins [4]. Therefore, three extracting solvents (water, citric acid-water or ethanol-water) were used. Citric acid-water solvent was prepared using 0.1 M citric acid and water (1:1). Ethanol-water was prepared diluting technical ethanol (96%) with equal volume of water. Then sample was transferred into microwave extraction apparatus [3]. Extraction was carried out under a controlled 600 W microwave power at 80 ^{o}C temperature for 4 min irradiation time [15]. After extraction, the flask was allowed to cool down room temperature before opening the cap. Aqueous infusion was extracted with first chloroform then ethyl acetate as described above.

2.1.4. Quantification of caffeine and catechins

In conventional and MAE quantification of caffeine and catechins were carried out by weighting the chloroform and ethyl acetate fractions after evaporation the solvent and drying the extract. Extract yields were calculated from the mass of extracts. HPLC analyses were carried out to determine the amount of each catechins present in the mixture [16]. The instruments used in this study was a HPLC system (Hitachi Elite Lachrom) equipped with a Shim-pack VP-ODS C18 column (5 mm, 4.6 x 250 mm, 35 °C) at 278 nm. Solvents A (water) and B (DMF-methanol-acetic acid mixture, 20:1:0.5) were run with 86% A for 13 minutes then its volume was decreased to 64% within next 15 minutes and finally back to initial concentration for another 6 min. Concentrations of catechins were quantified by their peak areas against those of standards prepared from original compounds.

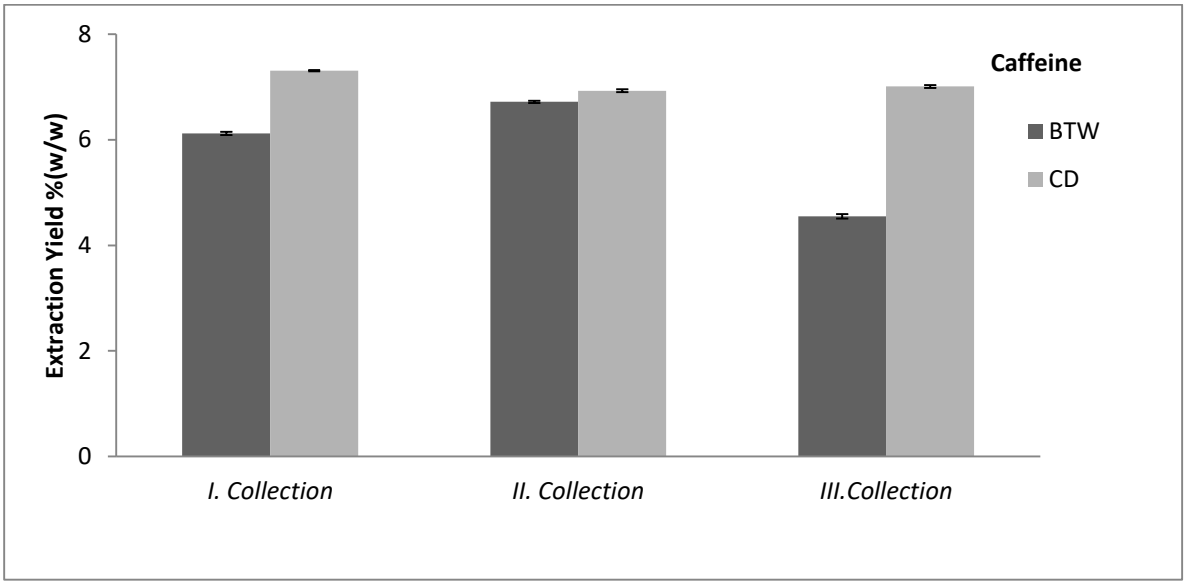
3. Results and Discussion

3.1. Extraction yields

3.1.1.Conventional Extraction

Waste materials were collected at three collection and processing times in 2013. The average caffeine and catechin yields from the first (in May, *I. Collection*), second (in June, *II. Collection*) and third collection period (in August-September, *III. Collection*) using conventional extraction method is given in Figure 1.

a)



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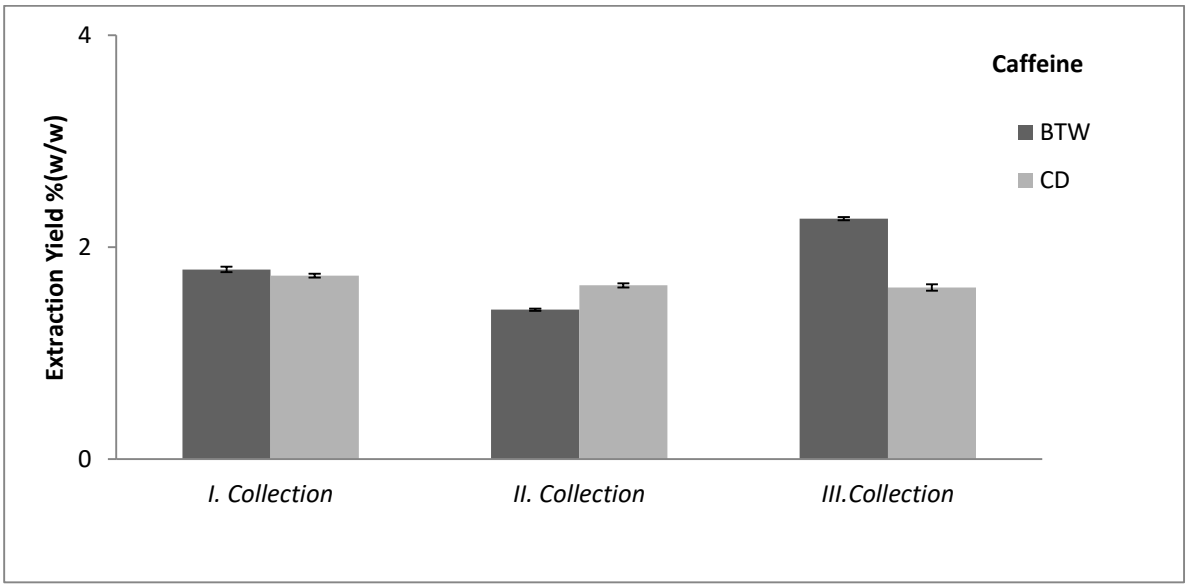


Figure 1. Caffeine and catechin yields of black tea waste (BTW) and caffeine dust (CD) in three collection periods. Data are expressed as the mean of three replicate.

Figure 1 shows that caffeine yields of both waste materials are quite high. Processed black tea usually contains 2-4% caffeine and Serpen et al. (2012) reported the caffeine content of black tea produced in the same region was between 1.5-1.8%. Their results were solely obtained from HPLC analysis and separation of caffeine from tea extract had not been carried out. In

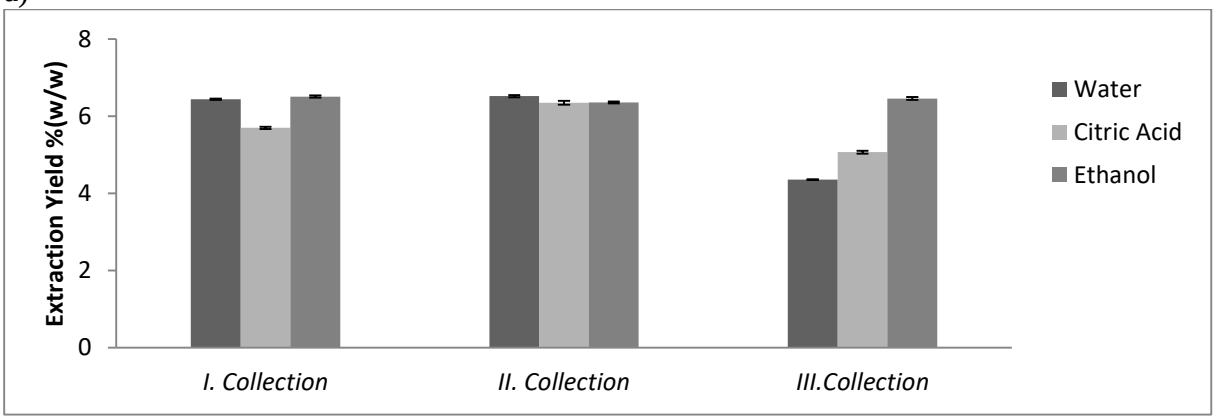
BTW caffeine yields were similar at first two collection periods (6.12 and 6.72% respectively). BTW collected in the third collection period gave lower caffeine extract (4.25%). CD gave higher caffeine yields than BTW at all collection periods (ranging between 6.72-7.31%).

Although catechin content of BTW was slightly higher than CD both waste materials have lower catechin yields. However, catechin yield was slightly higher in BTW collected in the third collection period (2.27%).

3.1.2 Microwave Extraction of BTW and CD

Waste material was subjected to MAE in different solvents for a 4 minutes exposure period. Extract yields (%, w/w) obtained from black tea waste and caffeine dust is given in Figure 2 and 3.

a)



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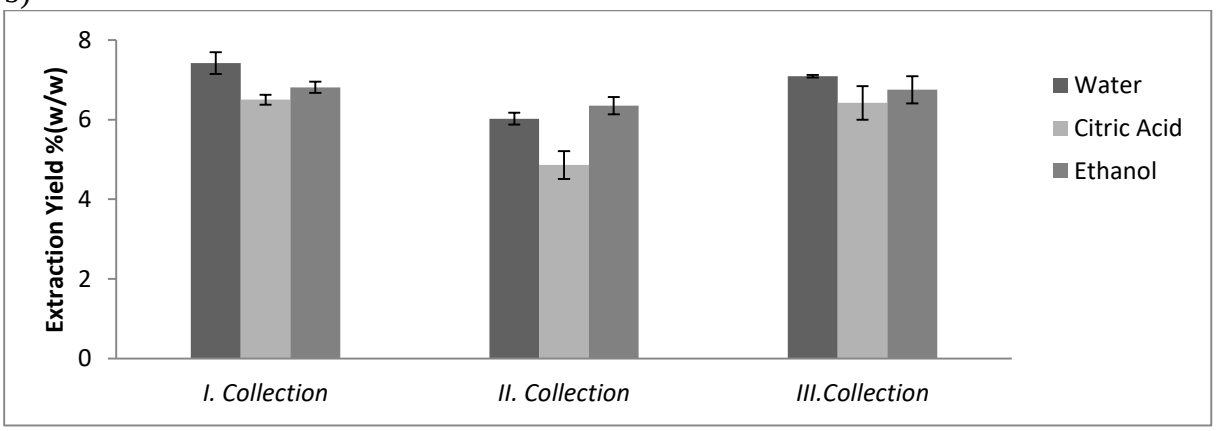


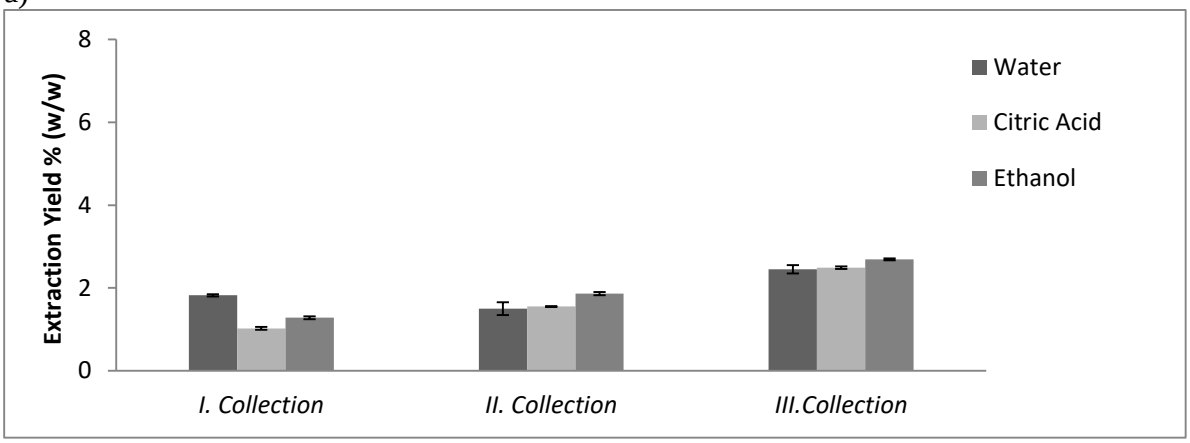
Figure 2. Caffeine yield of **a)** black tea waste (BTW) and **b)** caffeine dust (CD) in three collection periods. Data are expressed as the mean of three replicate.

First of all, caffeine yields in both waste materials were high as much as conventional hot water extraction. Extract yields varied between 4.36-7.42% in all collection periods and solvent systems. The mass of caffeine extracts was highest in CD reaching 7.42% (w/w, 74.2 mg/g tea waste). Caffeine content is usually between 2-4% in black or green tea that means 20-40 mg caffeine can be obttained from 1 gram of tea material [1]. In current literature there are published studies related to extraction of caffeine from green tea and tea waste. Water and supercritical carbon dioxide extraction (SFE) of caffeine from tea plant waste (mainly stalks) was reported by İçen and Gürü [12, 17, 18]. They reported that the maximum yield of caffeine from tea stalk wastes and fiber wastes were 14.95 mg/g and 18.92 mg/g, respectively. According to another study carried out by Shalmashi et al., (2010) supercritical water extraction

of Iranian tea waste produced 0.77% (w/w) of caffeine while conventional hot water extraction yielded only 0.46% (w/w). Our results show that caffeine content of tea waste used in this study is extremely high and should be considered as a good source for caffeine extraction. Using water as extraction solvent seems to be more appropriate providing the same amount of caffeine in a 4 minutes extraction period for all collection periods. Citric acid-water or ethanol-water mixture does not alter the extraction yields except for third collection period of BTW. Using ethanol as co solvent in MAE increased the caffeine yield to 6.46%.

BTW and CD yielded similar amount of catechin extracts (Figure 3). The yields of BTW were between 1.41-2.27% for conventional extraction and 1.82-2.45% for MAE in water. Using citric acid-water or ethanol-water solvents slightly increase the catechin yields. It was the highest in the third collection period.

a)



b)

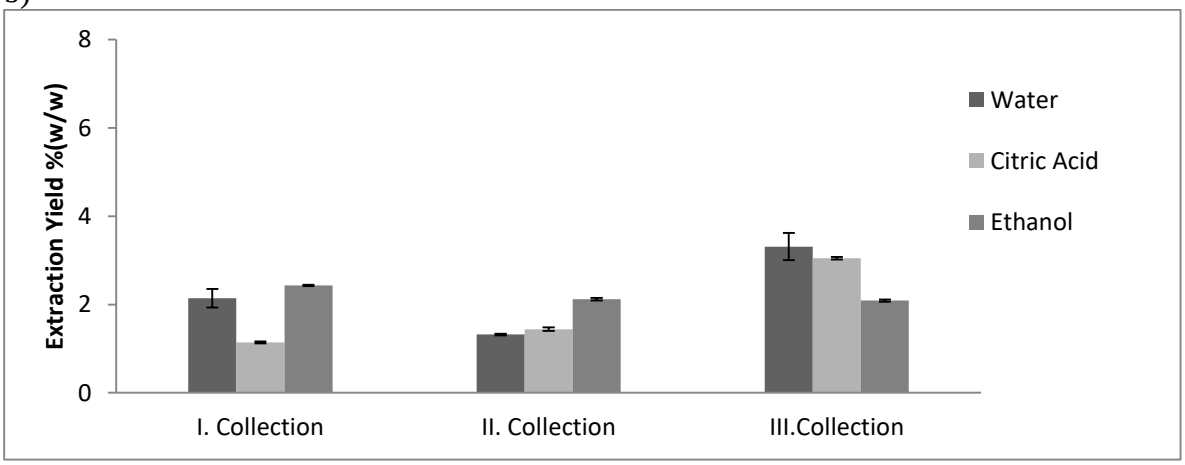


Figure 3. Catechin yield of **a)** black tea waste (BTW) and **b)** caffeine dust (CD) in three collection periods. Data are expressed as the mean of three replicate.

MAE of caffeine dust gave higher yields than conventional hot water extraction especially in third collection period. Ethanol-water solvent increased catechin yields in some collection periods but there was no constant improvement. But it is clear that extraction of catechins employing MAE is more promising since the same amount of extract was obtained after just 4 minutes treatment. Microwave seems to be facilitating the extraction of catechins into extracting solvent via heat zones formed in waste material (Oliveira and Franca, 2002). The temperature is localized in zones that ease the selective migration of target compounds from the material in shorter time.

Extraction yield calculated from extract mass can give a rough idea about how much caffeine and catechin can be isolated from extracting medium. Caffeine extraction with chloroform provides pure caffeine but separation of catechins by liquid-solid extraction is not purely selective and ethyl acetate can remove some other principles as well as catechins. Quantification of individual catechins present in the extract should be done for proper evaluation. Therefore, HPLC analyses were carried out to determine the real amount of each catechin present in the extracts.

3.1.3. HPLC analysis

Caffeine and catechin constituents of the extracts were determined by reversed-phase HPLC. A standard mixture is used for identification and quantification of individual catechin. HPLC chromatogram of standard mixture containing catechins, gallic acid and caffeine is given in Figure 4.

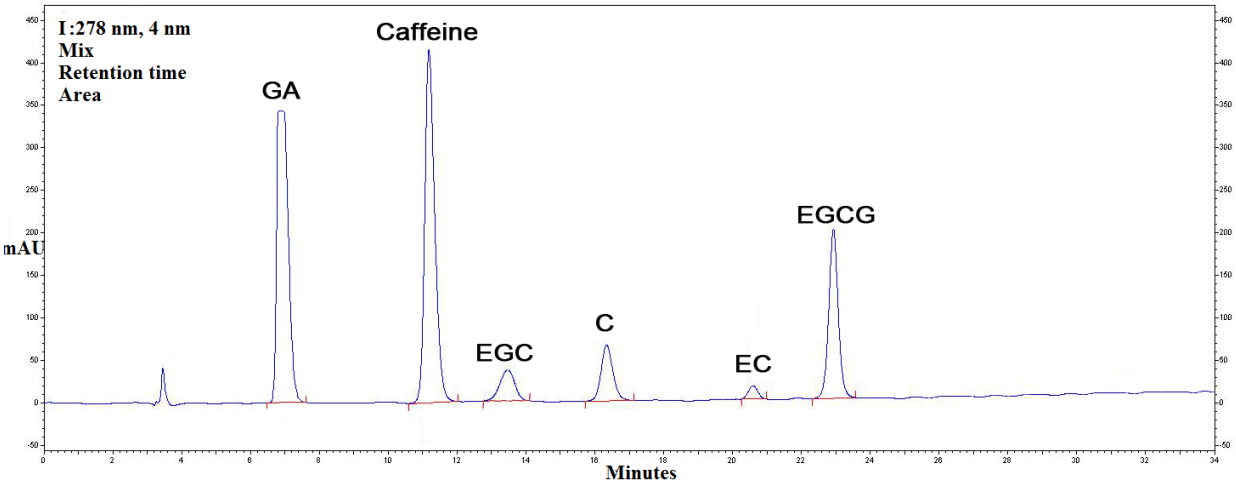
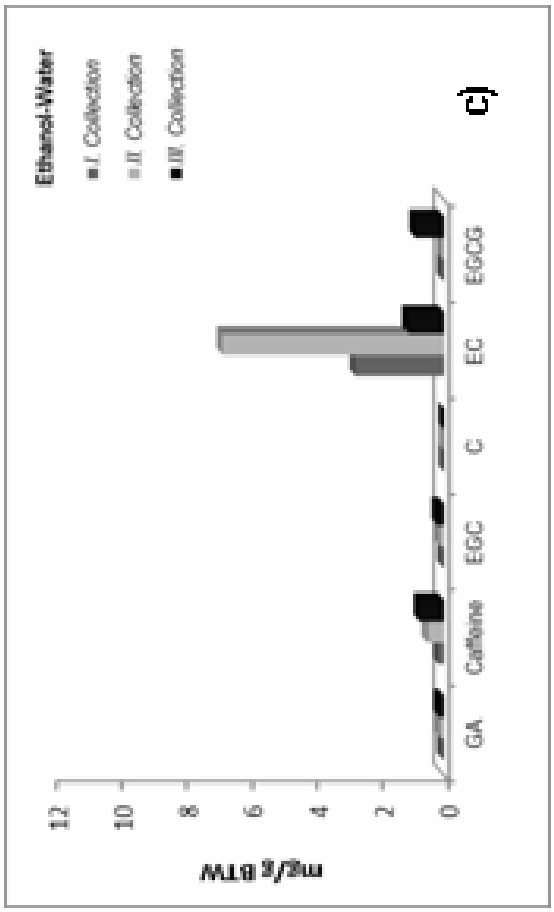
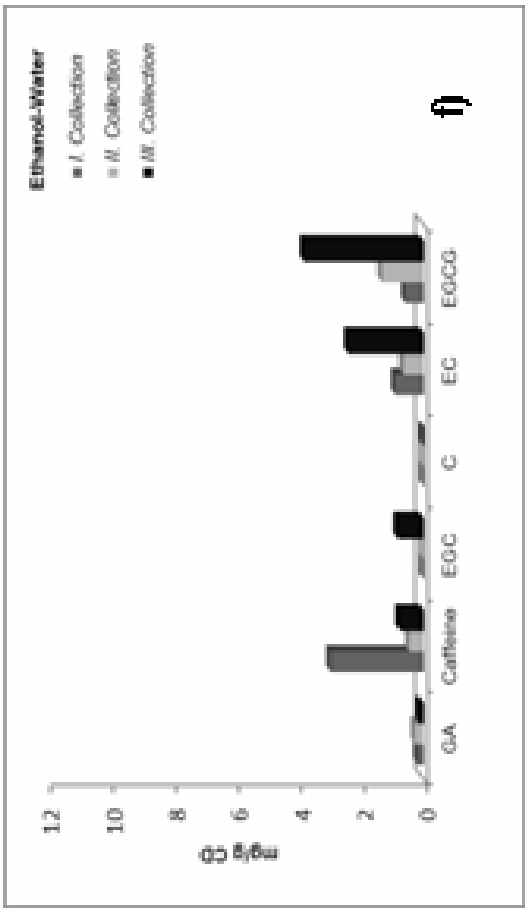


Figure 4. HPLC chromatogram of the catechin standard mixture. Gallic acid and caffeine were included into the standard mixture. Retention times are as following; gallic acid (GA): 7.00; caffeine: 11.03; epigallocatechin (EGC): 13.44; catechin (C): 16.05; epicatechin (EC): 20.44; epigallocatechin gallate (EGCG): 22.79 min. (λ=280 nm, injection volume: 20 µL).

The identification of catechins was carried out by comparing their retention times to standards and the amount of individual catechin was calculated from these chromatograms. The amounts of individual catechins present in the extract might give a good idea to compare extraction efficiency. Therefore quantified catechins from the HPLC chromatograms, along with gallic acid and caffeine residue, in the extracts obtained by MAE are given in Figure 5.


c)
Ethanol-Water
I. Collection
II. Collection
III. Collection
mg/g BTW
12
10
8
6
4
2
0
GA
Caffeine
EGC
C
EC
EGCG


f)
Ethanol-Water
I. Collection
II. Collection
III. Collection
mg/g CD
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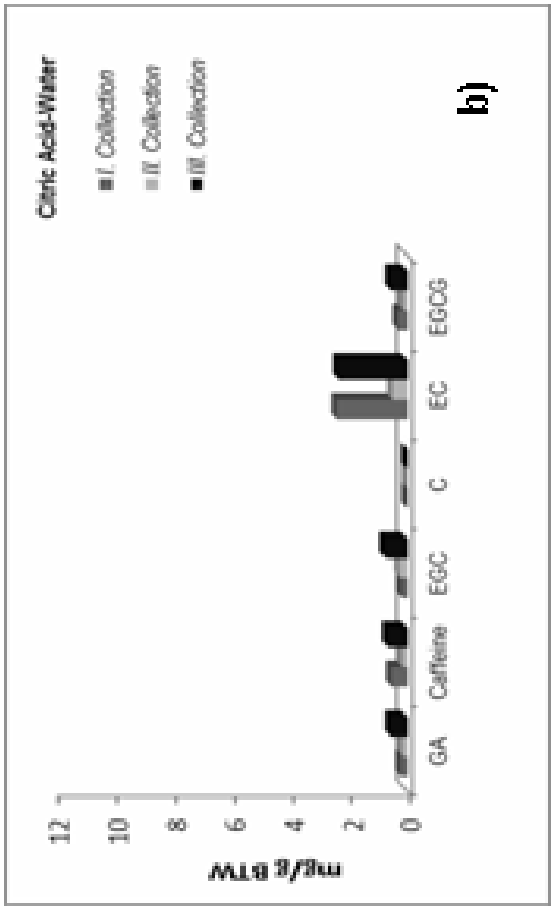
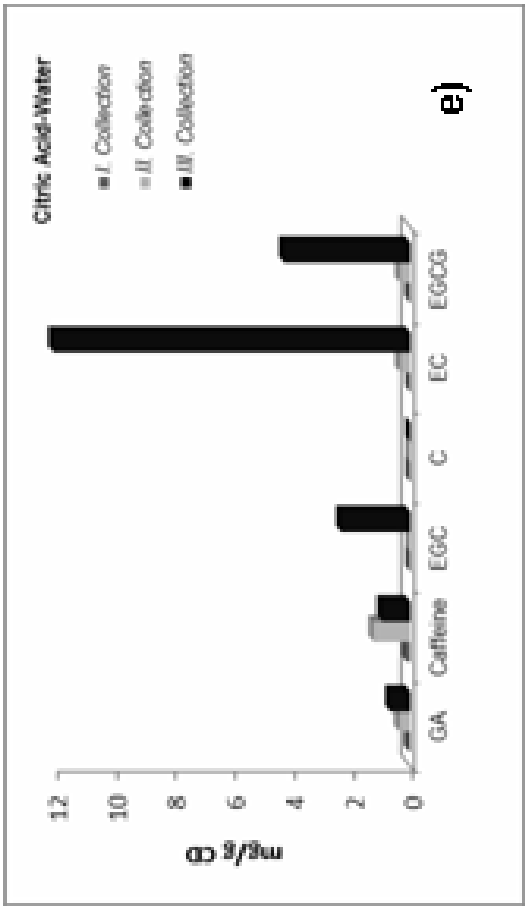
10
8
6
4
2
0
GA
Caffeine
EGC
C
EC
EGCG


b)
Citric Acid-Water
I. Collection
II. Collection
III. Collection
mg/g BTW
12
10
8
6
4
2
0
GA
Caffeine
EGC
C
EC
EGCG


e)
Citric Acid-Water
I. Collection
II. Collection
III. Collection
mg/g CD
12
10
8
6
4
2
0
GA
Caffeine
EGC
C
EC
EGCG

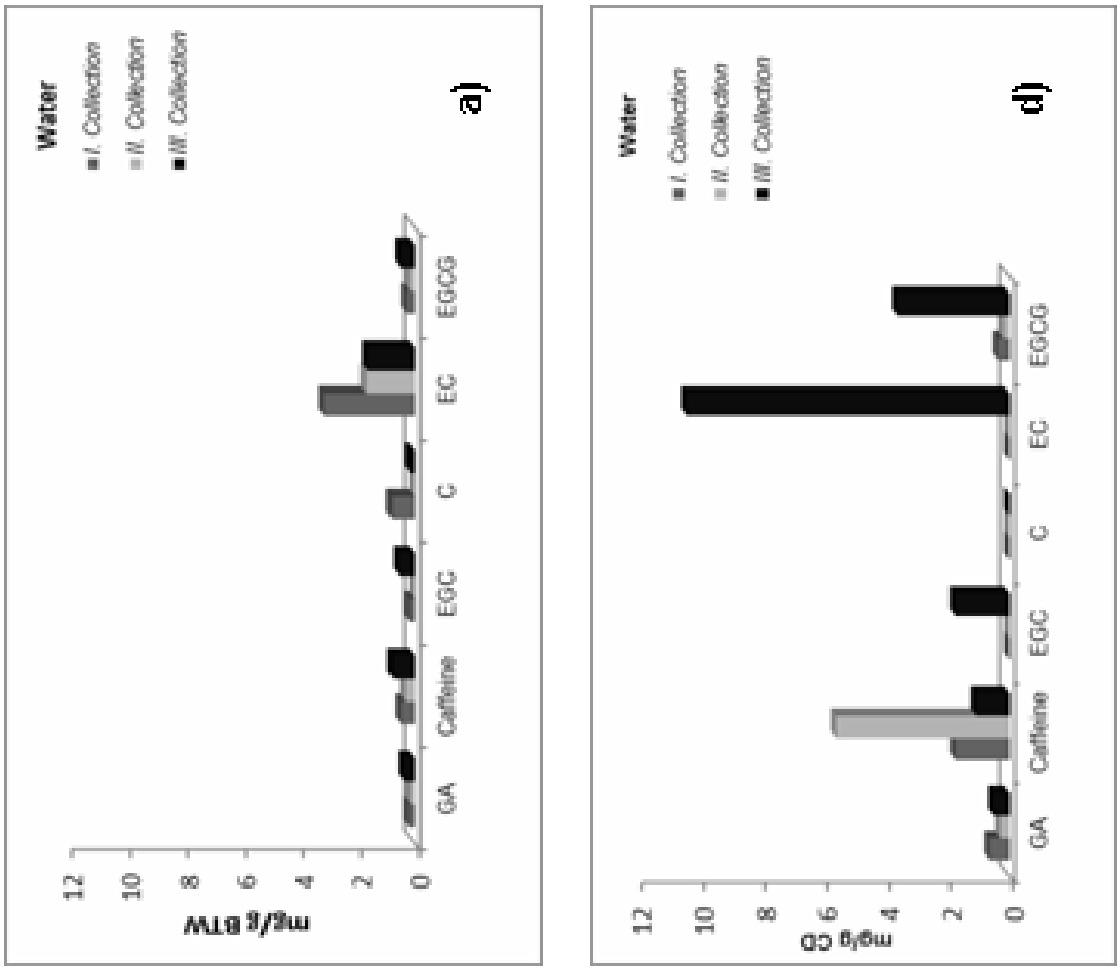
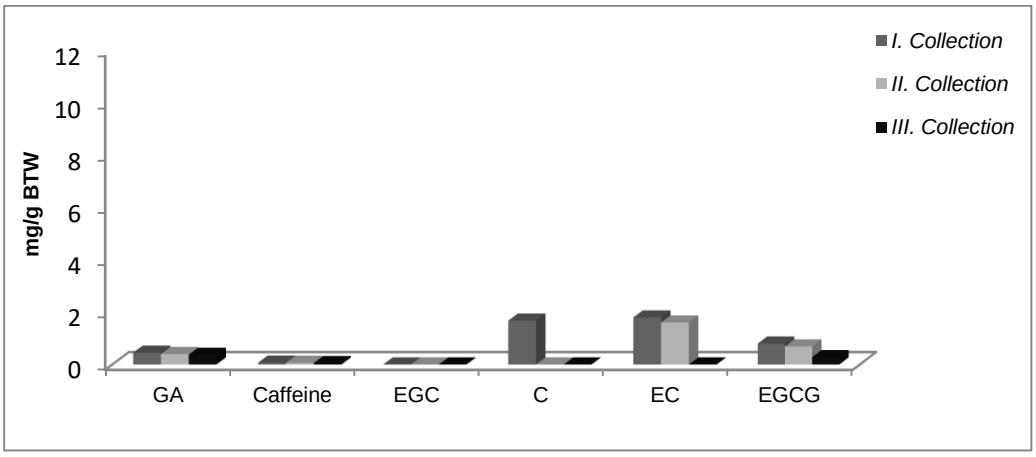


Figure 5. Quantification of catechins present in microwave extraction of BTW (a-c) and CD (d-f).

Quantification studies provided good information about which extraction solvent was more fruitful for effective extraction of individual catechins. As seen from Figure 5a-c BTW contains lower catechins than CD samples (Figure 5d-f). Concentration of EC is higher than other catechins in both waste materials but CD has also higher amount of other catechins such as EGC and EGCG. Extracted catechins were significantly high in the third collection period. The amounts of extracted catechins, especially EC and EGCG, are quite high in MAE in water and citric acid-water mediated systems. Citric acid must be providing an acidic medium for successful separation of EC and EGCG. It should be noted that citric acid-water solvent system extracts less caffeine than water mediated MAE. Therefore, it seems to be more selective to extraction of catechins. The amount of catechins obtained form conventional hot water extraction is given in Figure. 6.

a)



b)

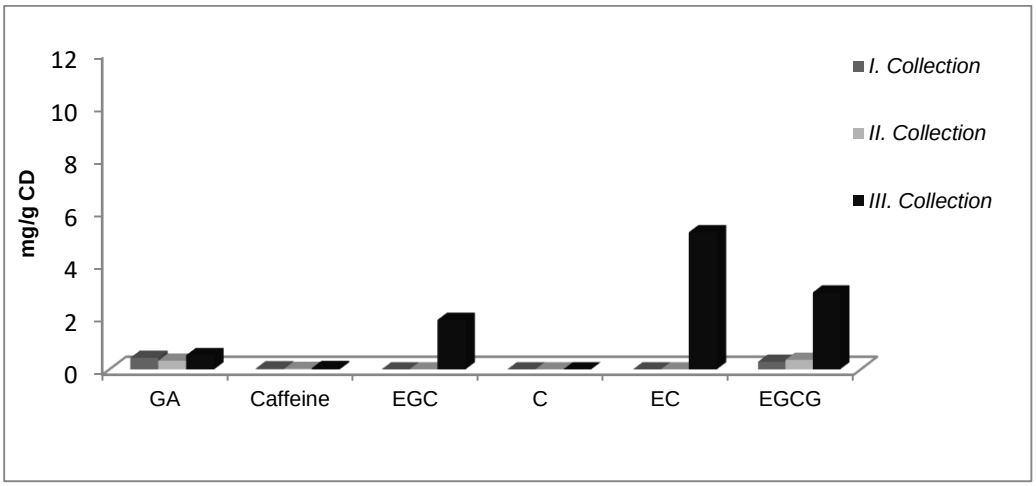


Figure 6. Quantification of catechins present in conventional hot water extract of **a)** BTW and **b)** CD.

Hot water extraction provided lower catechin constituent than MAE. Extract obtained from BTW has lower EC (ranged between 0.01-1.80 mg/g) and EGCG (0.28-0.82 mg/g) in all collection periods (Figure 6a). In CD extracts, EC concentration was extremely low in the first two collection periods. It increased to 5.21 mg/g in the third collection but still nearly half of MAE of the same sample (Figure 6b).

It is clear that extract yields are not solid evidence how extraction is successful for effective separation of these valuable chemicals. This study provides useful information related to effective extraction of tea waste employing microwave power. Separation of catechins was achieved in shorter extraction period (4 minutes), proposed MAE provide high extract yields and it is selective to EC and EGCG. MAE can be used as an effective, economical and time saving technique to obtain caffeine and catechins from tea wastes such as BTW and CD. Using citric acid-water for extraction in MAE enhanced the EC and EGCG constituent and might be used for selective extraction of these compounds.

The waste materials used in this study contain quite high amount of caffeine and remarkable amount of catechins. As a result, tea waste produced in Black Sea region can be considered as valuable bio-resource and MAE at large scale might be favored for energy cost industrial applications.

Acknowledgements

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Conflict of interest

This study does not have any conflict of interest.

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Research Article

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Bacterial Biodiversity of Industrial Soils from Aydın and Trabzon Province

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Abstract: The aim of this study was to determine bacterial biodiversity of industrial soils from Aydın and Trabzon Province using morphological, cultural, biochemical and molecular methods. Factory wastes, detergents, pesticides and gasoline are accepted as pollutants to the environment. In this study a total of 65 samples which can tolerate xylene, acetone, chloroform and methanol were acquired. According to the Bergey's Manual of Systematic Bacteriology morphological, cultural and biochemical tests, 48 of these samples were found to be *Bacillus sp*. and 17 of them were found to be *Pseudomonas sp*. For molecular identification 16S rDNA-PCR method was used. Seven different genera and a total of sixteen species were found as a result of the study.

Key Words: Bacteria, 16S rRNA, biodiversity soil, Aydın, Trabzon, Turkey

1. Introduction

Biodiversity is the foundation of ecosystem services to which human well-being is intimately linked [1]. It is one of the basic components of nature and it ensures the survival of earth by all means. Biodiversity depends on the climatic conditions and areal components of the region. The presence of the pollutants diminishes the bacterial biodiversity as well as overall biodiversity.

Soil biodiversity influences a huge range of ecosystem processes that contribute to the sustainability of life on earth [2]. Soil biodiversity maintains critical and key processes such as carbon storage, nutrient cycling, plant species diversity, soil fertility, soil erosion, nutrient uptake by plants, formation of soil organic matter, nitrogen fixation, biodegradation of organic materials, reducing hazardous waste, production of organic acids that weather rocks, and control of plant and insect populations through natural biocontrol [3, 4, 5]. Biodiversity and soil are strongly linked, because soil is the medium for a large variety of organisms, and interacts closely with the wider biosphere. Conversely, biological activity is a primary factor in the physical and chemical formation of soils [6]. The main role of soil microorganisms is to recycle organic matter which stemmed from in and above-ground organisms, even after their

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passing away. Both natural and agricultural vegetation boundaries correspond closely to soil boundaries, even at continental and global scales [7].

In our day soil pollution can be traced back to xenobiotic activities. The rapid development of the industry caused rapid disposal of the wastes and this caused serious problems for the environment. Factory wastes, detergents, pesticides and gasoline are the main pollutants of the environment. Various technologies have been developed for remediation of contaminated soil/ sediments [8]. One of these clean up options is the usage of biological elements such as bacteria. This process is called bioremediation [9]. Some microorganisms can digest certain organic chemicals. Bacteria that live in these kinds of soils can also be used to clean these pollutants from soil. The aim of this study is to determine bacterial biodiversity of industrial soils from Aydın and Trabzon Province of Turkey. Bacterial species which found in this study can be used as biological cleaners in polluted soils.

2. Material and Methods

2.1. Sample Collection

Samples were collected aseptically from industrial sites, sewer sites, city dump and factory dump sites. Locations are Değirmendere Industrial Site/TRABZON, Değirmendere Sewer/TRABZON, Değirmendere Site/TRABZON, Menderes River Site/AYDIN, Nazilli City Dump/AYDIN.Samples were taken in March, 2014. Samples were collected in the 50 ml sterile falcon tubes and kept in a portable refrigerator and brought to the laboratory. Samples were added in the enrichment media that were prepared in advance.

2.2. Bacterial Isolation

Enrichment of the bacteria was made by adding 1% of chloroform, methanol, xylene and acetone to the soil samples and leaving them to incubation at 30 ºC for five days. This process repeated three times. A series of dilutions were made from enriched samples up to 10^{-6}. Bacterial growth was realized on Plate Count Agar (PCA) and Pseudomonas Selective Agar at 30 ºC for 48 h. Samples which gives between 30-300 colonies were chosen. Each different colony was isolated and stocked in skim milk.

2.3. Classical Identification

Morphological, cultural and biochemical identifications were made according to the Bergey's Manual of Systematic Bacteriology [10]. For identification Gram staining, lactose, sucrose, mannitol, citrate, nitrate reduction, starch hydrolase and gelatin hydrolase tests were made.

2.4. Molecular Identification

DNA isolation of the samples was made according to De Boer and Ward (1995) [11]. After isolations DNA concentration and purity was measured with nanodrop spectrometer (Thermo Scientific). Their purity values were between the values of 1.73 and 2.20. For PCR 16S universal rDNA primers were used (27F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'-CGG TTA CCT TGT TAC GAC TT-3') [12]. 16S rRNA PCR reactions were carried out at initial denaturation 95 ºC 5 min, denaturation 94 ºC 40 sec, annealing 50 ºC 40 sec, extension 72 ºC 40 sec with 35 cycles and final extension at 72ºC 10dk. Reagents concentrations were 10X Taq Buffer, 0.5M dNTP mix, 10 pM from each primer, 7.5 mM $MgCl_2$ and 1U Taq polymerase with the final volume of 25 µl. PCR products were sent to the sequencing (GATC BioTech, Germany) after electrophoresis at 1.4% agarose jel at 90 V 40 min. Evolutionary analysis and tree construction were made with MEGA 7.0 software The evolutionary history was inferred using the Maximum Parsimony method [13-15].

3. Results and Discussion

3.1. Classical Identification

In the present study, a total of 65 samples that can tolerate xylene, acetone, chloroform and methanol were obtained. Consistent with morphological, cultural and biochemical tests, 48 of these samples were found to be *Bacillus sp*. and the rest were found to be *Pseudomonas sp*. *Pseudomonas sp*. is a Gr(-) rod shaped bacteria (Fig.1) with glycerol, lactose, sucrose and mannitol fermentation abilities, produces H_2S, hydrolyses gelatin and is citrate positive (Table 1, Fig.2) while *Bacillus sp*. were Gr (+) rod shaped (Fig.1), endospore forming bacteria with catalase, glucose, sucrose, mannitol, gelatin hydrolisation and citrate positive (Table 2, Fig.2).

Table 1. Classical identification of *Pseudomonas* species from Aydın and Trabzon Province industrial soils.

Source	Gram Staining	Cell Shape	G	L	S	M	NR	SH	H_2S	C	GH	Bacteria
T-3Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Chloroform	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Methanol	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T3Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-3Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
A-2Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Methanol	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Chloroform	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Methanol	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Chloroform	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-3Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.

G: Glycerol, L: Lactose, S: Sucrose, M: Mannitol C: Citrate, NR: Nitrate Reduction, SH: Starch Hydrolyse, GH: GelatinHydrolyse
T1:Değirmendere Industrial Site/TRABZON, T2:Değirmendere Sewer/TRABZON, T3:Değirmendere Site/TRABZONA1:Menderes River Site/AYDIN, A2:Nazilli City Dump/AYDIN,

Table 2. Classical identification of *Bacillus* species collected from Aydın and Trabzon Province.

Source	Gram Staining	Cell Shape	G	L	S	M	NR	SH	H_2S	C	GH	Ct	Bacteria
A-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.

G: Glycerol, L: Lactose, S: Sucrose, M: Mannitol, C: Citrate, NR: Nitrate Reduction, SH: Starch Hydrolyse, GH: GelatineHydrolyse, Ct: Cathalase

T1:Değirmendere Industrial Site/TRABZON, T2:Değirmendere Sewer/TRABZON, T3:Değirmendere Site/TRABZONA1:Menderes River Site/AYDIN, A2:Nazilli City Dump/AYDIN,

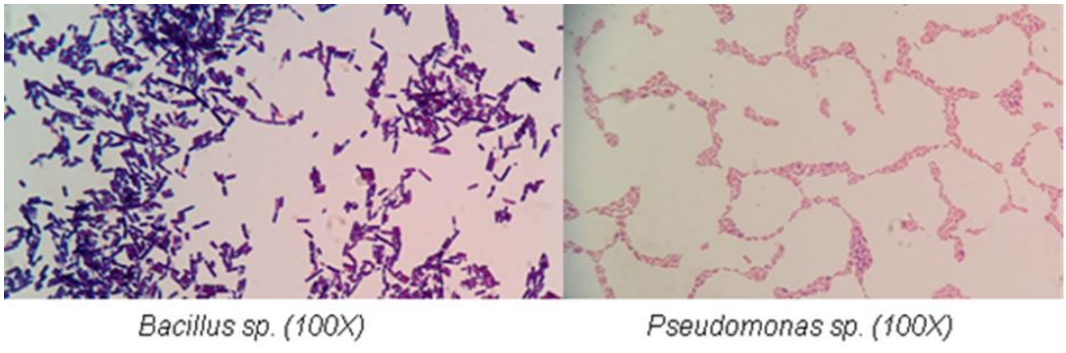

Fig. 1. Gram staining of *Bacillus* and *Pseudomonas* species

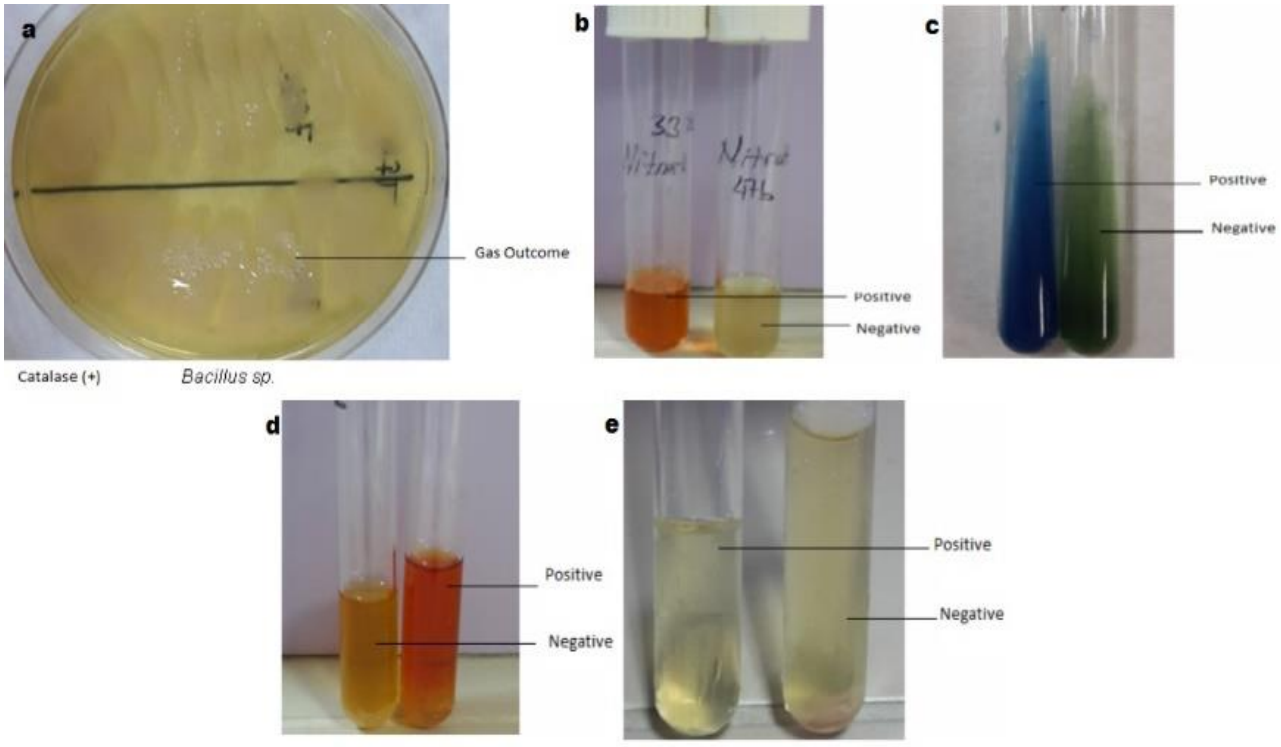

Fig. 2. Biochemical test results of the samples. (a: Catalase test, b: Nitrate reduction test, c: Citrate test, d: Voges-Proskauer test, e: Gelatin hydrolase test)

3.2. Molecular Identification

16S rDNA primers were used for molecular identification (Fig.3). After amplification and agarose gel electrophoresis PCR results of these samples were send to the sequencing (GATC BioTech, Germany).Molecular identification was made by comparing sequence results with GenBank using BLASTn software. Our analysis of the industrial soils of Trabzon and Aydın Province were showed that there were not only *Bacillus* and *Pseudomonas* species but seven different genera and sixteen species were present (Table 3).

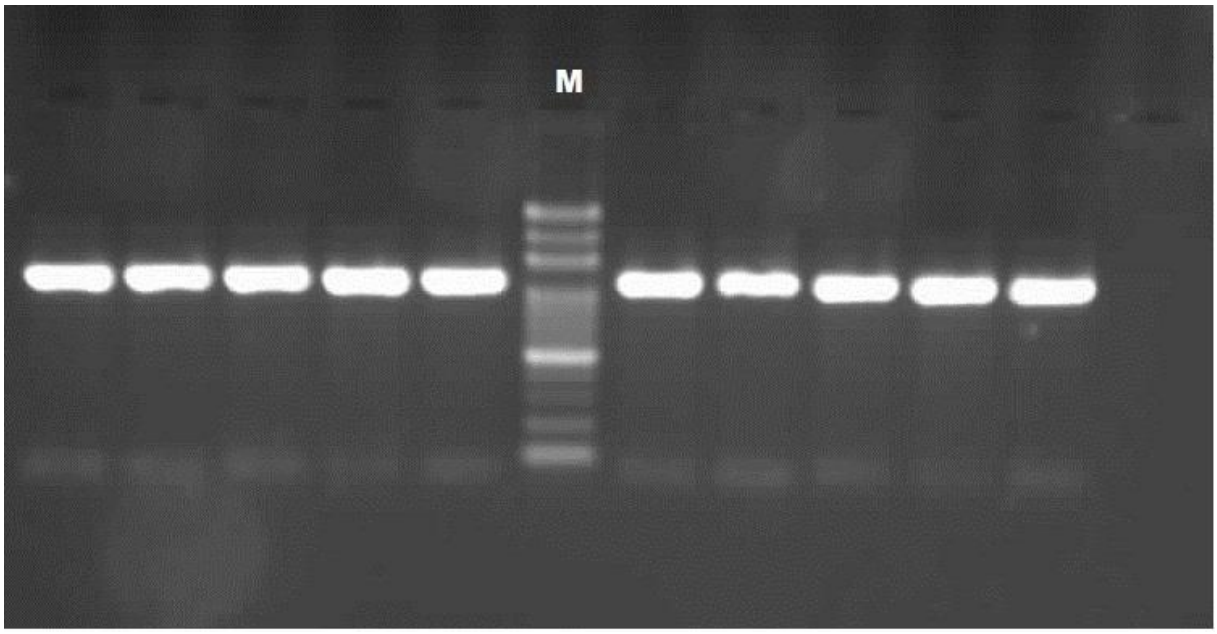

Fig. 3. 16SrDNA-PCR products. (M: 100 bp DNA ladder)

Table 3. Molecular identification of the species from Aydın and Trabzon Province industrial soils.

Name of The Species	Province	Number of Strains	Accession No
Bacillus stratosphericus	Trabzon	1	KC866366.1
Pseudomonas sp.	Trabzon	1	KT005263.1
Bacillus cereus	Trabzon	6	KF836529.1 JF736841.1 KF494191.1 KP729612.1 JX629271.1 JQ824137.1
Bacillus megaterium	Trabzon	1	KT222849.1
Arthrobacter oxydans	Trabzon	2	LN774368.1 KC934768.1
Bacillus anthracis	Trabzon	2	KP813675.1 KP813664.1
Bacillus thuringiensis	Trabzon	2	KT965080.1 KT986127.1
Corynebacterium tuberculostearicum	Trabzon	1	KT805279.1
Pseudomonas brassicacearum	Trabzon	1	KP851953.1
Bacillus pumilus	Trabzon	1	KP851957.1
Staphylococcus epidermidis	Trabzon	1	KX349995.1
Pseudomonas putida	Trabzon	1	KX349990.1
Pseudomonas plecoglossicida	Trabzon	1	KX082839.1
Bacillus megaterium	Aydın	4	KJ526882.1
Bacillus cereus	Aydın	3	KT897915.1 GQ855296.1 FJ763650.1
Bacillus subtilis	Aydın	1	KP184704.1
Bacillus subterraneus	Aydın	1	KT719810.1
Aerococcus viridans	Aydın	1	GQ161096.1
Microbacterium aerolatum	Aydın	1	LN774527.1
Staphylococcus hominis	Aydın	1	HM163532.1
Arthrobacter oxydans	Aydın	1	EU086783.1

MEGA 7 software was used for evolutionary analysis. Maximum parsimony method was used to infer evolutionary history. Maximum parsimony tree was shown in Figure 4. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).All positions with less than 95% site coverage were eliminated.

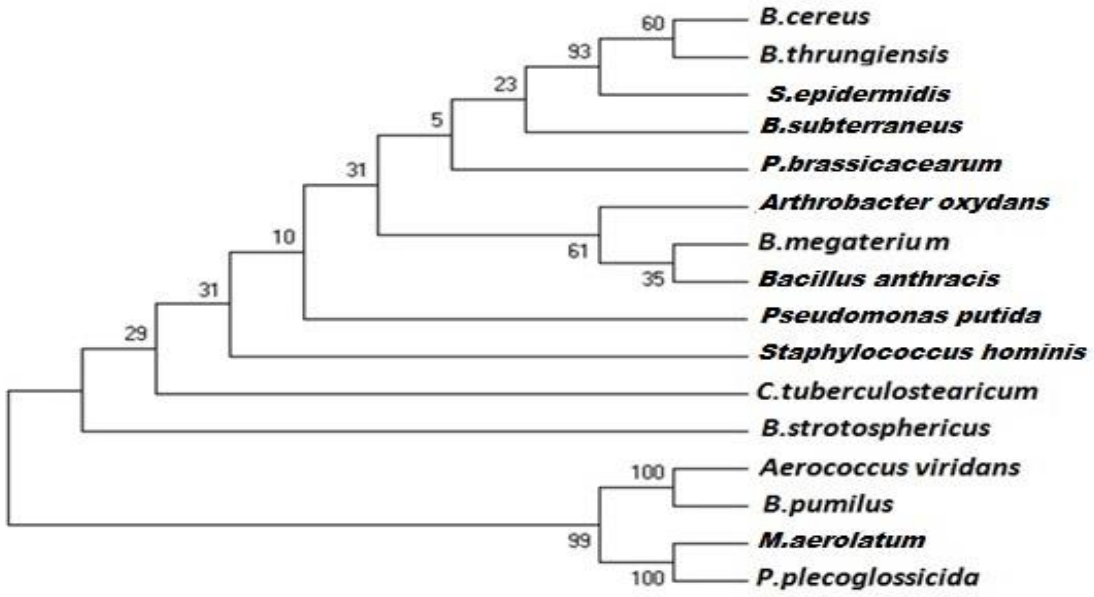

Fig. 4. Maximum Parsimony analysis of taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

It is a fact that the microbial diversity of the soil is immense but pollutants have an inhibitory effect on this diversity. In contrast to normal soil samples, Bornemann and Triplett (1997) found 98 bacterial and 2 archaea species in Eastern Amazonia, while Jesus et al. (2009) found 654 bacterial species in non-industrial soils [16, 17]. This can clearly be seen in our study. Bacterial diversity was reduced to seven genera and sixteen species because of the pollutants.

According to morphological, cultural and biochemical test only two genera can be identified but molecular identification showed that there is seven genera in fact. This dictates the importance of molecular methods in identification of organisms once more. Classical identification methods allowed us to identify on the genus level while molecular methods let us to identify on the species level. Once more this shows us the insufficiency of the classical identification methods and the necessity of molecular methods. The failure of the detection of *Arthrobacter oxydans* with classical methods is normal since this bacterium can show different properties, e.g. gram staining, cell shape, between young and old colonies [18].

Soil hosts a large variety of *Bacillus* species. Even in industrial soils *Bacillus* species are abundant as nine of the sixteen species belongs to the genus *Bacillus*. This can be inferred as the members of the genus *Bacillus* have high tolerance to the environmental conditions. This is due to the spore formation ability of the genus.

4. Conclusion

In this study we aimed to determine the bacterial biodiversity of industrial soils from the two Province of Turkey. We used morphological, biochemical and molecular methods for that purpose. Morphological and biochemical methods gave us two genera, *Bacillus* sp, and *Pseudomonas* sp. while molecular method led us to seven genera and sixteen species. Most abundant genus in the soils are *Bacillus* species as they can form spores.

It is not a surprise that molecular methods can give more accurate results than morphological and biochemical methods as they use DNA sequence knowledge. Since these bacterial species can live in polluted soils they can also be used as biological cleaners. More studies are required for that purpose.

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Research Note

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Effects of Nickel Contamination on Nutrient Contents of Daffodil (*Narcissus poeticus* L. c.v. *"Ice Folies"*)

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Abstract: The objective of this study was to determine the effects of nickel on nutrient contents of daffodil (*Narcissus poeticus* L. c.v. "*Ice Folies*") in nickel contaminated media. This research was carried out in a completely randomized experimental design with three replications in greenhouse conditions. Four different doses of nickel (control, 25 mg kg^{-1}, 50 mg kg^{-1}, 75 mg kg^{-1}) were applied to each pot having 500 g soil:sand mixture in 2:1 ratio. The distillate water was used in irrigation and ½ hoagland solution was applied for fertilization. At the end of experiment the highest K, Mg and Ca contents of daffodil bulbs were obtained as 0.90 %, 0.91 % and 2.72 % in control respectively. The highest Fe (27.42 mg kg^{-1}), Cu (7.62 mg kg^{-1}), and Zn (20.99 mg kg^{-1}) were in 50 mg kg-1, 75 mg kg^{-1} and 25 mg kg^{-1} nickel applications respectively. Similarly the highest K, Mg and Ca contents of daffodil leaves were obtained as 2.2 %, 1.72 % and 5.87 % in control. The highest Fe contents (66.62 mg kg^{-1}) was in 25 mg kg^{-1} nickel application, while Cu (41.29 mg kg^{-1}) and Zn contents (41.04 mg kg^{-1}) were in 75 mg kg^{-1} nickel application. Nickel applications increased micronutrients contents of daffodils except manganese.

Keywords: daffodil, nickel, nutrient content

1. INTRODUCTION

Environmental pollution through heavy metals is growing concern. The contamination of heavy metals as a by product of various human activities has been accompanied by large scale soil pollution. Heavy metals pollution in soils cause many environmental and human health problems. It reported that heavy metals are metalic elements with atomic number higher than 20 and widely known inhibitors of plant metabolism [1]. Heavy metals can be divided into two groups. First group cantains metals such as Fe, Cu, Zn, Co, Mo are necessary for plant metabolism as enzyme activitors, regulators, photosynthesis. The metals within second group such as Cd, Hg, Pb, Cr are not required for plant metabolism. Heavy metals are toxic to plants

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if their accumulation levels exceed the accetable levels of the plant tissues [2]. Nickel has been recognized as essential micronutrient in plant cells [3].

Nickel is also one of the more important metal pollutants contaminating the environment [4]. One of the most important factors in heavy metals influence on plant metabolism are their relationships with other mineral nutrients [5].

Narcissus spp. used as plant material in this study is an ornamental plant belongs on *Amaryllidaceae* family. It is known that some ornamental plants are hyperaccumulator plants and they are used for phytoremediation [6].

In this study, the effects of nickel applications on nutrient uptake of *Narcissus poeticus* in nickel contaminated media were investigated.

2. MATERIAL and METHODS

This research was conducted in a completely randomized experimental design with three replications in green house conditions. Four doses of nickel as control, 25 mg kg^{-1}, 50 mg kg^{-1}, 75 mg kg^{-1} were applied to each pot having 500 g soil:sand mixture in 2:1 ratio. Total number 60 plastic pots were used in the study. The distillate water was used in irrigation and ½ hoagland solution [7] was applied for fertilization. *Narcissus poeticus* belonging to the *Amaryllidaceae* family, is used as a plant variety. Daffodil bulbs were planted to each pot. The irrigation was made by distillate water and hoagland solution was applied for fertilization. The experiment was ended after four months. Harvested plant samples were washed, dried and crushed for macro and micro nutrient elements analysis. After wet digestion of plant samples, calcium, magnesium, potassium, iron, zinc, copper and manganese contents were analysed by using mixed of HNO_3+$HClO_4$ in 1:2 ratio reporetd by [8]. The macro and micro nutrients were determined in their extract solutions by using atomic absorption spectrophotometer. Statistical analyses was done using SPSS package programe to show difference among the mean values of nutrient contents from the different applications.

3. RESULTS and DISCUSSIONS

The effects of applications of nickel on nutrient contents of daffodil bulbs and leaves were found significant (Table 1, 2).

Table 1. Effects of Ni application on macro element contents in bulb and leaf parts of daffodil

Plant part	Ni doses mg kg^{-1}	K %	Mg %	Ca %
Bulb	0 (control)	0.89 b	0.09 c	0.27 d
	25	0.81 b	0.07 d	0.22 e
	50	0.75 b	0.08 d	0.22 e
	75	0.89 b	0.07 d	0.23 e
Leaf	0 (control)	2.20 a	0.17 a	0.59 a
	25	2.15 a	0.17 a	0.57 a
	50	2.19 a	0.14 b	0.48 c
	75	2.06 a	0.15 b	0.53 b
Sign. level		at 1%	at 1%	at 1%

Increasing nickel doses generally decreased macronutrient contents of daffodil bulbs and leaves. The highest Mg (0.09 %) and Ca (0.27 %) contents of bulbs were obtained in control while the highest. K (0.89 %) contents were in control and 75 mg kg^{-1} Ni applications.

Table 2. Effects of Ni application on micro element contents in bulb and leaf parts of daffodil

Plant part	Ni doses mg kg^{-1}	Fe mg kg^{-1}	Cu mg kg^{-1}	Zn mg kg^{-1}	Mn mg kg^{-1}
Bulb	0 (control)	23.0	6.3 c	18.9	10.3 d
	25	14.7	5.4 c	21.0	8.7 d
	50	27.4	5.5 c	19.2	9.1 d
	75	21.1	7.6 c	19.2	10.0 d
Leaf	0 (control)	56.5	15.1 b	32.6	48.8 a
	25	66.6	11.0 bc	35.9	37.5 b
	50	59.4	12.1 bc	28.7	30.1 c
	75	62.9	41.3 a	41.0	39.7 b
Sign. level		at 5%	at 1%	ns	at 1%

Similarly, the highest Mg and Ca contents of daffodil leaves were determined as 0.17 % and 0.59 % in both of control and 25 mg kg^{-1} Ni applications while the highest K content was in control as 2.20 %. The highest Fe (27.4 mg kg^{-1}), Cu (7.6 mg kg^{-1}), Zn (21.0 mg kg^{-1}) and Mn (10.3 mg kg^{-1}) contents of daffodil bulbs were obtained 50 mg kg^{-1} Ni, 75 mg kg^{-1} Ni, 25 mg kg^{-1} Ni and control applications respectively. Generally Ni applications increased micronutrient contents of daffodil bulbs except manganese contents.

The macronutrient contents of daffodil leaves generally decreased by increasing nickel doses. The highest means of K, Mg and Ca contents of leaves were obtained in control as 2.20 %, 0.17 % and 0.59 % respectively.

Zinc and copper contents of daffodil leaves increased by 75 mg kg^{-1} Ni application. Iron contents of daffodil also increased by nickel applications. Contrary, Mn contents of daffodil leaves decreased by nickel applications.

The highest Fe (66.6 mg kg^{-1}), Cu (41.30 mg kg^{-1}), Zn (41.0 mg kg^{-1}) and Mn (48.8 mg kg^{-1}) were in 25 mg kg^{-1} Ni application, 75 mg kg^{-1} Ni application, 75 mg kg^{-1} Ni application and control respectively.

The interactions among nickel doses and plant organs had significant effects on nutrient contents of daffodil except Zn contents. The nutrient contents of daffodil leaves were found higher than those in the bulbs.

In this study increasing nickel doses generally decreased macronutrients contents while micronutrient contents increased by nickel applications. It was reported that nickel toxicity was increased in the presence of Co, Cr, Zn, Mn, Mo [9].

It was reported that nickel toxicity decreased contents of divalent cations (Ca^{++} and Mg^{++}) in rice plant [10]. The results obtained in this study belong Zn and macronutrients contents showed correspond with presented literature knowledges. Many non spesific as well as spesific interactions between mineral nutrients of plants were reported by [11].

The nutrient levels determined in this study in nickel contaminated media can be related reported interactions, e.g. competition between nutrients at the celular level or replacement of one nutrient by another.

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2,4-Dimetilpirol'ün Başak Yanıklığı Hastalığı Etmeni Fusarium culmorum'un Üzerine Etkilerinin İncelenmesi

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Özet: Bitki patojeni *Fusarium culmorum* dünyada ve ülkemizde tahıllar üzerinde kök çürüklüğü ve başak yanıklığı başta olmak üzere çeşitli hastalıklara neden olmaktadır. Bu çalışmada *F. culmorum* ile mücadele kullanılabilecek yeni ve potansiyel bir ajan olarak 2,4-Dimetilpirol'ün patojen üzerindeki etkileri incelenmiştir. Bu amaçla farklı konsantrasyonlarda 2,4-Dimetilpirol (0, 0.5, 1, 2 ve 4 mg mL^{-1}) uygulanmış *F. culmorum* 20F izolatında artan konsantrasyonlarda doğrusal büyüme oranındaki (DBO) değişim incelenmiş ve kontrol ve deney grupları arasında anlamlı bir düşüş olduğu görülmüştür. Ayrıca seksüel üreme ve hücre çeperi bütünlüğünden sorumlu *Mgv1* geninin anlatımı gerçek zamanlı kantitatif polimeraz zincir reaksiyonu (k-PZR) ve ters transkripsiyon PZR (RT-PZR) ile incelenmiştir. Deney gruplarında kontrol gruplarına göre *Mgv1* geni için $5.21\pm0.05x10^2$ kat artma saptanmıştır. Elde edilen bulgulara göre 2,4-Dimetilpirol'ün, *F. culmorum* türünde potansiyel bir antifungal etkili ajan olabileceği gösterilmiştir. İleriki çalışmalarda bu ajanın tarladaki fungal biyokütlenin ve toksin üretiminin azaltılmasını sağlayarak hastalık ile mücadelede yeni bir yaklaşım olarak kullanılabileceği düşünülmektedir.

Anahtar Kelimeler: Antifungal; Başak Yanıklığı; *Fusarium*; 2,4-Dimetilpirol

Investigation of the Effects of 2,4-Dimethylpyrrole on *Fusarium culmorum* Causal Agent of Head Blight Disease

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Abstract: *Fusarium culmorum*, a phytopathogen, cause several diseases including root rot and head blight on cereals in worldwide and our country. In this study, effects of 2,4-Dimethylpyrrole, as a novel and potential agent on pathogen which can be used in fight with F. culmorum were investigated. For this purpose, alteration in linear growth rate (LGR) in *F. culmorum* 20F isolate, subjected to different concentrations of 2,4-Dimethylpyrrole (0, 0.5, 1, 2, 4 mg mL^{-1}) was investigated and the significant decrase between control and experiment sets was determined. Besides, expression of *Mgv1* gene, responsible for sexual stage and cell wall integrity, was investigated via quantitative real time polymerase chain reaction (q-PCR) and reverse transcription PCR (RT-PCR). In comparison to control groups, $5.21\pm0.05x10^2$ fold increases in experiment set for *Mgv1* gene was detected. According to findings obtained, it was shown that 2,4-Dimethylpyrrole could be a potential antifungal agent for *F. culmorum*. In further works, it is suggested that this agent could be used as a novel approach in disease control including inhibition of fungal biomass and toxin production in field.

Keywords: Antifungal; Antifungal; Head Blight; Fusarium; 2,4-Dimethylpyrrole

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1. GİRİŞ

Tahıllar tüm dünyada ekonomik açıdan büyük bir öneme sahip olup pek çok ülke için önemli bir geçim kaynağıdır. Food and Agriculture Organization (FAO) 2013 verilerine göre, dünya genelinde 2.780.666.068 ton $yıl^{-1}$ tahıl üretimine ulaşılmıştır [http://faostat.fao.org]. 2013 yılında ülkemizde ise 37.475.264 ton $yıl^{-1}$ tahıl üretimi gerçekleşmiştir. Biyotik ve/veya abiyotik stresler sonucunda tahıllarda meydana gelecek ekonomik kayıplar, ülke ekonomilerini doğrudan ilgilendirmektedir. Tahıllarda meydana gelen en önemli biyotik stres faktörü olan fungal hastalıklar ile mücadele bu kapsamda büyük önem taşımaktadır [1].

F. culmorum tahıllar başta olmak üzere çok çeşitli bitkileri enfekte edebilmektedir. Bu etmen tahıllarda başak yanıklığı ve kök çürüklüğüne sebep olmaktadır. Her iki hastalık için de *F. culmorum* tahıl üretilen bölgelerde *F. graminearum* ile birlikte iki önemli patojenden birisidir [2]. Bu hastalıklardan başak yanıklığı, ekonomik değere sahip tahıllarda global anlamda ciddi bir hastalık konumundadır. *F. culmorum* bu hastalığı sıklıkla ılıman bölgelerde oluşturmakla birlikte, serin ve nem oranı yüksek bölgelerde de son yıllarda hastalığa neden olduğu bildirilmiştir [3]. Başaklar beyazlaşır, tohumlarda kuruyup büzüşme ile gelişim geriliği olur. Ürün kayıpları Kanada, Amerika ve Çin'de milyon dolarlara kadar ulaşabilmektedir [4, 5]. *F. culmorum* tarafından oluşturulan diğer bir global hastalık kök çürüklüğüdür. Başak yanıklığının aksine hastalığın belirtileri enfeksiyon zamanına göre değişkenlik gösterir. Erken gelişim döneminde bitkilerde enfeksiyon meydana gelmişse koleoptiller, sap ve kökler renksizleşir, buna karşın, ileriki dönemlerde meydana gelen enfeksiyonlarda, ana sapta kahverengi lezyonlar meydana gelir ve yeni sürgünler gelişemez. Başak yanıklığında olduğu gibi hastalık nemli bölgelerde de görülmektedir. [6]. Hastalığın özellikle su stresinin olduğu kurak alanlarda Türkiye'de orta Anadolu'da en önemli kök boğazı çürüklük etmeni Fusarium türü olduğu bazı çalışmalarda ortaya konulmuştur [7, 8].

F. culmorum taksonomik olarak Ascomycota şubesinden Hypocreales takımının bir üyesidir. Esas habitatı toprak olan *F. culmorum,* nekrotrofik yaşam döngüsüne sahiptir. Dünya genelinde Avustralya, Kuzey Afrika, Avrupa, Batı Asya ve Amerika'da bu türün varlığı rapor edilmişken ülkemizde de Marmara, İç Anadolu, Ege, Karadeniz ve Akdeniz bölgelerinde var olduğu kayıtlara geçmiştir [2, 3, 8-10]. *F. culmorum* 'a ait genom projesi resmi olarak tamamlanmamıştır. Haploit kromozom sayısı n=4 olan bu türde, FcUK99 izolatının kromozomal düzeyde dizilim bilgisine ait çok sayıda veri toplanmıştır. Genom boyutu 39 Mbp olarak tahmin edilmekte ve öncül veriler, veri tabanlarına yüklenmiştir [5]. Ayrıca bu türdeki bazı özgün genlere (toksin üretimi, eşem tipi genleri vb.) ait veriler veri tabanlarında mevcuttur [http://www.embl.org; www.ncbi.nih.nlm.gov]. Günümüzde mevcut gen/genom dizilim bilgileri baz alınarak *F. culmorum* türündeki çalışmalar giderek artmakta ancak *F. graminearum* türüyle gerçekleştirilen çalışmalar ile karşılaştırıldığı zaman nispeten nicelik olarak daha düşük seviyede kalmaktadır. Bu sebeple *F. culmorum* türüyle gerçekleştirilecek genomik ve transkriptomik düzeydeki çalışmaların artması patojenin karakterizasyonu ve hastalıkları ile mücadelede önem arz etmektedir.

Yurdumuzda tanı, genotiplendirme ve kemotiplendirme araştırmaları ile arpa ve buğday bitkilerinde *F. culmorum*'un varlığı saptanmıştır [10-12]. Ancak yurdumuzda çalışmaların çoğunluğu genetik çeşitlilik ve mikotoksin analizleri üzerine yoğunlaşmıştır [2, 5]. Mikotoksin analizlerinde ise çoğunlukla *tri5* gen kümesindeki polimorfizm üzerinde durulmuştur [10, 12]. Bu patojen ve oluşturduğu hastalıklarla mücadele kapsamındaki çalışmalar özellikle *F. culmorum* türü baz alındığında sınırlı düzeyde kalmıştır. *F. culmorum* ve başak yanıklığı ile mücadele çalışmalarında patojene dirençli bitki çeşitlerinin geliştirilmesi önemli bir yaklaşımdır. Ancak bu süreçte sınırlı sayıda çeşit geliştirilebilmesi, geliştirilen çeşitlerin agronomik kalitesinin düşük nitelikte olabilmesi ve çalışmaların uzun süre gerektirmesi önemli olumsuzluklardandır. Ayrıca, antagonistik mikroorganizmalar ile mücadele ve geleneksel

olarak fungisit uygulaması da diğer yaklaşımlardır.Tritikonazol, tebukanozol ve carbendazim fungisitleri bu kapsamda Türkiye de dahil olmak üzere çeşitli ülkelerde uygulanmıştır [13-15]. *F. culmorum* türünde genetik çeşitlilik seviyesinin yüksek olması, paraseksüel üremenin varlığı ve fungusların potansiyel antifungallere direnç geliştirebilme yetenekleri, *Fusarium* türleriyle mücadelede muhtemel yeni yaklaşımların uygulanmasının gerekliliğini ortaya koymaktadır. *Fusarium* sp. tarafından üretilen toksinlerin üretiminin durdurulması hastalıkla mücadele stratejilerinin geliştirilmesi kapsamında moleküler genetik uygulamalarıyla gerçekleştirilmiştir [2, 16, 17]. Fungusun eşeysiz ve/veya paraseksüel üremesiyle ilişkilendirilmiş genlerin karakterizasyonu ise bu kapsamda incelenecek bir diğer alternatif yaklaşımdır. *Mgv1, FcStua, VelA, TopI* ve *chs1* genleri bu kapsamda ele alınabilecek en önemli potansiyel genler arasındadır. Bu genler arasından *Mgv1* geni, gen boyutu ve öncü dizilim bilgileri sebebiyle çeşitli bileşiklerin antifungal etkilerinin incelenmesi için muhtemel hedef gendir. *Mgv1* geni mitozu teşvik eden maddeler tarafından uyarılan ve fosforilasyonla aktive olan bir protein kinazı kodlamaktadır [18]. Genin boyutu 1543 bç olup 4 intron içermektedir. Genin protein ürünü toksin (butenolid ve trikotesen) üretimi, bitki enfeksiyonu ve hücre çeperi bütünlüğü açısından elzemdir. Bu noktada *Mgv1* geni ilişkili olduğu ciddi biyolojik süreçler vasıtasıyla muhtemel antifungal etkili bileşiklerin etkilerinin gösterilmesinde ve ispatlanmasında hedef bir gendir.

Bu çalışmada ilk kez bitki patojeni *F. culmorum'*da antifungal etkinliği araştırılmamış olan 2,4-Dimetilpirol incelenmiştir. 2,4-Dimetilpirol, çeşitli kompleks makrosiklik yapıların bileşeni olan pirollerin *N*-metilpirol ($C_4H_4NCH_3$) türevlerinden birisi olup C_6H_9N açık formülüne sahip bir moleküldür. 2,5-Dimetilpirol bileşiğinin antimikrobiyal etkileri *Candida tropicalis* ve *Aspergillus niger* üzerinde gösterilmiştir [19]. 2,4-Dimetilpirol'ün antifungal etkiye sahip olabileceği Arif ve diğ. (2009) tarafından ifade edilmiştir. Çalışma kapsamında 2,4-Dimetilpirol'ün fungus üzerindeki fenotipik ve transkriptomik etkileri *F. culmorum* 20F izolatında incelenmiştir. Doğrusal büyüme oranı (DBO) analizleri fenotipik düzeydeki incelemelerde kullanılırken, hücrede önemli biyolojik süreçlerin sürdürülmesinden sorumlu *Mgv1* geninin anlatım profilleri de transkriptomik araştırmalarda kullanılmıştır. Elde edilen verilerin sonucunda *F. culmorum*'a karşı mücadelede uygulanabilecek muhtemel etkili bir antifungal bileşiğin karakterizasyonu açısından önem arz etmektedir. Ayrıca farklı konsantrasyonlardaki 2,4-Dimetilpirol solüsyonlarının doğrudan uygulaması ve/veya püskürtme yaklaşımı ile tarlada hastalığın kontrol altında tutulabilmesine katkı sağlanabilecektir.

2. MATERYAL ve METOD

2.1. Patojen ve Fenotipik Testler

F. culmorum 20F izolatı Prof. Dr. Berna Tunalı (Samsun Ondokuz Mayıs Üniversitesi, Bitki Koruma Anabilim Dalı Kültür Koleksiyonun)'dan temin edilmiştir. Kontrol grubuna ait örnek patates dekstroz agar (PDA: Hi-Media, India) besi ortamında, deney grubunda ise PDA'ya ek 0, 0.5, 1, 2 ve 4 mg mL^{-1} artan konsantrasyonlarında 2,4-Dimetilpirol (Sigma, A.B.D.) içeren besi ortamında yedi gün süre ile 28°C'de inkübe edilmiştir. Araştırmada doğrusal büyüme oranını elde etmek ve minimum inhibisyon konsantrasyonunu hesaplamak için agar dilüsyon tekniği kullanılarak 0.25 cm^2 lik kültür plakları besi ortamlarına aktarılmıştır.

2.2. Total RNA İzolasyonu ve cDNA Çevrimi

Tri-Reagent (Sigma, A.B.D.) kullanılarak *F. culmorum* 20F izolatının yedi günlük katı kültürlerinden total RNA izolasyonu gerçekleştirilmiştir. Total RNA izolasyonu 0.5 mL Tri-Reagent için 5-10 mg miselyum kullanılarak tavsiye edilen protokole göre gerçekleştirilmiştir. Elde edilen total RNA molekülleri UV ışık altında, %1'lik agaroz jel elektroforezi ile

görüntülenerek analiz edilmiştir. Total RNA'ların kantitatif analizleri ise GelQuant yazılımı (Biochemlabsolutions, A.B.D.) ve spektrofotometre (Shimadzu, Japonya) ile gerçekleştirilmiştir.

cDNA molekülleri, iki aşamalı ticari kit ile kPZR ve RT-PZR tekniğinde kullanılmak üzere total RNA moleküllerinden (Applied Biosystems, İngiltere) sentez edilmiştir. cDNA sentezi tavsiye edilen protokole göre tüm örnekler için başlangıç miktarı eş olacak şekilde 2 µg RNA kullanılarak gerçekleştirilmiştir. Elde edilen cDNA'lar 1/4 oranında sulandırılarak gen anlatımı analizlerinde kullanılmıştır.

2.3. Gen Anlatım Analizleri

Mgv1, (ve içsel kontrol gen olarak *β-tubulin*) anlatımının analizinde RT-PZR ve kPZR uygulamaları kullanılmıştır. RT-PZR deneylerinde bileşenler konsantrasyonları 1 X PZR tamponu, 0.25 mM dNTP karışımı, 10 pmol ileri ve geri primerler (Tablo 1), 2.5 mM $MgCl_2$, 2 µg RNA'ya denk gelen cDNA'dan 5 µL ve 1 U *Taq* DNA polimeraz enzimi (Thermo, A.B.D.) olacak şekilde toplam 25 µL hacimde birleştirildi. Çoğaltım, 94°C'de 5 dakikalık ön denatürasyonu takiben 45 tekrardan oluşan 94 °C'de 20 saniye, 59 °C'de 20 saniye ve 72 °C'de 40 saniye basamakları ile tamamlanmıştır. Süreç 72 °C'de 5 dakikalık final uzama basamağı ile tamamlandı. Çoğaltım ürünleri %1.7'lik agaroz jel ile gerçekleştirilmiştir.

Tablo 1. Gen anlatımı çalışmalarında kullanılan primer molekülleri.

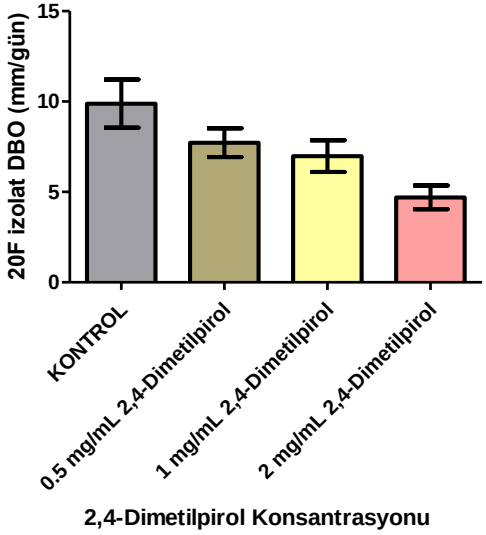
Primer	**Primer Dizisi (5'-3')**	**Band Boyutu (bç)**
QPCRF	AGGGTCATTACACCGAGGGT	121
QPCRR	GTACCACCACCAAGAGAGTGG	
MGVRTF	AGGTTCAACGATTCCGACAG	100
MGVRTR	GACCATTACCCTGAGGCAGA	

Gerçek zamanlı gen anlatımı analizlerinde *β-tubulin* geni içsel kontrol, *Mgv1* geni hedef gen olarak kullanılmış olup; oransal değişimlerinin analizi için kPZR cihazına ait yazılımından elde edilen veriler kullanılmıştır (Roche LightCycler 480 II, İsviçre). kPZR işleminde Eva Green (Biorad, Fransa) floresan boya olarak kullanılmıştır. Toplam hacim 20µl olacak şekilde kPZR bileşenleri 1 X Eva Green karışımı, 5 pmol ileri primer, 5 pmol geri primer ve 1 µg RNA'ya denk gelen miktarda cDNA olacak şekilde mikrotüplerde bir araya getirilmiştir. PZR koşulları 95 ºC'de 10 dakika ile ilk denatürasyonu takiben 45 tekrardan oluşan 95ºC'de 20 saniye, 59 ºC'de 20 saniye, 72ºC'de 40 saniye bekleme süresinden ve son olarak da 40ºC'de 30 saniyelik soğutma aşamasından oluşmaktadır. Erime eğrisi analizleri ve 4 logaritmik fazdan oluşan standart serileri, PZR etkinliğinin ve doğruluğunun analizi için gerçekleştirilmiştir. Gen anlatım profilleri $2^{-\Delta\Delta CT}$ normalizasyon değerleri göre elde edilmiştir [21]. Çalışmalar en az iki tekrar ile gerçekleştirilmiştir. İstatistiksel analizler GraphPad Prism 5.0 yazılımı kullanılarak (Tukey Testi, Tek Yönlü ANOVA Analizi) gerçekleştirilmiştir.

3. BULGULAR

3.1. *In vitro* Üreme ve 2,4-Dimetilpirol Uygulaması

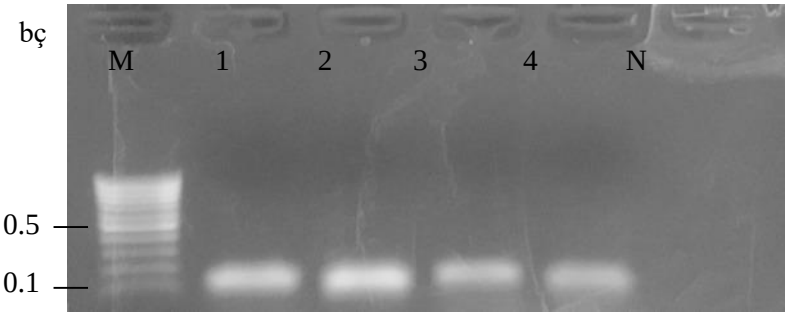
F. culmorum 20F izolatı 2,4-Dimetilpirol içeren ve içermeyen (0, 0.5, 1, 2 ve 4 mg mL^{-1} 2,4-Dimetilpirol) PDA besi ortamlarında üreme göstermiştir. Üremenin görülmediği 2,4-Dimetilpirol konsantrasyonu 4 mg mL^{-1} 2,4-Dimetilpirol olarak saptandığından IC_{50} konsantrasyonu 2 mg mL^{-1} 2,4-Dimetilpirol olarak belirlenmiş ve gen anlatım çalışmalarında bu deney seti kullanılmıştır. Deney ve kontrol grupları arasında DBO açısından anlamlı bir farklılık ($p<0.01$) görülmüştür (Şekil 1). DBO değerleri kontrol grubunda 9.88±0.54 iken, bu değerin deney setlerinde 4.7±0.27 – 7.72-0.32 arasında olduğu saptanmıştır.



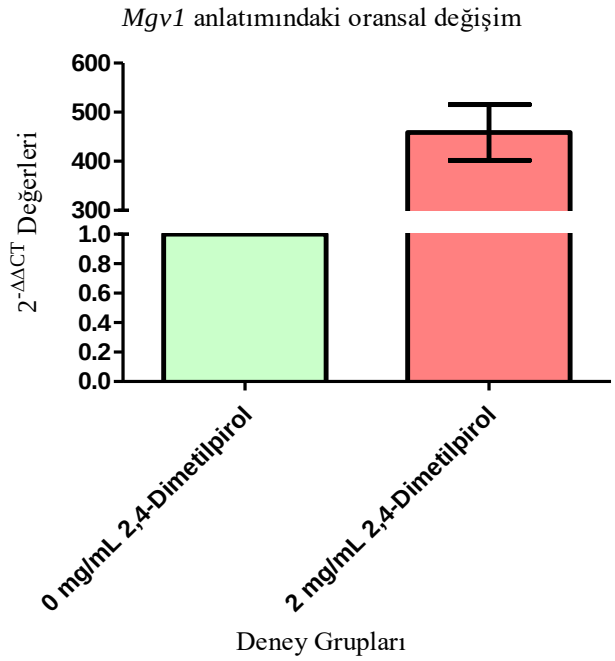
Şekil 1. Farklı konsantrasyonlarda 2,4-Dimetilpirol içeren besi ortamlarında üretilen 20F izolatının doğrusal büyüme oranı verilerine ait grafik.

3.2. Gen Anlatımı Analizleri

20F izolatına ait deney ve kontrol setlerinde yüksek kalite ($\Delta_{260/280}$ = 1.9-2.0) ve miktarda (0.5-2 µg μL^{-1}) total RNA molekülleri izole edilmiştir. Total RNA moleküllerinden çevrilen cDNA molekülleri RT-PZR ve kPZR çalışmalarında kullanılmıştır. Normalizasyon, hem deney setlerinde hem de kontrol gruplarında *β-tubulin* amplifikasyonu ile sağlanmıştır. RT-PZR analizlerinde, agaroz jel elektroforezi sonucunda içsel kontrol gene ait 121 bç'lik, *Mgv1* genine ait 100 bç'lik amplifikasyon ürünü elde edilmiştir (Şekil 2). Bulgular kPZR ile doğrulanmıştır. kPZR analizlerinde, $\bar{X}E$ (ortalama etkinlik) değeri 2.04±0.03 olarak saptanmıştır. Ortalama erime eğrisi skorları içsel kontrol gen ve *Mgv1* geni için sırasıyla 0.94±0.01 ve 0.97±0.01 olarak belirlenmiştir. Bu veriler kPZR işleminin etkin bir şekilde gerçekleştiğini göstermiştir. $\bar{X}\Delta$Cp (ortalama eşik döngüsü farkı) değerleri deney grubu için 1.48±0.015 iken, kontrol grubu için 10.51±0.03 olduğu belirlenmiştir. Normalizasyon bulgularına göre; deney setleri kontrol grubu ile kıyaslandığında $\bar{X}2^{-\Delta\Delta CT}$ (ortalama gen anlatımındaki oransal değişim) değeri *Mgv1* geni için $5.21\pm0.05x10^2$ olarak belirlenmiştir. Bu durum gen anlatımındaki oransal değişimlerin yaklaşık $5x10^2$ kat (Şekil 3) oranında arttığını göstermiştir. İstatistiksel verilere göre gen anlatımındaki bu oransal değişimler anlamlı farklılık göstermektedir ($p<0.001$).



Şekil 2. 20F izolatının hedef (1, 2) ve içsel kontrol gene (3,4) ait RT-PZR profili. M: 100 bç DNA boyut markırı (Sibenzyme, Rusya), N: negatif kontrol, 1 ve 3 sırasıyla kontrol grubu, 2 ve 4 ise deney grubuna ait amplikonlar.



Şekil 3. *Mgv1* gen anlatımındaki oransal değişim profili.

4. TARTIŞMA ve SONUÇ

Bitki patojeni funguslar tahıllarda en önemli stres faktörlerinden birisidir [1]. Bitki patojenlerinin mısır ve buğday başta olmak üzere ekonomik öneme sahip bitkilerde kayıp meydana getirmesinin yanı sıra üretilen mikotoksinlerin bu bitkilerde birikmesi; dolaylı olarak insan ve hayvan sağlığını olumsuz etkilemektedir [2, 22]. Fusarium başak yanıklığı ve kök çürüklüğü hastalıkları tahıllarda görülen ve ülkemizde de varlığı tespit edilen hastalıklar arasında yer almaktadır [10]. Bu çalışmalarda detaylı olarak patojenlerin genotiplendirmeleri ve mikotoksin üretme potansiyelleri genetik ve analitik metotlar ile araştırılmıştır. Bu şekilde bu çalışmalarla birlikte hastalıkla mücadeledeki elzem bir başlangıç basamağı olan patojenin tanımlanması gerçekleştirilmiştir [2, 10, 12].

Fusarium başak yanıklığı ve kök çürüklüğü ile ilgili kapsamlı tarımsal biyoteknoloji ve moleküler genetik çalışmalarında; patojenlerinin tanımlanmasını genellikle patojenin meydana getirdiği zararların indirgenmesi ve hatta mümkünse bu hasarların ortadan kaldırılması takip eder. Deoksinivalenol mikotoksininin *F. graminearum* ve *F. culmorum* türlerinde 'quelling' süreci ile üretiminin durdurulması bu bağlamda gerçekleştirilmiştir [16, 17]. Ancak, bu güncel

teknoloji kullanılarak gerçekleştirilen çalışmalarda hem gen anlatımı hem de toksin üretiminde beklenilenin aksine trajik seviyelerde artış da gözlemlendiğinden global anlamda etkili bir çözüme ulaşılamamıştır. Benzer şekilde çeşitli fungusit uygulamaları başak yanıklığı ve kök çürüklüğü etmenleri üzerinde denenmiş patojen üzerinde %80 civarında etkili oldukları gösterilmiştir [13, 14]. Son yıllarda farklı tipte potansiyel antifungal niteliği taşıyan bitki ve mikroorganizma türevli metabolitler *Fusarium* türleri üzerinde denenmektedir. *Fusarium* türleri ile ilişkili çalışmaların çoğunluğu genom projeleri tamamlanmış iki tür, *F. graminearum* ve *F. oxysporum* üzerinde yoğunlaşmıştır. Buna karşın *F. culmorum* üzerinde gerçekleştirilen çalışmalar nispeten daha sınırlıdır [23, 24]. Hastalıkla mücadelede yeni bakış açılarının elde edilmesinde, *F. culmorum* üzerinde yapılacak antifungal aktivite çalışmaları, kapsamlı ve yararlı verilere ulaşılmasını sağlayacaktır.

Pirol içeren bazı çeşitli bileşikler bitkilerde, hayvanlarda çok fazla sayıda biyolojik bileşiğin bir parçası olarak bulunmaktadır. Pigmentasyon, antimikrobiyal etki, enzimatik süreçler ve diğer bazı önemli biyolojik faaliyetlerde rol oynayan moleküllerde yer alırlar. Hint yağı bitkisi, su yasemini ve diğer bazı bitkilerden elde edilebilen dimetilpirol formlarının ilaç ve antimikrobiyal etkileri günümüzde araştırılmaya başlanmıştır [20, 25, 26]. Bununla beraber dimetilpirol formlarının *F. culmorum* üzerine etkisi bilinmemektedir. Bu sebeple bu çalışmada çeşitli bitkilerden elde edilebilen 2,4-Dimetilpirol bileşiğinin saf molekülünün potansiyel antifungal etkileri, daha önceki çalışmalarda tür düzeyinde tanımlanmış ve toksin (deoksinivalenol) üretme yeteneği araştırılmış olan *F. culmorum* 20F izolatı üzerinde gerçekleştirilmiştir [12]. Yoshi ve diğ. (2013) farklı mikroorganizmalarda 2,5-Dimetilpirol'ün IC_{50} değer aralığının 0,5-250 μg mL^{-1} arasında değiştiğini rapor etmiştir. Bu çalışmada ise *F. culmorum* 20F izolatında IC_{50} değerinin 2 mg mL^{-1} olarak belirlenmiştir. DBO verileri bu bileşiğin *F. culmorum* türü için potansiyel bir antifungal ajan olabileceğini göstermektedir. Ayrıca DBO'ya ait bilimsel olarak anlamlı farklılıklar, bitki enfeksiyonu, hücre çeperi bütünlüğünde iş gören ve seksüel/aseksüel üremede görevli Mgv1 genine ait normalizasyon verileri ile desteklenmiştir.

Fungusun üremesiyle ilişkili genlerin anlatımının incelenmesi, herhangi bir antimikrobiyal bileşiğin fungusta oluşturacağı değişiklikleri araştırılmasına yönelik fenotipik testlerin doğrulanması için potansiyel bir yaklaşımdır. Mgv1, FcStua ve VelA genleri bu açıdan incelenebilecek en önemli potansiyel genler arasındadır. Mgv1 geni veri tabanlarında dizilim bilgilerinin yer almasından dolayı bileşiklerin antifungal etkilerinin incelenmesinde olası bir hedef gendir. Mgv1 geni mitojen ile aktive edilen bir protein kinazı kodlamaktadır [18]. Genin protein ürünü toksin üretimi, bitki enfeksiyonu ve hücre çeperi bütünlüğü açısından anlatımı elzem bir gendir. Çalışmada 2,4-Dimetilpirol uygulanmamış örnek ile gerçekleştirilen normalizasyon verilerine göre Mgv1 anlatımı 5x102 kat artış göstermiştir. Bu durum, potansiyel bir antifungal uygulaması sonucunda funguslarda görülen ve anlatımı yaşam çevriminin devamlılığı için gerekli olan genlerin anlatımındaki artışa bir örnektir [28]. Antimikrobiyal bileşiğin varlığı hücrede abiyotik bir stres olarak tanımlanmış ve yaşamın devamlılığı için Mgv1 geninin anlatımında potansiyel bir artış olduğu saptanmıştır. Veriler başak yanıklığı ve kök çürüklüğü hastalık etmenleri ile mücadelede potansiyel ve yeni bir uygulama sunması açısından önemlidir. Bu çalışmadan kullanılan bileşiğin daha önce *F. culmorum* üzerindeki etkilerinin incelenmemiş olması çalışma sonunda yeni veriler sunmuştur. Çalışma daha önceden bu bileşiğin *F. culmorum* üzerindeki fenotipik ve transkriptomik etkilerinin incelenmemesi açısından yeni veriler sunmuştur. Ayrıca *Mgv1* geninin eşeyli üremede görev aldığı bilindiğinden eşeyli üreme görülen diğer *Fusarium* spp.' de de, bu bileşik, potansiyel bir ajan olarak kullanılabilir. 2,4-Dimetilpirol uygulanmış fungusta görülen ve dramatik seviyelere ulaşan gen anlatımındaki aşırı artış, bu kimyasalın fungusun homeostasisinde kuvvetli değişim meydana getirebildiğini göstermektedir. Bu durum bileşiğin

potansiyel bir biyolojik mücadele ajanı olduğunu göstermektedir. Bu bileşiğin diğer bazı nekrotrofik ve biyotrofik *Fusarium* türlerinde uygulanmasını içeren ileriki çalışmalar gelecek uygulamaları başak yanıklığı ve kök çürüklüğü ile mücadelede önemli verilerin ulaşılmasını sağlayabilir. *Fusarium* spp.'de de, bu bileşik, potansiyel bir ajan olarak kullanılabilir. Çalışmadan elde edilen veriler *F. culmorum*'un tahıllarda meydana getirdiği hastalıklarla mücadele için yeni stratejilerinin geliştirilmesi bakımından önem arz etmektedir.

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SUMMARY

Purpose

Fusarium spp. is one of the major mycotoxin producing phytopathogenic fungi. Presence of more than 10 phytopathogenic species of this genus are reported in Turkey. *Fusarium culmorum* is a phytopathogenic species cause several diseases on all small grain cereals, especially on wheat, barley, oat and rye which are economically important crops, in Turkey and worldwide. This species lead to severe yield loses in crops including reduction in crop quality and quantity. Contamination of food by mycotoxins, especially class B-trichothecenes and zearalenon, produced by *F. culmorum* have been detected wordwide as well as in Turkey. *F. culmorum* is a causal agent of head blight and root rot in wheat and barley. Struggle with *F. culmorum* has critical importance in terms of development of novel disease management stratgies worldwide. Genetically modified plants or *Fusarium* resistant cultivars usage, antogonistics approaches and gene silencing technologies are currently used in fight with *F. culmorum* diseases. Each approach has several disadventages, whereas investigating the effects of novel or unexperienced antifungal compounds for *F. culmorum* present promising strategy in disease management. For this purpose, effects of 2,4-Dimethylpyrrole on *F. culmorum* 20F isolate which was obtained from diseased plants in Turkey was tested via phenotypic and transcriptomics analysis in this study.

Results and Discussion

In this study, phenotypics and transcriptomics effects of 2,4-Dimethylpyrrole on Turkish *F. culmorum* 20F isolate were investigated. *F. culmorum* 20F isolate was subjected to 2,4-Dimethylpyrrole with different concentrations (0.5, 1, 2 ve 4 mg mL^{-1}). Seven-day-old cultures were used in Minimum inhibitory concentration (MIC) value determination. IC_{50} value was determined at PDA medium supplemented with 2 mg mL^{-1} 2,4-Dimethylpyrrole. Moreover, incereased concentrations of 2,4-Dimethylpyrrole led to the decrease in the linear growth rate (LGR) values. The significant difference ($p<0.01$) was detected among control and each experiment set as well as in the increased concentrations for experiments sets in LGR values. Experiment set with IC_{50} value was used in transcriptomics analysis. First, total RNA molecules were extracted from control and experiment sets and then total RNAs were immediately converted to cDNA molecules. Expression of *Mgv1* gene, responsible for sexual stage and cell wall integrity, was investigated via quantitative real time polymerase chain reaction (q-PCR) and reverse transcription PCR (RT-PCR). β-tubulin gene was used as endogenous control. qPCR analysis were conducted on Eva Green dye detection on Roche LightCycler 480 II

system. Except values were as 2.04±0.03, mean melting scores were ranged from 0.94±0.01 to 0.97±0.01 for endogenous and target genes. Mean ΔCp values were ranged from 1.48±0.015 to 10.51±0.03. In comparison to control groups, $5.21\pm0.05x10^2$ fold increases in experiment set for *Mgv1* gene was detected. In RT-PCR analysis, 121 bp and 100 bp amplicons belonging to endogenous and target genes, respectively, were obtained from control and experiment sets. According to findings obtained, it was shown that 2,4-Dimethylpyrrole could be a potential antifungal agent for *F. culmorum.* In further works, it is suggested that this agent could be used as a novel approach in disease control including inhibition of fungal biomass and toxin production in field.

Conclusion

In current study, significant changes in LGR and fold changes in gene expression for *Mgv1* were determined. Presence of 2,4-Dimethylpyrrole at high level of concentrations (up to 2 mg mL^{-1}) could be described as a potential antimicrobial against *F. culmorum*. Expecially dramatic increase in *Mgv1* gene expression obtained from relative quantification analysis supported that 2,4-Dimethylpyrrole could be a potential antifungal compund to *F. culmorum*. Further analysis including 2,4-Dimethylpyrrole exposure to necrotrophic and biotrophic *Fusarium* sp. could provide a detailed and comprehensive strategy to fight with head blight and root rot diseases.

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Total Phenolics, Antioxidant, Antibacterial and Cytotoxic Activity Studies of Ethanolic Extracts *Arisarum vulgare* O.Targ.Tozz. and *Dracunculus vulgaris* Schott.

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Abstract: The present study reports the antioxidant, total phenolics, antibacterial and cytotoxic activities of *Arisarum vulgare* and *Dracunculus vulgaris* of aerial and under ground ethanol extracts. The antioxidant activity was determined by DPPH radical scavenging activity and β-carotene linoleic acid assays. Folin-Ciocalteu assay was used to detect total phenolic contents in the extract. *In vitro* cytotoxic activity was determined by the Brine shrimp lethality test. Antibacterial activity was investigated with the microdilution method. As a result, under ground parts of the extract showed higher antioxidant activity than aerial parts of the extract. Total phenolics ranged from 14.5±2.02 to 53.4±2.01 mg GAE/g, and the antioxidant activity according to the β-carotene/linoleic acid assay ranged from 70.76±1.08 % to 85.43±1.05 and according to the DPPH assay IC_{50} values ranged from 0.089±1.02 to 1.095±1.07mg/ml. The *A. vulgare* under ground extract was tested against 2 and showed a good antibacterial activity at a concentration of <50 µg/mL Minimum inhibitory concentration (MIC) values for the bacteria *S.aureus.* The results of the cytotoxic activities showed a very high activity of the extracts, *D.vulgaris* under ground extract (LC_{50} = 10.6 µg/mL).

Keywords: Antioxidant, antibacterial, cytotoxic, *Arisarum vulgare*, *Dracunculus vulgaris*,

1. Introduction

Free radical induced oxidative damage has long been thought to be the most important cause of many diseases such as diabetes, stroke, cancer, arteriosclerosis and cardiovascular diseases [1, 2]. Oxidative stress affects the prooxidants and antioxidants equilibrium in biological system which leads to the modification of DNA, proteins, carbohydrates, and lipids. Hydroxyl radicals, superoxide anion radicals, and singlet oxygen are the examples of free radicals that attack the unsaturated fatty acids in the biomembranes resulting in lipid peroxidation, decrease in fluidity, loss of enzymes and receptor activity, and damage to membrane proteins and ultimately leading to cell inactivation. Lipid peroxidation is also strongly associated with aging and carcinogenesis [1-3]. Antioxidants mitigate oxidative stress, the adverse effects of free radica. Plant products are rich sources of phytochemicals and have been found to possess variety of biological activities including antioxidant, cytotoxic, and

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hepatoprotective potential. They act as reducing agents and reverse oxidation by donating electrons and/or hydrogen ions [4, 5].

Interest in toxic plants is increasing because it is recognised that these plants contain bioactive compounds and then, have medicinal virtues. So, medicinal plants are of great importance to the health. Also, many papers have reported that the major pharmacological proprieties of medicinal plants are associated and attributed to their antioxidant activity, then; much attention is being paid to antioxidant compounds [6]. Among plant secondary metabolites, polyphenols are known to have a high capacity to scavenge free radicals due to the hydrogen and electron transfer abilities and in that way are considered as prevailing responsible for antioxidant action. High content of non-phenolic compounds (monoterpenes and sesquiterpenes) in most essential oils might be related to their weak antioxidant activity [7].

Arisarum vulgare (Araceae) is very toxic, but their tubers are eaten in times of scarcity after boiling in water. It is also known for its uses in traditional medicine to treat various diseases. For example and according to ethnobotanical investigations, it is observed that in some regions of Algeria, *A. vulgare* is used for the treatment of several diseases such as headaches, asthma, flu and it promotes healing of early wound skin lesions. [8].

The genus *Dracunculus* (Araceae) is represented with only one taxon (*D. vulgaris* Schott) in Turkey, and tubers of this plant are used external in the treatment of rheumatism and hemorrhoids [9, 10]. Leaves and tubers of the *Dracunculus* genus contain saponin and conicine alkaloids, estragole, phelandrine, methyl cavicol, iodine, rutin, tanin, flavonoid and coumarin [11]. Seed oil of this genus contains also palmitic acid, oleic acid, cis-Vaccenic acid, stearic acid and arachidic acid [12]. *D. vulgaris* Schott is a poisonous plant, and the leaves and tubers of this plant have a toxic effect on humans and animals. In the light of all informations mentioned above, this study aimed to investigate total phenolic contents, the potential antioxidant, antibacterial and cytotoxic activities of aerial and under ground parts of the ethanolic extract of *D. vulgaris and A.vulgare.*

2. Material and Methods

2.1. Plant Material and Preparations of Extracts

A. vulgare and *D. vulgaris* species were collected in October 2015 from Denizli-Turkey (the campus of Pamukkale University). The fresh aerial and under ground parts of the plants samples were cleaned and dried in the shadow for extraction. Dried plant parts (under ground and above ground) were pulverized. Each ground sample was transferred into a beaker. Ethanol was added in the ratio of 1:10 and they were put in water bath at 55ºC for 6 h [13]. The extraction mixture was separated from the residue by filtration through Whatman No: 1 filter paper. After the filtration two extracts were combined. The residual solvent of ethanol extracts of samples were removed under reduced pressure at 48-49°C using a rotary evaporator (Rotavapor IKA VB 10, Germany). The water extract was lyophilized using a freeze dryer (Thermosavant Modulyo D, USA). Extracts were produced in duplicates and used to assay the biological activity.

2.2. Plant Extracts

Arisarum (A), under ground-ethanol (AUE), above ground-ethanol (AAE). *Dracunculus* (D), under ground-ethanol (DUE), above ground-ethanol (DAE),

2.3. Antioxidant Activities

2.3.1. β-Carotene-Linoleic Acid Assay

This test was carried out according to a described procedure [15], based on the aptitude of various extracts to decrease the oxidative discoloration of β-carotene in an emulsion. A stock solution of β-carotene-linoleic acid mixture was prepared as following: 0.5mg β-carotene was dissolved in 1 mL chloroform. 25 µL linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 mL of distilled water was added with vigorous shaking. Also, 2.5 mL of this reaction mixture was dispensed into test tubes and 350 µL portion (1mg/mL) of the extract was added and the emulsion system was incubated for up 2 h at 50ºC. The same process was done again with synthetic antioxidant, BHT, as positive control and a blank. The absorbance of the mixtures was measured with a spectrophotometer (Shimadzu UV- 1601, Japanese) at 490 nm after the incubation period, and inhibition ratio was calculated. The antioxidant activity was measured in terms of successful bleaching of β-carotene by using the following equation [15]. The measurements were made using the equation below:

AA: [1- (A0-At / A0o - Ato) x 100]

Where AA is the total antioxidant activity, A0 is the initial absorbance of the sample, At is the initial absorbance of the control, A0o is the sample's absorbance after 120 min, and Ato is the control's absorbance after 120 min.

2.3.2. DPPH Free Radical Scavenging Activity Assay

The method of Wu et al. [16] was used for determination of scavenging activity of DPPH free radical. 4 ml of the DPPH's 0.004% metanolic solution was mixed with 1 mL (0.2-1.0 mg/mL) of the extracts, and their absorbances were measured to be at 517 nm after incubation for 30 min at room temperature the absorbance value of the samples were evaluated against empty control group (where all determinants except the test compound were present). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Every test was treated three times and the averages as determined. Free radical scavenging activity was measured using the equation below:

Scavenging activity = $[(A_0 - A_1 / A_0) \times 100)]$

Where A0 is the absorbance of the control (blank, without extract) and A1 is the absorbance in the presence of the extract. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration.

2.3.3. Determination of Total Phenolic Content

The total phenolic content of extracts were determined with Folin- Ciocalteau reagent, according to the method of Slinkard and Singleton [14]. Briefly, 0.75 mL of Folin–Ciocalteu reagent (1:9; Folin-Ciocalteu reagent: distilled water) and 100 mL of sample (5 mg/mL) were put into a test tube. The mixture was allowed to stand at room temperature for 5 min. 0.75 mL of 6% (w/v) Na_2CO_3 was added to the mixture and then mixed gently. The mixture was homogenized and allowed to stand at room temperature for 90 min. Total polyphenol content was determined using a spectrophotometer at 760 nm. The Standard calibration (0.01–0.05 mg/mL) curve was plotted using gallic acid. The total phenolic content was expressed as Gallic Acid Equivalents (GAE) in mg/mL plant extract. The estimation was performed in triplicate, and the results were expressed as mean ± SD.

2.4. Cytotoxic Activity

Brine shrimp lethality test (BSLT) was applied to analyze the possible cytotoxic activity of the extracts. *A. salina* eggs (10mg) were incubated in 500mL of seawater under artificial light at 28ºC, pH 7-8. After incubation for 24h, nauplii were collected with a pasteur pipette and kept for an additional 24h under the same conditions to reach the metanauplii (mature larvae) stage. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5mL of brine solution. In each experiment, 0.5mL of the plant extract was added to 4.5mL of brine solution and maintained at room temperature for 24h under the light and then dead nauplii were counted [17]. Experiments were conducted along with control and five different concentrations (10-1000µg/mL) of the extract in a set of three tubes per dose. Analysis of the data was performed by EPA Probit Analysis Program (version 1.5) to determine the LC_{50} values.

2.5. Determination of Antibacterial Activity

The microorganism strains used in this study were *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). The microorganism strains used were provided by the Department of Medical Genetics of the Medicine Faculty of Pamukkale University.

The antibacterial effects of ethanolic extracts were examined using the minimum inhibition concentration with (MIC). The broth micro dilution method microdilution method was used to determine the minimum inhibitory concentrations (MICs) of the plant extracts using 96 well microtitration plates as previously described by Satyajit et al. [18]. Bacterial strains were streaked on Mueller Hinton agar plates using sterile cotton swabs. Five microlitres of dimethylsulphoxide (DMSO), loaded on sterile blank disks were placed on the agar plates and were incubated at 37ºC for 24 h. One hundred and eighty-five microliter (200 µl) of the broth was added into each well in the first row of microtitration plate and 100 µl to the rest of the wells from the second row down wards. Fifteen microliter (15 µl) of the plant extracts was then added into each well on the first row (row A), starting with the positive control (Gentamicin for bacteria and Floconazole for yeast, all the antibiotics were from MAST), followed by the negative control (the 20% DMSO used to dissolve the plant extracts) and the plant extracts in the rest of the wells on that row. A twofold serial dilution was done by mixing the contents in each well of the first row and transferring 100 µl to the second well of the same column and the same was done up to the last well of the same column and the last 100 µl from the last well was discarded. Then 100 µl of yeast suspensions was added. The results were observed after 24 h incubation at 37°C, followed by the addition of 40 µl of a 0.2% Iodo Nitro Tetra the lowest concentration at which color change occurred was taken as the MIC value. All measurements of MIC values were repeated in triplicate and the most representative values were used.

3. Results and Discussion

3.1. Antioxidant Activities

The total phenolic content content of the plant extracts is shown in Table 1. The total phenolic contents of the extracts, as estimated by the Folin-Ciocalteu reagent method, ranged from 14.5±2.02 to 53.4±2.01mg GAE/g extract. The phenolic assay involving an electron-transfer reaction was evaluated by using Folin-Ciocalteu reagent [19]. Among all plant extracts, DAE had the highest phenolic content (53.4±2.01 mg GAE/g extract), followed by DUE (37.8±1.02 mg GAE/g extract).

Table1. Total phenolic contents (mean ± SD) of extracts from *A. vulgare* and *D. vulgaris*

Plant extracts	Total phenolic contents (mg GAE/g extract)
AUE	37.8±1.02
AAE	53.4±2.01
DUE	14.5±2.02
DAE	18.8±3.01

The total antioxidant activity of the extracts from *A. vulgare* and *D. vulgaris* plants were determined using β-carotene linoleic acid system. This system is based on the fact that β-carotene discolors when no antioxidant is present as a result of free radicals that form hydroperoxide from linoleic acid.

Under ground-Ethanol (AUE) and (DUE) extracts showed the highest antioxidant activity. The mean antioxidant activity of AUE and DUE were 85.43±1.05% and 83.43± 1.03%, respectively. Both plants extracts showed slightly low, but above ground-ethanol extracts showed lowest antioxidant activity (Table 2). These results indicated that the under ground and over ground parts of the plants have the same amount of phenolic compounds during the flowering time.

Table 2. The total antioxidant activities and IC_{50} values of plant extracts

Plant extracts	β-carotene linoleic acid bleaching assay Total antioxidant activity (%)	DPPH assay IC_{50} (mg/mL)
AUE	85.43±1.05	0.089±1.02
AAE	80.21±1.03	1.075±1.04
DUE	83.43±1.03	1.064±1.03
DAE	70.76±1.08	1.095±1.07
BHT	95.64±1.01	0.012±1.03

The total antioxidant activity of *A. vulgare* was observed to be higher than that of the *D. vulgaris* extracts tested in this study. The DPPH• is a stable radical and gives maximum absorbance at 517 nm. When reduced to the hydrazine derivative by an antioxidant via electron or hydrogen atom transfer reactions, this absorption maximum decreases [20]. Percent inhibition values estimated for all the extracts are presented in Table 3. The higher percent inhibition value means the higher antioxidant activity [21].

Kadri et al., reported that the methanol-water extract of *A. vulgare* seeds possesses strong antioxidative properties *in vitro.* Results confirmed by high polyphenols and flavonoids contents and corroborated by HPLC identifications [8].

Table 3. DPPH radical scavenging activity % of the *A. vulgare* and *D. vulgaris* extracts

	Concentrations			
Extracts	0.2 mg/mL	0.4 mg/mL	0.8 mg/mL	1 mg/mL
AUE	43.98	54.32	73.09	84.32
AAE	25.87	45.46	64.08	78.43
DUE	65.67	70.65	74.87	78.86
DAE	34.75	43.76	56.98	63.54
BHT	68.34	78.98	84.79	96.93

DPPH radical scavenging activities of the ethanol extracts are shown in the Table 3. DPPH free radical scavenging activities of extracts at 0.2-1 mg/mL concentrations were compared with BHT. Additionally, the concentrations of the extracts required to scavenge 50% of the DPPH radicals, the IC_{50} values, were calculated (Table 2). The most active radical scavenger was AUE (IC_{50} = 0.089±1.02 mg/mL). DAE exhibited the weakest antiradical activity (IC_{50} = 1.095±1.07mg/mL) in this study. The results demonstrated that there is a correlation between higher radical scavenging activity and larger amount of total phenolics in the extracts. This data is supported by previous reports, which showed that phenolic compounds generally correlate with antioxidant capacities measured by DPPH assay [22-24]. Öztürk, Aslantürk *et al.* reported that potential antioxidantactivity and anticancer effect of extracts from *D. vulgaris* on mcf-7 breast cancer cells [25, 26]. Previous reports suggest that the leaves and tubers of the *Dracunculus* genus contain saponin and conicine alkaloids, estragole, phelandrine, methyl cavicol, iodine, rutin, tanin, flavonoid and coumarin [12].

3.2. Antibacterial Activity

Antibacterial activity of plant extracts against Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-negative *Escherichia coli* (ATCC 25922) was determined using broth micro dilution method. The results of the analysis of the antibacterial activity of investigated ethanol extracts, obtained by the dilution method are given in Table 4. The obtained results showed that the tested extracts possessed different antibacterial activity within the concentration range from 50 to 100 μg/mL. Increased concentrations of extracts caused decrease in survival of bacterial cells. Very strong reduction of gram-positive *Staphylococcus aureus* growth was observed during incubation of bacteria in *A. vulgare* under-ground extracts (MIC was < 50 μg/mL). Lower antibacterial effect was demonstrated against gram-negative strains *E. coli* (MIC was >100 μg/mL) in *D. vulgaris* extracts.

Table 4. Antibacterial activity of *A. vulgare* and *D. vulgaris* extracts

Extracts	MIC (μg/mL)	
	S.aureus	*E.coli*
AUE	<50 μg/mL	75 μg/mL
AAE	100 μg/mL	100 μg/mL
DUE	<100 μg/mL	> 100 μg/mL
DAE	100 μg/mL	70 μg/mL
Gentamycin	10 μg/mL	-
Ampicillin	-	20 μg/mL

Previous studies have suggested that piperazirum alkaloid isolated from n-butanol fraction of water extract of *A. palaestinum* leaves, which is from the same family as *D. vulgaris* has a significant cytotoxic effect on human lung cancer (A549), ovarian cancer (Skov-3), melanoma (SK-MEL-2) and colon cancer (HCT- 15) cells. Also, the cytotoxic activity of the extracts from this plant on MCF-7 cells in our study may be due to the presence of these chemicals, especially the presence of flavonoids and alkaloids in extracts [28].

3.3. Cytotoxic Activity

The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tümor properties. Based on the results, the ethanol extract (DUE) of *D. vulgaris* has showed good toxic to brine shrimp nauplii, with LC_{50} of 10.60 µg/ml. In addition, the degree of lethality was found to be directly proportional to the concentration of the extract (Fig. 1).

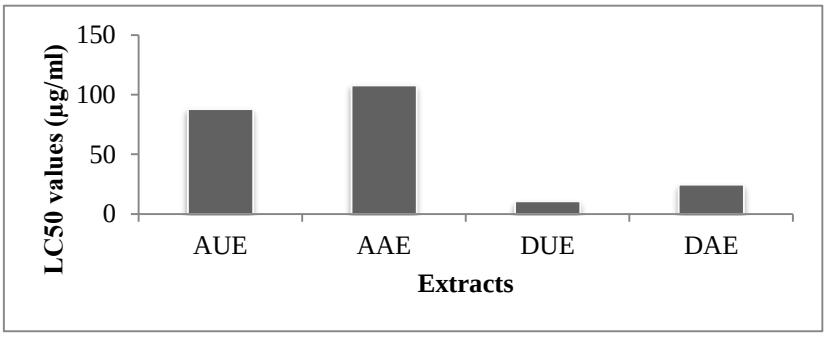

Fig 1. Cytotoxics activity of extracts by LC_{50} values

4. Conclusion

In this study we aimed to determine the antioxidant, total phenolics, antibacterial and cytotoxic activities of *Arisarum vulgare* and *Dracunculus vulgaris* of some parts of (aerial and under ground) ethanol extracts. A further study is needed to isolate and identify the active compounds that are responsible for antioxidant activity. This study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index.

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Review Article

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Tarımsal Üretimde Yararlanılan *Trichoderma* Ürünleri ve Metabolitleri

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Özet: Fitopatojenik ajanların sebep olduğu bitki hastalıklarıyla, önemli ölçüde ürün kayıpları meydana gelmektedir. Dünya üzerindeki birçok ülkede, tarımda pestisit uygulamalarını ve bağımlılığını azaltacak, tüketici ve çevre güvenliğini arttıracak yasal yaptırımlar uygulanmaya başlamıştır. Yönetmelikler ile integre zararlı düzenlemesinin uygulamaya konulmasını teşvik edecek gerekli şartların oluşumuna ve aynı zamanda ticari ürünlerin güvenliğinin sağlanmasına da çalışılmaktadır. *Trichoderma* spp. mikrobiyal biyokontrol ajanı olarak en yaygın kullanılan ve çalışılan funguslar arasındadır. *Trichoderma* ürünleri, biyopestisit, biyofungusit, biyoinokulant, biyo-stimulant, biyodekompoze edici, biyofertilize edici ve bitki büyüme teşvikleyicileri olarak kullanılmaktadır. Yararlı etkileri için bitkilerle muamele edilmelerinin altında yatan başlıca mekanizmalar; mikoparazitizm veya hiperparazitizm, antibiyozis, kompetisyon, hücre duvarlı litik enzim aktivitesi, bitki büyümesinin arttırılması, toprakta bulunan besin elementlerinin kazanımı ve bitki savunma cevaplarının indüklenmesi olarak sayılabilir. Bu derlemede, tarımsal üretimde yararlanılan *Trichoderma* türleri ve başlıca sekonder metabolitleri ile fungal patojenler veya bitkiler ile arasındaki interaksiyonlar üzerine odaklanılmıştır.

Anahtar Kelimeler: *Trichoderma*, biyokontrol, sekonder metabolit

Trichoderma–based Products and Metabolites Used in Agricultural Production

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Abstract: Phytopathogenic agents cause considerable crop losses incurred by plant diseases worldwide. The most goverments in World, have started to implement legislative mandates in order to reduce the pesticide applications and dependency in agriculture and increase the environmental and consumers safety. By implementing integrated pest management [IPM], it is aimed to establish the necessary conditions to employ these practices. *Trichoderma* is among the best known and widely used fungal genera as biocontrol agent. Products of *Trichoderma* and itself, are promoted as bio-pesticide, bio-fungicide, bio-inoculant, bio-stimulant, bio-decomposer, bio-fertilizer, plant growth stimulator. Main mechanisms underlying its beneficial properties are; mycoparasitism, hyperparasitism, antibiosis, competition, cell wall lytic enzymes, enhancement of plant growth, acquisiton of soil nutrients, induction of plant defense responses. In this review, we focused on the *Trichoderma* and its secondary metabolites that are used in agriculture and their interactions between phytopathogens and plants.

Keywords: *Trichoderma*, biocontrol, secondary metabolite

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1. GİRİŞ

Geleneksel olarak bitki koruma, bitki hastalıklarını ve böcekleri kontrol etmek amacıyla kimyasal pestisitlerin kullanımına dayanmaktadır. Ancak bu uygulamalar, son kullanıcı ve agro-ekosistem açısından, pollinatörlerin, yararlı predatör/parazitoidlerin ve faydalı mikrobiyal kommünitelerin inhibisyonu gibi negatif etkilere sebep olabilmektedir [1]. Sentetik pestisitler hem maliyetlidir, çevreyi kirletir hem de potansiyel olarak hayvanlara ve insanlara zararlıdır. Ayrıca uzun vadede sürekli olarak kullanımları, kimyasallara dayanıklı patojenlerin gelişimini teşvik etmektedir [2]. Sadece Avrupa'da, sentetik agro-kimyasalların yıllık tüketimi 250k ton olup, bunun 180 k tonu fungusittir [1].

Türkiye'de pestisit kullanımı daha çok polikültür tarımın yapıldığı Akdeniz ve Ege bölgelerinde yoğunlaşmaktadır. Entansif tarım yapılan bu bölgelerde pestisit kullanımının ülke ortalamasının çok üzerinde olduğu ve bu bölgelerde tüketimin gelişmiş ülkeler düzeyine ulaştığı söylenebilir. Yoğun pestisit tüketilen Ege ve Akdeniz bölgeleri, beslenmede büyük yeri olan sebze ve meyvelerin entansif biçimde yetiştirildiği alanlar olmasının yanı sıra, ihracata yönelik gıda endüstrisinin hammaddeleri de büyük ölçüde bu bölgelerimizden sağlanmaktadır. Türkiye'de pestisit tüketiminde sırasıyla en fazla fungusit (%45), herbisit (%18) ve insektisit (%15) yer almaktadır [3].

Tarımda zararlıların mücadelesinde mikroorganizmaların kullanımı, biyolojik mücadelenin en etkin stratejilerinden biridir. Yararlı mikroorganizmaları kullanmanın başarısı mikrobiyal ırka bağlıdır ve ilgili bitki ile olan avantajları şunları içermektedir: (1) Rizosferde antagonistik mikrobiyal komünitenin kurulması (2) Patojenlerin baskı altına alınması, (3) Bitki sağlığının iyileştirilmesi (4) Büyümenin teşvik edilmesi, (5) Artan besin elde edilebilirliği ve alınımı, (6) Biyotik ve abiyotik streslere karşı artmış konukçu direnci [4-6].

Mikrobiyal biyolojik kontrol ajanları biyopestisit, biofertilizer, büyüme teşvik ediciler ve doğal bağışıklığı stimüle ediciler olarak pazarlanmaktadır. *Trichoderma* temelli preparasyonlar, dünya çapında çeşitli bitki patojenlerine karşı ürün korunmasında veya çeşitli ürünlerde, örneğin tarla, sera veya bahçelerde bitki büyüme veya verimliliğini arttırma amaçlı kullanılmaktadır. *Trichoderma* genusu, *Ascomycota* filumundan *Hypocreales* ordosuna ait rhizo kompeten bir fungus olup, bütün Dünya'da çok çeşitli ekosistemlerde yayılış göstermektedir [7]. *Trichoderma* genusuna ait funguslar, bitki hastalıklarına kaşı 1930'lardan beri biyolojik kontrol ajanları olarak bilinmekte olup, 1990'lardan itibaren ticari tarımda bu amaçla hem gelişmiş hem gelişmekte olan ülkelerde oldukça yaygın kullanılmaktadır. Bununla birlikte, etki mekanizmaları ve potansiyel kullanımlarına dair bilgiler henüz yeterli değildir [8].

1.1. Trichoderma'nın fungal patojenler ve bitkilerle etkileşimleri

Trichoderma diğer mikroorganizmalarla fakat özellikle de patojenik funguslarla interaksiyon kurar. Bu interaksiyonlar hiperparazitizm, antibiyozis ve rekabeti içermektedir [9]. Hiperparazitizm, antagonistin br patojenle direkt kontak kurması ve patojenin tanıması, saldırı, dereceli penetrasyon ve ölümle sonuçlanan safhalara sahip bir ilişkidir [6].

Antibiyozis *Trichoderma* spp. tarafından yaygın olarak bitki fungal patojenlerine karşı kullanılan, halen tam olarak anlaşılamamış mekanizmaya sahip bir stratejidir [10]. Evrim sürecinde, *Trichoderma* türleri diğer fungal türler ile rekabet edebilecek özel mekanizmalar geliştirmiştir [11]. Bu tip rekabet aktiviteleri için genomik bilginin daha fazla depolanmasına gerek duyulmaktadır. Bu durum oldukça parazitik türler olan *T. virens* ve *T. atroviride*'nin genomlarının daha geniş olduğunu ve daha ılımlı türlere göre neden 3000'den fazla geni içerdiğini açıklamaktadır [12]. Rekabet ve mikoparazitizmde, çevresel anlamda miselyal gelişim için uygun olmayan durumlarda bile *Trichoderma*'nın ekstraselüler enzim sistemleri aktif kalabilmekte ve bu durum da daha iyi stres toleransı için strainlerin geliştirilebileceği

imkanını düşündürmektedir [13]. Biyokontrol ajanlarının farklı tarım topraklarında ve klimatik koşullarda verimli bir şekilde kullanımlarının olması için toprak sıcaklığı, radyal gelişimi ve rekabetçi kolonizasyonu etkileyen önemli bir parametredir. Doğal olarak var olan askomisetlerin maksimum büyüme sıcaklığı 30-35 °C aralığında iken, termotolerant *Trichoderma* strainlerinin en yüksek büyüme oranı 37 °C'dir [14]. *Trichoderma* spp. ve fitopatojenler arasında enfeksiyon bölgeleri ve besinler için, nişden uzaklaştırma olarak bilinen bir yarış vardır. *Trichoderma*'da kök yüzeyine adherens ve kolonizasyonun başlaması, hidrofobinlerle yürütülmektedir. Bunlar, fungal hücre yüzeyini çevreleyen, en dıştaki hücre duvarı tabakasının küçük hidrofobik proteinleri ve hücre duvarı gelişimi ile ilgili expansin-benzeri proteinlerdir. *Trichoderma*'ya karşı konukçu bitki cevabı potansiyel fungal penetrasyon bölgesinin ilerisinde gözlenmiştir. Bitki hücre duvarı güçlendirmesini indüklemeyen *Piriformosa indica* kolonizasyonundan farklı olarak, kalloz-zengin duvar appozisyonları epidermisin ve korteksin interselüler alanlara fungal büyümenin sınırlandırılmasında ve vasküler içine doğru girişinin engellenmesinde oldukça etkin olduğu gözlenmiştir. Bitkiler aynı zamanda fungal invazyona, antimikrobiyal bileşikleri sentezleyerek ve akümüle ederek karşılık verir. Her strainin bitki köklerini kolonize edebilme yeteneği bu bileşikleri tolere edebilme kapasitesi ile bağlantılıdır. *Trichoderma*'da bu rezistan, ABC taşıyıcı sistemlerinin varlığı ile ilişkilidir. Bu sistem potansiyel olarak toksik veya antagonistik bir ortamda *Trichoderma* biyo kontrol strainleri tarafından diğer mikroorganizmalar ile kurulan çoklu interaksiyonlarda anahtar faktördür. Bitkilerden salınan fenolik bileşiklerin hızlı degrade edilmesinde, fitoaleksinlerin üretiminin supresyonu da rol oynamaktadır [15]. Son zamanlarda yapılan proteomik bir çalışmada, farklı *Trichoderma* spp. lerdeçeşitli bitki ve fungal patojenlerle interaksionda, birçok sayıca ABC taşıyıcı genlerin upregüle edildikleri ve bunun da hem antagonistik aktiviteyi hem de kök kolonizasyonunu desteklediklerini belirlenmiştir [16]. Ruocco et al (2009) tarafından, *Trichoderma* genusundan ilk kez ABC taşıyıcı geni tam olarak dizilenmiş ve karakterize edilmiş ve *Taabc2* olarak isimlendirilmiştir. *H. atroviridis*'in ABC taşıyıcı kodlayan genlerinin birinde gerçekleştirilen knock-out denemesinde, kontrol denemesine göre *R. solani*'nin biyokontrolünde düşüş olduğu, bu sonucun da mikoparazitizmde detoksifikasyon proseslerinde rolünü desteklediği de gösterilmiştir [17]. *Trichoderma* spp.'nin ekolojik başarısının altında yatan anahtar faktörlerden biri, ekzojen ve endojen toksik bileşiklere dayanıklılığıdır [18]. Bu funguslar, metil bromid ile sterilizasyon sonrası dahi, toprağı rekolonize edebilen ilk mikroorganizmalardır. Bu anlamda Entegre Zararlı Düzenlemesi Uygulamaları kapsamında fungal bitki hastalıklarının kontrolünde, biyolojik ve kimyasal metodların birleştirilmesinde önerilmektedir. Ayrıca, birçok ticari olarak uygulanan *Trichoderma* strainlerinin çeşitli fungusitler ile örneğin; Captan, Chlorothalonil, Iprodione, Thiophanate methyl, Mtalaxyl, Chloropyrifos, Vinclozolin gibi, uyumlu oldukları bildirilmiştir [17].

Trichoderma genusunun bazı izolatları iyi bilinen biyokontrol ajanları olarak birçok fungal patojene karşı örneğin; *Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum, Sclerotium* spp.*, Pythium ultimum, Phytophthora* spp.*, Armillaria* spp.*, Fusarium oxysporum, Verticillium* spp. ve *Gauemannomyces graminis* gibi, antagonistik kapasiteye sahiptir. Biyokontrolde en çok kullanılan türler, *T. harzianum, T. atroviride, T. asperellum, T. polysporum, T. viride*'dir. Biyolojik ürünlerin büyük çoğunluğu *T. harzianum* (%83) ile kombine *T. viride* (%55) ve *T. koningii*' (%28)'den oluşmaktadır [1].

Trichoderma strainleri birçok kök ekosisteminde gelişir. Kökten salgılanan müsijel tabakanın oldukça hidrate polisakkaritleri ve bitki köklerince rizosfere salgılanan mono ve disakkaritler, fungusun büyümesini teşvikler. Bitkiden kaynaklanan sükroz, *Trichoderma* hücrelerine kök kolonizasyonunu kolaylaştırmada, savunma mekanizmalarının koordinasyonunda ve yaprak fotosentezinin artmasında önemli bir kaynak oluşturur. Kök

eksüdalarının kazanılmasında iş gören solut taşıyıcılar örneğin; di/tripeptid taşıyıcı ve bir permeaz/intraselüler invertaz sistemi *Trichoderma*'da tanımlanmıştır [15].

Trichoderma uygulamalarının, kök büyümesinin teşvik etmesi, aynı zamanda toprak üstü vejetatif büyümenin örneğin gövde uzunluğu ve kalınlığı, yaprak yüzeyi, klorofil içeriği ve verimi anlamında da dikkat çekici etkisi olduğu görülmüştür. Bu gözlemleri açıklayabilmek için birçok hipotez önerilmektedir. Örneğin; kimyasalların çözünürlüğü, metal bağlayabilme/elde edilebilirliği [siderofor üretimi], bitki tarafından besin alınımını arttırması, aynı zamanda fito hormonların da birlikte etkisi öneriler arasındadır. Fungus aynı zamanda çevreyi asitleştirerek de konukçu bitkiye yardımcı olur. *Trichoderma* zararsız bir şekilde köklerin dış yüzeyini kolonize ettiğinden, bitkinin sinyal yolaklarını aktive eden savunma mekanizmalarının dikkatini çeker. *Trichoderma* tarafından sağlanan bu dayanıklılık, patojene karşı daha hızlı ve güçlü yanıt verebilen savunma mekanizmalarının akışını tetikler [4].

1.2. Trichoderma metabolitleri, bitki yapısı ve metabolizmasına etkileri

Fungal metabolitler, bitkilerle interaksiyonlarda önemli rol oynar. Bitki patojenlerinin bir bölümü virülensliklerini [kısmen veya tamamen] konukçu-spesifik toksinlere borçludur. Analitik sonuçlara göre, *Trichoderma* spp., rapor edilen 100'den fazla bileşiğin profilik olarak üreticisidir. Bunlar içerisinde, düşük moleküler ağırlıklı non-polar bileşikler örneğin, pironlar, terpenoidler, steroidler ve poliketidler yer almaktadır. Diğer Askomisetler ile benzer olarak, non-ribozomal peptidleri örneğin; epipolythiodiozopiperazinleri [ETP' ler] ve sideroforları üretmektedir. Bu genusun bilinen üyeleri Peptaiboller olarak bilinen peptaibiyotiklerin bir alt grubunu da üretmektedir [19].

1.2.1. Virulans faktörleri

Bitkiler patojen-türevli molekülleri [elisitör veya virulans faktörleri de denilen] tanıdıkları zaman savunma cevapları tetiklenir. Patojenler bu savunma cevapları ile bitki tarafında tanınmadan veya konukçu savunmasını nötralize den cevaplarla başarılı olur. Tanınmadan kaçış mekanizmasına en iyi örnek domates ve patojenik fungus *Cladosporium fulvum* arasında gösterilmiştir. Domates *Cf* dayanıklılık gen ürünleri, fungal virulans geni [*Avr*] ürünlerinin tanınması işini, 'gen-gen için' anlamında yönetir [20]. *Trichoderma* spp.'de ve aynı zamanda diğer mikoparazitik türlerde sekrete edilen birçok 'saldırgan' bileşik birçok sayıda farklı enzimleri [özellikle kitinazlar, glukanazlar, proteazlar ve selülazlar], 200' den fazla antibiyotik ve mikotoksinleri içermektedir [21-24].

Proteom analizlerinde, 'Küçük sekrete sistein zengin proteinler' [SSCP], daha önce *Cladosporium fulvum*'dan tanımlanan virulans protein olan Avr4'ün homoloğu olarak, *T. harzianum* ve *T. atroviride*'den identifiye edilmiştir [5]. *C. fulvum*'dan Avr4 virulans genini taşıyan *Trichoderma* transformantları, Cf4 rezistan genine sahip domates bitkilerinde, konstitiütif ve indüklenebilir promotorların kontrolünde test edilmiştir. *Cladosporium*-domates interaksiyonunda beliren semptomlara benzer nekroz ve suberifikasyon zonları gözlenmiştir. Bu durum, *Trichoderma* strainlerinin metabolizmayı, hastalık dayanıklılığını belirgin şekilde etkileyen bitki moleküllerini transfer edebildiğini göstermiştir. Bu nedenle, bu yararlı funguslar kullanışlı bileşiklere sahip ürün çeşitlerinin elde edilmesinde biyoteknolojik araçlar olarak değerlendirilebilir. Ayrıca, *in vitro* kompetisyon testlerinde, transformantlar, yabani tipe göre *A. alternata* antagonistleri olarak çok efektif bulunmuşlardır [17].

Avr4'ün fungusları bitkisel kitinazların parçalayıcı etkisinde koruyabildikleri, bitki kitinazları ve β-1,3 glukanazlarına karşı duyarlı *T. viride* ve *F. solani* f.sp. *phaseoli* strainleri ile gösterilmiştir. 0.3 μM ChiI varlığında hemen tüm *T. viride* sporları 24 saat içerisinde parçalanırken, kitinaz ilavesinden önce Avr4 ilave edilmiş *T. viride* sporlarının büyümesi

ChiI'ye karşı korunmuştur. Avr4'ün kitine bağlanmasının, *Trichoderma*'yı bitkisel kitinazlara karşı koruyabileceği belirtilmiştir [20].

Önceden oluşturulmuş fiziksel ve kimyasal bariyerlere ilaveten, bitkiler mikroorganizmalardaki korunmuş yapısal özellikler ve domainleri tanıyabilecek bir immün sisteme sahiptir. Bunlara 'patojen veya mikroorganizma ilişkili moleküler patternler' adı verilmektedir (PAMP veya MAMP'ler). MAMP tetiklemiş bitki cevapları hızlı ve geçici olarak yönetilir. Erken MAMP cevapları plazma membranı boyunca iyon akışlarını içermektedir. Bugüne kadar birçok bitki-faydalı funguslarca üretilen MAMP üreticisi *Trichoderma* strainleri bulunmuştur [25]. İlk tanınan *Trichoderma* MAMP'ı, ET- indükleyen ksilanaz (Xyn2/Eix) olup, *Trichoderma* tarafından spesifik domates ve tütün kültivarlarında bitki savunma cevaplarının potansiyel elisitörü olarak üretilmektedir. Bitkilerce tanınan Eix epitopu, enzim aktivitesinde iş görmeyen 5 adet yüzeye maruz aminoasitten oluşmaktadır. *Trichoderma* aktifleştirilmiş veya ısı ile denatüre edilmiş selülazlar SA ve ET sinyal yolizlerinin aktivasyonu ile kavunda savunmayı kolaylaştırmaktadır. Kök kolonizasyonunda iş gören *Trichoderma* proteinleri aynı zamanda MAMP olarak da iş görmektedir. Swollenin TasSwo salatalık kökleri ve yapraklarında savunma cevaplarını teşvik eder ve fungus ve bakterilere karşı lokal korumayı sağlar. SSCP cerato-platanin aliesinin ortologları – *T. virens*' ten Sm1 ve *T. atroviride*' den Epl1- kolonizasyon sırasında hifte akümüle olur ve mısır ve pamukta MAMP olarak iş görür [15].

Kitin polisakkaritleri de bitkilerde savunma cevaplarının elisitörleri olarak iş görür. Böyle oligomerlerin süpürülmesi fungal patojenler için konukçularını kolonizasyonunu takiben hayatidir [26]. Kitinin algılanması için bir mekanizma olarak bitkiler, istilacı fungusların hücre duvarlarından aktif polimerlerin açığa çıkmasını sağlayacak kitinazları geliştirmişlerdir. Bu şekilde savunma cevaplarını tetikleyebilmektedirler. Böylece, *Trichoderma* kitinazlarının mikotrofik aktivitesi kitooligosakkaritlerin salınmasına ve indirekt olarak savunma mekanizmalarının indüksiyonuna katkı sağlayabilmektedir [15].

T. atroviride'nin endo (*ech*42) ve exo- (*nag*70) kitinazlarının elmada ko-ekspresyonu, *Venturia inaequalis*'te artmış rezistan ile korele edilmiştir. Benzer şekilde, iki kitinaz (*ech* 42 ve *nag* 70) ve bir B-1,3- glukanaz (*gluc*78) kodlayan pirinç transgenlerinin çoklu ekspresyonu, pirinçte *Rhizoctonia solani* ve *Magnaportshe grisea*'ya karşı artmış rezistan ile sonuçlanmıştır [27].

Trichoderma tarafında üretilen bazı sekonder metabolitler yüksek dozlarda antimikrobiyal etki gösterir fakat düşük konsantrasyonlarda MAMP' ler ve auxin benzeri bileşikler olarak iş görür. 1 ppm de 6- pentil piron, harzianolid ve harzianopiridon bitki savunma mekanizmalarını aktive eder ve bezelye, domates ve kanolada bitki büyümesini düzenler [6].

Primer metabolik prosesler açısından esansiyel olmamakla birlikte, mikroorganizmalar özellikle de funguslar, endüstriyel ve ekonomik öneme sahip çeşitli sekonder metabolitleri (SM) üretirler. SM' ler kimyasal olarak düşük moleküler ağırlığa sahip (< 3kDa), genellikle mikroorganizmalar ve bitkilerce üretilen, tipik olarak üretici genus, tür veya ırklar ile ilgili, değişik doğal bileşiklerdir. SM' ler özelleşmiş yol izlerinde örneğin Asetil CoA'dan türevlenen mevalonat yolizleri veya aminoasitler gibi primer metabolitlerden üretilir ve bazı genler ile birlikte küme halindedir. Bu genlerin ekspresyonu bir veya birkaç global regülatör tarafından indüklenmektedir. SM' ler muhtemelen organizmanın yaşamsal fonksiyonları ile ilişkili çeşitli biyolojik aktiviteler gösterir örneğin; diğer mikro ve makroorganizmalara karşı yarışma, simbiyozis, metal transportu sayılabilir. Fungal SM' ler insan tedavisinde de, önemli ilaçlar olarak örneğin; antibiyotikler, immunosupresan (siklosporin), antihiperkolesterolomik ajanlar (lovastatin ve kompaktin) şeklinde kullanılmaktadır. Peptaibollerin mitokondriyal ATPaz inhibisyonunu, oksidatif fosforilasyonun ayrılmasını teşvik ettikleri, immunosupresyon,

platelet agregasyonunun inhibisyonu ve fungal morfojenezisi indükledikleri rapor edilmiştir [27, 28].

Funguslarda SM' lerin üretimi morfolojik farklılaşmanın spesifik safhalarına ve aktif büyümenin fazlarına korelasyon göstermektedir. Dağılım aralığı belirli sayıda türden tek bir straine göre değişiklik göstermektedir. Bu durum evrensel bir fonksiyonu olmadığını göstermektedir. Çeşitli biyolojik fonksiyonlar gösterirler ve organizmalar arasında etkileşimleri düzenlemede önemli rol oynarlar. Bazı örnekleri; fitotoksinler (bitkilere saldıran fungal patojenlerce üretilen SMler], mikotoksinler (ürünleri kolonize eden funguslarca üretilen insan ve diğer hayvanlarda hastalık ve ölüme sebep olabilen SMler], pigmentler (aynı zamanda antioksidan aktiviteye sahip renkli bileşikler) ve antibiyotikler (mikrobiyal kompetitörleri öldürme veya inhibe etme yeteneğine sahip doğal ürünler) dir. Bununla birlikte biyolojik aktiviteler spesifik bir grup veya tek metabolitler ile sınırlandırılamaz. SM'lerin bolluğu ve çeşitliliği, genomda bulunan biyosentetik genlerin varlığına ve onların ekspresyonunu indükleyen koşullara bağlıdır. SM biyosentezi, intrinsik veya eksternal koşullara cevaben açılıp kapanabilmektedir. Daha ilginç olarak, fungal SMler, bitkilerin büyüme ve metabolizmasını modifiye edebilmektedir [29].

Çeşitli biyolojik aktivitelerde rol alan mikrobiyal metabolitler tahıl üretiminde önemli sonuçlara sahiptir. *F. graminearum* tarafından oluşturulan Buğday Kök Boğazı Çürüklüğü'ne karşı biyolojik ajanlar olarak *T. harzianum* ve *T. virens* strainleri ile yapılan çalışmada, *F. graminearum* B-63 strainin miselyal büyümesinin, *T. harzianum* ve *T. viride* strainleri tarafından üretilen volatil metabolitlerce sırasıyla %23,12 ve %43,22 oranlarında düşürüldüğü bildirilmiştir [30]. *T. harzianum*'dan elde edilen volatil ve non volatil bileşiklerin de biberlerde *Colletotrichum capsici* miselyal büyümesini kontrol grubuna göre %30-67 oranında inhibe ettiği bildirilmiştir [31].

2.2.2. Peptaiboller

Peptaiboller, proteinojenik olmayan aminoasitlerce (ör; α- aminoisobütirik asit ve isovalin) zengin, lineer, N-terminal grup asetillenmiş, C ucunda aminoalkol bulunan (ör; fenilalaninol, valinol, lösinol, isolösinol veya triptofanol içeren), hücre degrade edici enzimlerle özellikle de kitinaz, glukanaz ve peptidazlar ile sinerjitik etki gösteren, özellikle toprak kökenli ve bitki patojenik türlerce mikroheterojen bir doğada yoğun olarak sentezlenen, membran modifiye edici veya por oluşturucu peptidlerdir [23,32]. 1966'da izolasyonlarından beri, hücre membranlarını parçalama yoluyla patojenlerle mücadelede anahtar molekül olarak tanımlanmışlardır. Antimikrobiyal ve anti-kanser özellikleri ile aynı zamanda mikrobiyal saldırıya karşı sistemik rezistansı indükleyebilme yeteneklerinden dolayı ticari ve ekolojik anlamda önemlidir. Lorito et al. konukçu fungusta β- glukan sentaz aktivitesini inhibe ettiklerini, *T. harzianum* β- glukanazları ile sinerjitik olarak etki ettiklerini göstermiştir. Glukan sentazın inhibisyonu, patojen hücre duvarlarının yeniden oluşturulmasını önlemiştir böylece β-glukanazın bozucu etkisini kolaylaştırmıştır. Petaibiyotik ailesinin ilk üyeleri, Suzukacillin ve Alamethicin, 1960'ların sonlarında *Trichoderma* strainlerinden izole edilmiştir. En geniş çapta bilinen peptaibol *T. viride* Alamethicin'idir. Alamethicin, *T. viride* tarafından üretilen, her biri 20 aminoasit rezidüsü içeren 12 bileşikten oluşan bir karışımdır. Bu peptaibolün, lima fasulyesinde, homoterpenlerin ve metilsalisilatın üretimini kolaylaştırdığı gösterilmiştir. Eksprese edilen bütün peptaibollerin spektrometrik yöntemlerle örneğin; LC/ESI-MS veya bütün hücre MALDI-TOF yöntemiyle analizi gerçekleştirilmektedir. Peptaiboller genellikle üretici strain ile isimlendirilir örnek olarak; atroviridin, harzianin, longibrachin, koningin verilebilir [33].

Büyük multifonksiyonel peptid sentetazlar olarak bilinen enzimler peptaibolleri biraraya getirir. *Trichoderma* genomlarında 18 ve 14 modülleri olmak üzere 2 peptaibol sentetaz

bulunmaktadır. Gen bozundurma kullanılarak, 18-modül peptaibol sentetaz *Tex*1 in trichovirinII-tip-18 rezidü peptaibol üretiminden sorumlu olduğu gösterilmiştir. Öte yandan, 14 modül enzimi *T. virens*'te 14-birim ve 11- birim rezidülü peptaibolleri birleştirir. Özellikle ilgi çekici olan tek bir sentetazın çok sayıda peptaibol üretebilmesidir. Örneğin, *T. virens*'in 14-modül NRPS'si, 11 ve 14 rezidülü en azından 88 non-ribozomal peptid üretmektedir [34]. Bu peptidlerin birçoğu yeni form olarak bulunmuştur. Bu durum da, zirai ve ticari uygulamalar için ticari yararlanmada çok sayıda fırsat çeşitliliğinin olduğunu göstermektedir [19].

Peptaiboller amfipatik doğadadır ve membranlarda voltaj-bağımlı iyon kanalları oluşturmak üzere kendi kendilerine biraraya gelirler. Bu yetenek, genellikle bu bileşiklerin antibiyotik özelliklerinin altında yatan temel sebeptir. Peptaibiyotikler ve onların üreticileri sadece akademik araştırıcılar anlamında değil, aynı zamanda endüstride, pazarlanan antibiyotiklere direnç ve bitki korumada sentetik pestisitlere alternatif kullanım kaynakları olarak da ilgi çekmektedir. O zamandan beri sürekli olarak artan sayıda peptaibiyotik belirlenmiş, tanımlanmış ve yakın zamanda 'Kapsamlı Peptabiyotik Veritabanı-Comprehensive Peptaibiotics Database'de özetlenmiştir [32]. Bu ücretsiz olarak elde edilebilir veritabanı geçerli olarak 1043 peptaibiyotik bilgisi içermektedir. Peptaibiyotik bilgisi olarak peptid kategorisi, peptidin ait olduğu grup ismi, aminoasit sekansı, sekans uzunluğu, üreticisi fungus, peptid yapısal formülü, moleküler formülü ve monoizotopik kütle verilmektedir. Tahminlere göre yaklaşık olarak Dünya üzerinde 1,5 milyon fungal türün olduğu, bunun ancak 90 bininin şu ana kadar tanımlanabildiği bildirilmiştir. Özellikle Dünya üzerinde keşfedilmemiş bölgelerde ve ekolojik nişlerde yeni fungal türlerin keşfi ve karakterizasyonları ile yeni üreticiler, yeni bileşenler ve dolayısıyla yararlı biyomoleküller ortaya çıkarılabilme potansiyeli oldukça yüksektir [35].

T. virens'ten Trichovirin II peptaibolünün rolü, salatalık bitkilerinde gösterilmiştir. *Tex* 1 geni bozulmuş mutantlarla büyüyen bitkiler, foliar patojenlere karşı düşük sistemik rezistan ve düşük fenolik bileşik üretimi göstermiştir. Ayrıca 2 izoformu, sistemik korumayı indükleyebilmiş ve savunma genlerinin üst regülasyonunu gerçekleştirebilmiştir. Bu bileşikler, patojen yapılara girişi teşvik eden hidrolitik enzimlerle sinerjitik etki gösterebilir, bu durum da bitki patojenlerinin antagonizminde rolü olduğunu düşündürmektedir [21].

1.2.3. Poliketidler

Poliketidler, birçok organizma ve aynı zamanda filamentöz funguslarca üretilen SMlerden bir gruptur. Birçok poliketid klinik açıdan antimikrobiyal, anti kanser ve immunosupresif özellikleri nedeniyle önemlidir. Bunun dışında, üretici organizma açısından, substrat için rekabeti ve organizmalar arası iletişimi kolaylaştırdığından dolayı önemlidir [36]. *Trichoderma* genomları PKSler açısından zengindir. gliP ve diğer sekonder metabolizma-ilgili genler gibi, *T. virens*'te PKSler velvet protein kompleksi Vel1 ile regüle edilir PKS geninin mikoparazitizmdeki muhtemel rolü gösterilmektedir [37].

Poliketid sınıfına dahil 10 üyeli laktonlar, Malimerca, ve ark. tarafından (2015), *tri*-5 geni bozulmuş *T. arundinaceum* mutantından izole edilmiştir. Mikroorganizma yabani-tip strainde saptanamayan Aspinolid B ve C ve 4 yeni aspinolid üretmiştir. Aspinolid C *B. cinerea* ve *Fusarium sporotrichoides*'e karşı antibiyotik etkiye sahiptir, Aspinolid B'nin bu iki bitki patojenine karşı bir aktivitesi gösterilememiştir Aspinolid B nin 50 µg/ml oranında domates tohumları ile muamele edilmesi yan köklerin proliferasyonunu indüklemişken, Aspinolid C'nin kontrole göre yan köklerin sayısında ve filizlerin boyunda önemli bir düşüşe neden olduğu belirlenmiştir. Ayrıca Aspinolid C'nin daha yüksek konsantrasyonları [250 µg/ml] bitkinin gelişiminde önemli bir indirgemeye neden olmuştur. Sonuç olarak Aspinolid C domateste domates savunma ile ilgili genlerin aktivasyonunda rolü olan SA- ilgili genlerin ekspresyonunu indüklemiştir [2].

6-pently-2H-pyran-2-on (6-PP) farklı *Trichoderma* türlerinin kültür filtratlarından genellikle saflaştırılmış olan bir metabolittir. Aksenik olarak geliştirilmiş kolonilerde açığa çıkan Hindistan cevizi aromasından sorumlu, biyokontrol açısından en çok çalışılmış SMlerden biridir [6]. 6PP'nin hem *in vivo* hem *in vitro* antifungal aktiviteleri gösterilmiştir. Bitki büyüme teşvik edici aktiviteleri bulunmaktadır. 6-PP için biyosentetik yolizi tam olarak aydınlatılamamıştır. Fakat *T. atroviride*'ye özgü lipooksijenaz geni içerilmektedir Bu bileşik *R. solani, F. oxysporum* f. sp. *lycopersici, B. cinerea* ve *F. moniliforme*'ye karşı geniş çapta antimikrobiyal karakteristikler göstermiştir. Cytosporon S *in vitro* olarak çeşitli bakterilere ve funguslara karşı antibiyotik aktiviteli olarak gösterilmiştir [12].

1.2.4. Peptidler

Gliotoksin ve Gliovirin ETP peptid sınıfına aittir. Gliotoksin, *Trichoderma*'dan tanımlanan ilk metabolittir. Fungistatik bir molekülür ve *Rhizoctonia* antagonizminde belirtilmiştir. Toprak kökenli fungal patojenlerin biyo kontrolünde oldukça dikkat çekmiştir. Bununla birlikte, kültürel ve çevresel koşullarda biyo kontrolde önemine dair karşıt görüşlü raporlar bulunmaktadır. Buna rağmen, oldukça güçlü bir antimikrobiyal olarak, gliotoksinin doğal koşullar altında mikrobiyal rekabetteki önemi göz ardı edilemez. Gliotoksin, *T. virens*'in 'Q' strainlerince üretilir ve *T. virens*'in 'P'strainleri ile *T. atroviride*'de bulunmaz. Gliotoksin üretemeyen *T. viride* 'P' strainleri, onun yerine Gliovirin üretmektedir. Gliovirin, antimikrobiyal etkiye sahip bir diğer ETP bileşiğidir. Fungistatik ve antikanser sterodial bileşik olan Viridin, *T. virens*'in hem 'P' hem 'Q' strainlerince üretilmektedir. Deneysel bulgular Viridinlerin, furanosteroidlerin bir grubu olduğunu, *T. virens* tarafından Viridiol'e indirgendiğini ve Viridiolun herbisidal özelliklere sahip olduğu belirtilmektedir. Terpenoidlere ilaveten diğer uçucu metabolitler katı-faz mikroekstraksiyon ile *T. atroviride* kültürlerinden saptanmıştır Trichodermin *T. brevicompactum* tarafından üretilen oldukça fungitoksik ve fitotoksik terpenoid bir toksindir. Üretiminden sorumlu olan gen Trichodien sentaz (*tri*5) geninin aşırı ekspresyonu *T. brevicompactum*'da Trichodermin'in aşırı üretimi ile sonuçlanmıştır [38].

Fungal SM üretimi genellikle aseksüel sporulasyon ile koreledir. Işık sinyalleri, 'velvet' kompleksi ve baş regülatör *Lae*A *Aspergillus*'ta konidiasyon ve sekonder metabolizmayı birlikte regüle etmektedir. *T. virens*'te *vel*1 geninin rolü knockout mutantlarla çalışılmıştır. v*el*1'in delesyonu katı ortamda konidiumların, zengin ortamda klamidosporların üretimini bozmuştur. v*el*1 ayrıca *gli*P geninin biyosentezinde ve diğer sekonder metabolizma genlerinin düzenlenmesinde iş görmektedir [19].

1.3. Trichoderma'da çevresel koşullara uyum ve dayanıklılık

Trichoderma toprak besleyicilerini taşıma ve alma kapasitesi açısından daha güçlüdür ve bu durum onu diğer toprak mikroorganizmalarından daha etkin ve yarışmacı yapmaktadır. Bu proses aynı zamanda organik asitlerin örneğin; glukonik, sitrik ve fumarik asitler gibi, üretimleri ile de ilgilidir ve toprak pH'ını düşürerek fosfatların, demir, manganez ve magnezyum gibi mineral katyonların ve mikronutrientlerin çözünürleşmesine imkan verir. Mikrobiyal aktivitelerin yoğun olduğu özellikle rizosfer çevresinde mikrobiyal rekabette demir kazanımı önemli bir bileşendir. Hücre içi siderofor Ferricrocin demirin depolanmasından sorumludur ve oksidatif stresten hücrelerin korunmasında rol oynar [39]. Sideroforlar demir sınırlaması olduğunda ($<10^{-6}$ mol/L) Fe^{3+}'ya bağlanabilen ve böylelikle diğer fungusları durdurarak etki gösteren taşıyıcı proteinlerdir [40]. Siderofor salgılayabilen mikroorganizmalar, demirce fakir doğal ortamlarda rezidüel olarak demiri kullanarak büyüme özelliğine sahiptir. Birçok fungus çeşitli sideroforlar üretir ve bu olumsuz koşullarla başa çıkabilir. Mikrobiyal sideroforların üretimi bitkiye 2 yönlü fayda sağlayabilir: (i): sideroforlar bitki için elde edilebilir olmayan demiri çözünürleştirir (ii): patojen olmayan

mikroorganizmalarca siderofor üretimi bitki patojenlerinin büyümesini, demir kaynaklarından onları mahrum bırakarak suprese edebilir [41].

İntrasellüler siderofor Ferrirocin, demirin depolanmasından ve hücrelerin oksidatif stresten korunmasında rol oynamaktadır. *Trichoderma* spp.'nin fusigen ve koprogen ailelerinin ekstrasellüler sideroforları ürettiği bilinmektedir. *Trichoderma virens* ve *T. reesei*'nin her ikisinin 2 putatif gen kümeleri içerdikleri ve ortologlarının (*sid*D ve *NPS*6) siderofor biyosentezinde rol oynadıkları bilinmektedir. Öte yandan, *T. atroviride* genomu, sadece *NPS*6 ortologunu içerir. Bu ekstraselüler sideroforların biyokontrol özelliklerindeki rolleri, mikrobiyal rekabet ve biyokontrolde siderofor-yardımlı demir kazanımının önemini aydınlatmaya yardımcı olacaktır. Demir için bu rekabetin, *T. asperellum* tarafından *Fusarium* domates solgunluğunun kontrolünde önemli bir rol oynadığı gösterilmiştir [42].

1.4. Trichoderma formülasyonları ve tarımsal üretimde kullanımları

Uluslararası pazarlarda piyasaya sunulan *Trichoderma* içeren ürünler, 250'den fazla olup son beş yılda eksponansiyel bir şekilde büyümektedir *Trichoderma* biyolojik formülasyonları dünya çapında bütün coğrafik bölgelerde büyük bir dağılım göstermektedir. Asya pazarında, *Trichoderma* biyolojik ürünlerinin %90'ını Hindistan üretmektedir. Ancak bunların sadece %1'i mikrobiyal fungusit olarak kayıtlıdır [1]. Bu ürünler hastalıkların gelişimini azaltır, bitki büyümesini stimüle eder, stres rezistansını arttırır ve gübreleşmeyi hızlandırıcı etki gösterir [4]. Azaltılmış dozda fungusid ile birlikte uygulamaların bitki sağılığını koruyucu ve arttırıcı etkileri olduğu, kültivasyon maliyetlerini önemli ölçüde aşağı çektiği ve çevresel anlamda pozitif etkilerinin olduğu da vurgulanmaktadır [43, 44]. Uygulamada canlı fungal sporlar çeşitli formülasyonlarda, yüzeysel spreyler ile, ekim öncesi uygulamalarla, budama sonrası muamelelerle, tohumlama sırasında ilaveler ile, sulama ile veya kök drenajı gibi hem geleneksel hem yenilikçi yaklaşımlarla birleştirilerek yapılabilmektedir.

Trichoderma'nın tarımsal üretimde kullanımı ticari pazarlarda yer alan ürünlerle sınırlı değildir. Fungal sporlar katı kültür fermentasyon yöntemiyle steril pirinç, mısır ve diğer tanelerde üretilebilir ve daha sonra ürünlere veya toprağa *Trichoderma* ile kolonize olmuş substratın kullanımı ile direkt olarak uygulanabilir yada sporlar elenerek ve suda tekrar süspande edilerek uygulanabilir. Birçok *Trichoderma* formülasyonları ıslatılabilir tozlar şeklinde ticarileştirilmiştir. İkinci olarak en sık kullanılan formülasyon, granüler daha sonra ise sıvı ve *Trichoderma* sporlanıncaya kadar büyümesine destek olacak katı substratı da içeren formülasyonlar gelmektedir. Bir diğer teknik *Trichoderma*'nın sıvı kültürde üretimini içerir. Burada sporlar, miselyum, litik enzimler, metabolitler vd. gibi fungal bir karışım direkt olarak biyolojik kontrol için tarlaya uygulanır. Venezuela ve Küba gibi bazı ülkelerde *Trichoderma* temelli ürünlerin kullanımı ve geliştirilmesi hükümet destekli ve resmi olarak teşviklenen bir tarımsal uygulamadır [1].

Biyokontrol ürünlerinin sunulması, modern teknolojileri içeren araştırmalar ile örneğin; kitinaz aktivitesi ekspresyonu arttırılmış Yeşil Floresan protein (GFP) mutantlarının oluşturulması gibi, ilerletilmektedir. Günümüzde *Trichoderma* temelli biyokontrol ürünlerinin geliştirilmesi, daha geniş çapta sebzelerin örneğin soğan, havuç, yonca, kırmızı pancar, dereotu, turp gibi kapsamlı korunmasında ve daha küçük çapta da zirai ürünler korunmasında yer almaktadır [9].

Ülkemiz Gıda Tarım ve Hayvancılık Bakanlığı Gıda ve Kontrol Genel Müdürlüğü Bitki Koruma Ürünleri Daire Başkanlığı veri tabanında belirtildiği üzere (https://bku.tarim.gov.tr/), *Trichoderma asperellum* ırk ICC 012 ve *T. gamsii* ırk ICC 080 aktif içeriğe sahip ithal ürünün, fungusit olarak biber, çilek, domateste ruhsatlı kullanımına izin verilmektedir. 2009 yılında, bitki koruma ürünlerinin piyasaya arzı ile ilgili 1107/2009 No'lu düzenleme ve 'Kimyasal mücadeleye alternatif teknikler ve entegre mücadele yöntemlerinin kullanımının teşviki ile

pestisitlerin sürdürülebilir kullanımını' sağlamak ile ilgili olan 2009/128/EC No'lu direktifin yayınlanması Avrupa Birliği'nde bir dönüm noktası olmuştur [3].

Bu mevzuatların yürürlüğe girmesi pestisit kullanımı ile ilgili riskleri azaltmak için alternatif yaklaşım ve teknikleri kapsayan "Entegre Mücadele Yönetiminin" benimseneceği anlamındadır. Bu yeni politikanın belirlenmesi ile birlikte Avrupa Birliği'nde "Pestisit Riskleri ve Kullanımının Azaltılmasına" yönelik çalışma ve araştırmaların ön plana geçeceği düşünülmektedir.

Ekonomik anlamda ticari pazarda rekabetçi olabilmek, kimyasal ürünlere eşdeğer veya daha üstün etkinliğe sahip olabilmek için, *Trichoderma*'nın yeni formülasyonlarının geliştirilmesine ihtiyaç vardır. Endüstriyel üretim maliyetleri, gelişmiş katı ve sıvı fermantasyon prosesleri kullanılarak önemli derecede düşürülebilir. Gıda üretim ve işleme endüstrilerinden geri dönüştürülen materyallerden veya pirinç gibi düşük maliyetli taneler üzerinde fungal spor üretimi, yüksek teknolojik ekipmanlara, yüksek kalifiye işçilere ve kompleks araçlara gerek kalmadan gerçekleştirilmektedir. Bu üretimlerin, tarımsal üretimi sınırlandıran patojenlerle mücadelede ve bitki gelişimine yardımcı olmada, biyoaktif metabolitlere dayanan yeni pestisitler ve biofertilizerlerin gelişime katkı sunmasına yardımcı olacağı görülmektedir.

2. SONUÇ

Yapılan çalışmalar *Trichoderma* orjinli bileşiklerin, *Trichoderma*-patojen interaksiyonunda antifungal, aynı zamanda bitki büyüme promotorları olarak davrandıklarını göstermiştir. Bitki metabolizmasını etkileyen böyle bileşikler, yararlı mikroorganizmalar ve diğer mikroorganizmalarla rizosferde kurulan bu birliktelikler, çevresel etkileşimlerde önemli rol oynayabilmektedir. Kimyasal ve biyolojik özelliklerinden dolayı, medikal, farmasötik ve zirai amaçlarla kullanım alanlarına sahip, antibiyotiklerden uçucu ve uçucu olmayan bileşiklere kadar geniş bir aralıkta çeşitlilik gösteren SM' lerin çeşitliliği ve üretim miktarları, genomdaki biyosentetik genler ve onların ekspresyonunu indükleyecek koşullar ile bağlantılıdır.

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SUMMARY

Purpose

Conventional agriculture/crop production is based on the use of chemical pesticides in order to control plant diseases and pathogens. However, these applications may have adverse effects from the point of end-users, pollinators, inhibition of beneficial predator/parasitioids and helpful microbial communities.

Microbial biocontrol agents/biopesticides have emerged as an alternative to chemical pesticides to solve/prevent these problems. They are marketed as biopesticides, biofertilizers, plant growth stimulators and native immunity supplements. Among these *Trichoderma* based preparations are marketed worldwide for the crop protection against to several plant pathogens also to enhance the yield and growth of the plants.

Microorganisms particularly fungi, produce secondary metabolites which are not essential in primary metabolic processes but have industrial and economic importance. These metabolites are natural compounds which have low molecular weights [<3kDa], produced by microorganisms and plants and related to genus, species and race.

Results

Genus *Trichoderma*, from phylum *Ascomycota* order *Hypocreales* is a rhizo-competent fungus that has a worldwide distribution. *Trichoderma* species are well known to produce secondary metabolites including volatile and non-volatile compounds such as pyrones, terpenoids, steroids and polyketides and a group of antibiotics that are called peptaibiotics which have different antagonistic activities against to pathogens.

Since 1930's the members of this genus are known as biocontrol agents against to plant diseases and widely used in agriculture in both developed and developing countries since 1990's. The pathogens that against to are *Botrytris cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*,

Sclerotium spp., *Phythium ultimum*, *Phytophtora* spp, *Armillaria* spp., *Fusarium oxysporum*, *Verticillium* spp. and *Gauemannomyces graminis*. However, their action mechanisms and potential applications are not known fairly.

Discussion

In order to the compete in commercial market and also to have equivalent to/superior features than chemical formulations, there is a demand for the development of new formulations. Industrial production costs can be fairly reduced by using developed solid and submerged fermentation systems. Using recyclable materials from food production and processing industries or on the low cost substrates such as rice grains, fungal spores can be produced without using developed equipments or qualified workers. These processes can be helpful to the development for challenging to the pathogens and also to support to development of new pesticides and biofertilizers that base on bioactive metabolites.

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Essential Oil Composition of Endemic *Sideritis leptoclada* O. Schwarz& P. H. Davis (Lamiaceae) from Turkey by Using Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry (GCxGC-TOF/MS)

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Abstract: *Sideritis* genus is present by 46 species in Turkey with high endemism rate (ca. 82%). The chemical composition of essential oil obtained from the aerial parts of endemic *Sideritis leptoclada* O. Schwarz & P. H. Davis was investigated. The chemical composition of *S. leptoclada* from the Southern Turkey is reported for the first time by GCxGC-TOF/MS technique. Among the sixteen constituent representing 96.74% of the *S. leptoclada* oil, major components of *S. leptoclada* were found as α-pinene (24.84%), trans-β-caryophyllene (22.99%), β-pinene (15.14%) and caryophyllene oxide (6.65%). The results were discussed with the genus pattern in means of medicinal purpose and plant essential oils.

Keywords: Essential oil, GCxGC-TOF/MS, monoterpenes, sesquiterpenes, *Sideritis leptoclada*,

1. Introduction

The genus *Sideritis* L. (Lamiaceae) with its nearly 150 species distributed in Northern hemisphere, occuring generally in the Mediterranean area [1-3]. The *Sideritis* name derives from the Greek word 'sideros' (iron) in reference to these vulnerary plants that heal the wounds [4]. Species of this genus, like *Sideritis leptoclada* O. Schwarz & P. H. Davis possess significant pharmacologic as well as economic values. Local people use this plant as herbal tea. *Sideritis* species are mainly named as mountain tea (*dağ çayı* in Turkish) in Turkey and comprises one of the most frequently traded herbs in bazaars. The genus *Sideritis* representing by 46 species and is an important species among the other Lamiaceae genera because the ratio of endemism (*ca*. 80%) in Turkey [5]. *Sideritis* species are frequently used in folk medicine due to their anti-inflammatory, antimicrobial, anti-spasmodic, anti-rheumatic, digestive and diuretic activities [6]. Recently, several studies have been reported on the chemical composition of *Sideritis* oils of different origins [7-12]. However, there are no studies on the *Sideritis* oil were conducted by GCxGC-TOF/MS. GCxGC with TOF/MS is highly desirable for identification and increases

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sensitivity of volatile compounds. Therefore, the purpose of the this study was to investigate the content and composition of essential oil in the leaves of *Sideritis leptoclada* O. Schwarz & P. H. Davis (Lamiaceae), a local endemic species in Turkey.

2. Material and Methods

2.1. Plant Material

The samples of *S. leptoclada* were collected from different locations from Sandras Mountain-Turkey. Voucher specimens were deposited in the Herbarium of the University of Pamukkale (Denizli, Turkey). Air-dried aerial parts were cut in small pieces (ca.10 mgr) and subjected to GCxGC-TOF/MS.

2.2. Direct Thermal Desorption (DTD) and GCxGC-TOF/MS analysis

The volatile compounds in *S. leptoclada* were analysed using DTD followed by GCXGC-TOF/MS. A GCXGC-TOF/MS system was used with a dual stage commercial thermal desorption injector. This incorporated a thermal desorption unit (TDU) which was connected, using a heated transfer line, to a programmable-temperature vaporisation (PTV) injector, CIS-4 plus (Gerstel, Mulheim an der Ruhr, Germany). The injector was equipped with a Gerstel MPS autosampler. Empty glass thermodesorption tubes were conditioned for 2h before use at a temperature of 400 °C [13]. Approximately 10 mg of *S. leptoclada* was placed into the quartz microfiber filter (QM-A sheets, Whatman, VWR) and loaded into the thermodesorption tubes. To keep the sample in position, glass wool was employed. Initial desorption of the sample was effected by heating the TDU from 40 °C (initial time 0.2 min) to 150 °C at a rate of 120 °C min^{-1} with a final hold time of 5 minutes under 1.5 mL min^{-1} helium flow in splitless mode. Volatile analytes emanating from this were cryo-focused at -40 °C in the CIS which had been cooled by liquid nitrogen prior to injection. The CIS was then heated at a rate of 10 °C s^{-1} to a final temperature of 150 °C. During this CIS temperature ramp, analytes were transferred to the GC column [13].

2.3. Chromatographic Analysis

The GCxGC–TOF/MS system comprised an HP 6890 (Agilent Technologies, Palo Alto, CA, USA) GC and a Pegasus III TOF/MS (Leco, St Joseph, MI, USA). The first column was a non-polar DB5 (30 m x 0.32 mm i.d. x 0.25 μm) and the second column a DB17 (1.9 m x 0.10 mm i.d. x 0.10 μm). Both columns were purchased from J&W Scientific (Folsom, CA, USA). The columns were connected using a press-fit connector [14]. The first dimensional separation is based on separation by volatility, whilst the second dimensional separation is based on separation by polarity [15]. The modulator secondary oven was operated at +15 °C higher than the GC oven temperature. The modulation time was 5 s and helium was employed as a carrier gas. The initial temperature of the first-dimension column was 60 ºC for 1 min; the temperature was then increased at 5 °C min^{-1} to 250 ºC, which was held for a 1 min. The initial temperature of the second-dimension column was 75 ºC for 1 min; the temperature was then raised at 5 °C min^{-1} to 265 ºC and held for a 1 min [16]. TOF/MS with electron-impact ionisation was used to identify peaks. Analytes were identified by employing GC-MS software; according to the NIST mass spectral library, and also by comparing their Kovats retention indices.

3. Results and Discussion

The chemical composition of essential oil from *S. leptoclada*, an endemic species from the Southwestern Anatolia region of Turkey, was studied for the first time using GCXGC-TOF/MS. Table 1 represents the chemical composition of the essential oil from *S. leptoclada*. As can be seen from this table, 16 compounds, representing about 99.99% of the essential oil, were characterized. The major components are as follows: α-pinene (24.84%), trans-β-

caryophyllene (22.99%), β-pinene (15.14%) and caryophyllene oxide (6.65%). Some of the *Sideritis* species of Turkey have been collected and their oils have been analysed by GC-MS techniques [9, 10, 12, 17, 18]. Current literatures showed that α-pinene and β-pinene were already proposed as the main constituents of essential oils from certain other *Sideritis* species such as *S. bilgerena* P. H. Davis (51.2% and 30.2%, respectively), *S. congesta* P. H. Davis et Hub.-Mor. (19.5% and 28.8%, respectively), *S. argyrea* P. H. Davis (16.5% and 23.9%, respectively) and *S. lycia* Boiss. et Heldr. (21.6% and 32.2%, respectively) [8, 11, 18-20]. *Sideritis* species classified as 6 groups; monoterpene, oxygenated monoterpene, sesquiterpene, oxygenated sesquiterpene, diterpene and others [21]. 57% of the *Sideritis* species existing in Turkey belong to the "monoterpene hydrocarbon-rich" group as shown in Tabanca et al. [22]. For our results, *Sideritis leptoclada* is also included in this group. But Başer [23,24] and Kırımer [20] classified *Sideritis* essential oils based on their main components, and *S. leptoclada* was included in the sesquiterpene-rich group, however, its whole essential composition was not presented previously; only the major component was given β-caryophyllene. In fact, *Sideritis* species are not rich in essential oil, but their smell and aroma are pleasant [25]. The percentage of trans-β-caryophyllene was found as 22.99% in the *S. leptoclada* studied. The result is different from the other *Sideritis* species. These differences might have been derived both from sampling time and, climatic/seasonal factors particularly genetical features (different chemotype).

Table 1. Percentage composition of components identified in the leaves of *S. leptoclada.*

No	Compound	RI	Percentage (%)
1	Pentanal	675	0,05
2	(E)-2-Hexenal	827	0,35
3	(E,E)-2,4-Hexadienal	858	0,12
4	Heptanal	879	0,16
5	α-Thujene	924	1,18
6	α-Pinene	933	15,14
7	β-Pinene	972	24,84
8	Limonene	1023	4,25
9	trans-β-Ocimene	1032	1,82
10	cis-Geraniol	1237	3,61
11	α-Terpinyl acetate	1333	2,29
12	D-Longifolene	1400	4,19
13	Trans-β-Caryophyllene	1405	22,99
14	Aromadendrene oxide	1440	4,40
15	α-Bisabolene	1496	4,69
16	Caryophyllene oxide	1573	6,65
	Unknown		3.26
TOTAL			100

4. Conclusions

The results showed that the species was rich by monoterpene constituents than sesquiterpenes. Various factors such as genetic, environmental, physiological and edaphic factors may affect the composition of the essential oil of *S. leptoclada*. *Sideritis* species are of great commercial interest for local people, becuase they collect this species from natural populations and use them in their life and they also sell in local bazaars for healty purposes. However, certain wild species from different environments (under different edaphic, climate and polluted sites) have not yet been studied. The essential oils were described as natural products preventing the growth of pathogens or other organisms in the test systems. Due to increasing demand on this species, further works are necessary to find the efficacy and suitable concentrations of these essential oils in folk use. The results of our work can be provided also useful data for the chemotaxonomy of *Sideritis* species.

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Research Article

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Phenolic Contents and Antioxidant Activity of Jojoba (*Simmondsia chinensis* (Link). Schindler

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Abstract: The ethanol and methanol extracts of Jojoba (*Simmondsia chinensis* (Link). Schnider) leaves and seeds were screened for antioxidant activity. The antioxidative potential of ethanol and methanol extracts of Jojoba (*Simmondsia chinensis*) were investigated for the first time using DPPH 2,2-diphenyl-1-picrylhdrazyl, total phenolic contents, antioxidant activity. Total phenolic substance amounts were calculated according to Folin-Ciocalteu method, substance concentrations in mg/GAE g, equivalent to gallic acid based on mg/ml gallic acid equivalent (GAE). The phenolic substance amounts in the leaves extracts (jojoba leaves: 313 mg/g GAE). Free radical clearance activities of the extracts were determined by using DPPH free radical. The Phenolic substances were calculated in highest jojoba leaves and lowest jojoba seeds. When DPPH radical clearance activity results were analyzed, it was seen that the highest activity was exhibited by jojoba leaf extract (% 43.20, 0.5 mg/ml concentration). The antioxidant activities of extracts were calculated in (nmol/g) via ascorbic acid system. When the activity scores are analyzed, higher scores were found in the ethanol extracts of jojoba leaves. The antioxidant activity was lower in the extracts with methanol.

Keywords: Phenolic contents, Antioxidant, Jojoba, leaves, seeds,

1. Introduction

Jojoba (*Simmondsia chinensis* (Link). Schnider) originates from the Sonoran desert and is grown in different place as a commercial crop [1]. There are about 720 ha of jojoba plantations in La Rioja reported information about methods for the elimination of appetite suppressant compounds constitute a good raw material for animal feed [2]. Due to the fact that these seeds have a high percentage of wax and proteins, they represent valuable raw material for various industries such as the jojoba wax producer and the animal food manufacturer, respectively [3]. The chemical quality parameters of damaged jojoba seeds were unknown. In this study, we followed phenolic contents and antioxidant properties of jojoba plant. Phenolic compounds are a complex, but important group of naturally occurring products in plants and are present in the pharmacological products which includes jojoba seed and leaves. Phenolic compounds are plant secondary metabolites, which play important roles in disease resistance [4], protection aganist pests and species dissemination. The interest on these compounds is related with their antioxidant activity and promotion of health benefits. Antioxidant activity of several plant materials has recently been described [5-8], and a number of plant products,

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including polyphenols, flavonoids and terpens, exert an antioxidant action. Plant species belong to several botanical families, such as Labiatae, Compositae, Umbelliferae, Asteracae, polygonacae. Many species have been investigated for their antioxidant properties.

The purpose of this work was to study the main phenolic contents present in jojoba tree leaves, seeds obtained from jojoba of Turkey cultivars. The total antioxidant activities of these samples were evaluated by the extent of their capabilities.

2. Material and Methods

2.1. Plant Materials and Extracts preparations

The orginal jojoba *Simmondsia chinensis* (L.) Schneider seeds and leaves were brought from Arizona, USA, in 1991 and transplanted to Sarıcasu town, Kumluca, Turkey in 1994. The jojoba seeds and leaves variety of Arizona A42 were used for experiments. The fresh under ground and above ground parts of the plant materials were cleaned and dried in the shadow for extraction. Jojoba seeds and leaves were extracted through ethanol and methanol with the help of soxhlet device (GFL TYP1042) and were added in the ratio of 1/10 and then mixture was filtered by a filter paper (whatman No:1), and the solvents were evaporated in a rotary evaporator (IKA R 10) at 50 °C. Each process of this experiment was carried out with 4 replications and repeated twice. Extracts were obtained from leaves and seeds of *S. chinensis.* These extracts are: *Simmondsia* Seed-Methanol (SSM), *Simmondsia* Seed-Ethanol (SSE) *Simmondsia* Leaf-Methanol (SLM), *Simmondsia* Leaf Ethanol (SLE).

2.2. Antioxidant capacities (activity)

In this article, antioxidant activities (capacity) was identified by measuring the conjugated drene methanol (MERCK, Germany) sodium merhylate methilat hydroperoxides arising from linoleic oxidation [13-14]. A stock solution of linoleic acid was prepared as follows: A preparation step was necessary prior to introduction of the oil into the GS/MS (Shimadzu 17A-GS/MS Q P5050, Kyoto, JAPAN) and auto sampler (Shimadzu AOC 20 i, Kyoto, JAPAN) for the individual determination of linoleic acid ingredient FAME (Fatty acid methyl esters) s were obtained by transesterification with sodium methylate in methanol 0.5 ml of a 0.5% (W/v) solution of sodium methylate in methanol and 100 µl oil were mixed [15]. Antioxidant capacity (AC) was measured in terms of successful beaching of lineoleic acid by using a slightly modified version of the formula from [16] and the absorbance was measured during.

2.3. Determination of total phenolic content

The total phenolic contents of ethanol, methanol seeds and leaves of *S. chinensis* were determiend using Folin- Ciocalteu's (FC) reagent according to the method of Singleton et al. [17]. Crude ethanol and methanol extracts (40 µL) of plant materials (2 gmL^{-1}) were mixed with 200 µL FC reagent (Sigma Aldrich, Steinheim, Germany) and 760 µL of distilled water. After shaking, the mixture was incubated for 7.5 min. at room temperature. Then, 600 µL of 20 % Na_2CO_3 solution was added. The mixture was shaken for 2 h at room temperature. The absorbance of the solution was measured at 765 nm aganist a blank. Gallic acid was used as a standart. The concentration of total phenolic compounds in *S. chinensis* was determined as a µg of gallic acid equivalents per 1 mg of extract using the following equation obtained from a standard gallic acid graph (R^2= 0.9999).

Absorbance = 0.0024 x gallic acid (µg).

Spectrophometric analysis was performed by using a five point calibration curve generated with pure gallic acid. Gallic acid was obtained from sigma, Aldrich.

2.4. Free radical scavenging activity

Free radical scavenging activity of ethanol and methanol extracts of S. chinensis was measured by (1,1- DPPH dipphenyl-2- picryhydrazyl (DPPH; Sigma Aldich, Steinheim, Germany) using the of Shimada et al (1992), Briefly, a 0.1 mM solution of DPPH in ethanol and methanol were prepared. Then, 1 mL of this solutions were incubated with varying concentrations of *S. chinensis* leaves and seeds (1-500 µg/ mL). The reaction mixtures were then shaken well and incubated for 30 min. in dark at room temperature.

*Statistical Analysis***:** All data presented are means of four replicates along with standart deviations. Correlation coefficients were determined between antioxidant capacity and phenolic constituents.

3. Results and Discussion

Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, antiartherogenic, antiflammatory, antimicrobial, antioxidant effects [9-10]. These compounds including flavanoids and phenolic acids are known to be responsible for antioxidant capacities in fruits and the fruits with higher phenolic contents generally show stronger antioxidant capacities [11]. The *Hypericum* family seems to be a rich source of plant species containing large amounts of phenolic acids, so it is considered to be a promising source of natural antioxidants [12]. We found that, the phenolic content ofthe ethanolic extracts are higher than the methanolic extracts. The phenolic contents of the extracts as follow:

The absorbance of the *S. chinensis* plant extracts at 695 nm was evaluated using absornance of the sample that was measured every 30 min. using UV spectrophotometer (Pelkin Elmer, Japan). Measurements were carried out in triplicate and the mean values of the measurements were calculated. Methanolic and ethanolic extracts of *S. chinensis* presented different absorbance values. The highest initial absorbance value is detected as 31.60 for SLE (*Simmonsia chinensis*, leaves, Ethanol) and the lowest initial absorbance value is detected as 19.93 for SSM (*Simmonsia chinensis*, Seed, Methanol). The antioxidant activities of the plant extracts were detected as follows: SLE (50.43+1.2%)> SSE (24.16± 0.71%) > SLM (40.32± 0.92%) > SSM (13.87± 0.02%). The antioxidant activities of extracts were calculated in (nmol/g) via ascorbic acid system (Fig. 1).

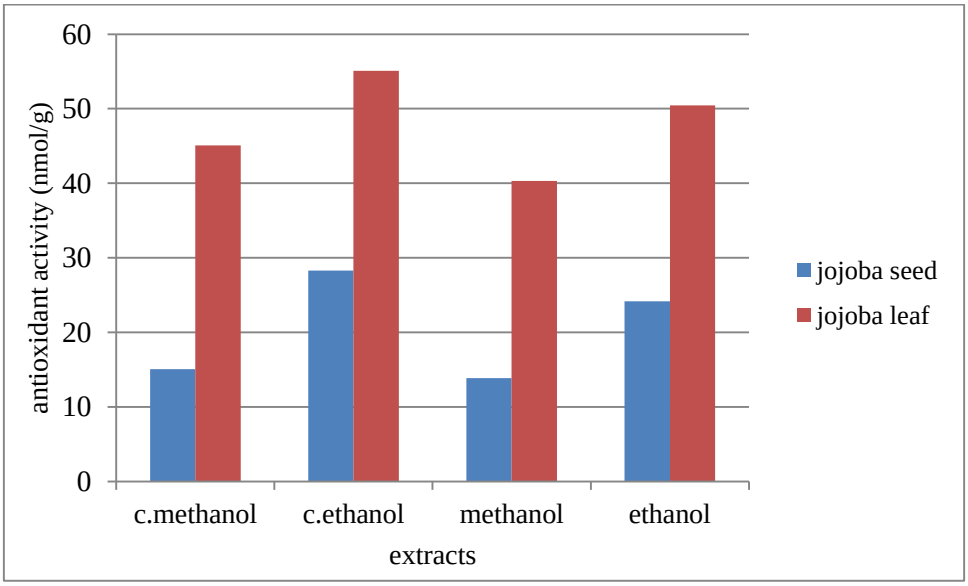

Fig. 1. The antioxidant activity in the methanol and ethanol extracts.

The free scaveging capacities of *S. chinensis* leaf and methanolic and ethanolic extracts of seeds were evaluated and determined as follows (Table-1). Results showed that lavander plant leaves has high phenolic contents. Because of that, it exhibited the high antioxidant and free radical scavering activities [12].

Table 1. The free radical scavering activity by DPPH method in *Simmondsia chinensis* leaf and seed.

Jojoba Seed				Jojoba Leaf			
Methanol		Ethanol		Methanol		Ethanol	
The sample quantity (µg/ml)	inhibition %	µg/ml	Inhibition %	The sample quantity (µg/ml)	inhibition %	µg/ml	Inhibition %
500	36.43±0.05	500	43.20±0.09	500	37.23±0.06	500	37.45±2.99
400	32.10±0.04	400	37.79±0.18	400	32.95±0.05	400	38.12±0.18
300	29.12±0.03	300	38.97±1.42	300	30.00±0.03	300	31.25±0.04
200	26.50±0.2	200	27.24±0.27	200	27.42±0.02	200	20.24±0.00
100	15.62±0.1	100	7.58±0.62	100	16.67±0.01	100	10.43±0.36

The amount of total phenolics, measured by Folin-Ciocalteu method, varied widely in help materials and ranged from 0.00 to 0.00 mg GAE/g dry material. The highest level of phenolics was found in *Echinacea purpurea*, while the lowest was in *Carum c*arvi [18]. The amount of total phenolics varied widely in plant materials and ranged from 0.59 to 313 mg GAE/g dry material (Fig. 2). The highest level of phenolics was found in *S. chinensis*, leaf, while the lowest was in *S. chinensis seed.* Total phenolic substance amounts were calculated according to Folin-Ciocalteu method, substance concentrations in mg/GAE g, equivalent to gallic acid based on mg/ml gallic acid equivalent (GAE). The phenolic substance amounts in the leaf extracts (jojoba leaf: 313 mg/g GAE). Nawar et. al. (1984) *Zizyphus spina Christi* (l.) Wild is a wild tree, with spiny branches and small, orange-yellow fruits, commonly found in Jordan, Israel and Egypt, known in Egypt, where it is used to treat the blisters, bruises, chest pains, dandruff, fractures, and headache. The fresh leaves are applied on swollen eye at night.

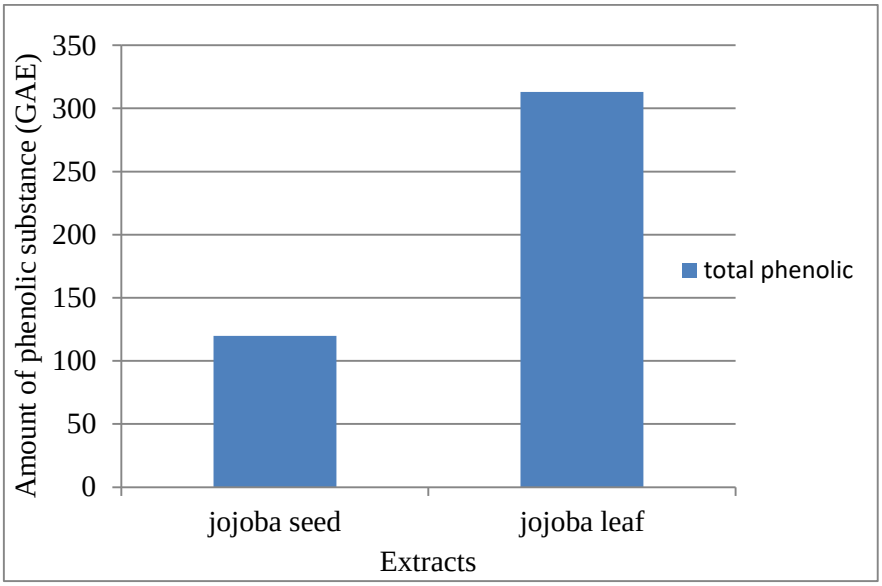

Fig. 2. Total phenolic substance amounts of the extracts

4. Conclusion

Our results demonstrated that all extracts of *S. chinensis* have efficient phenolic compounds. The methanol and ethanol extracts of *S. chinensis* leaves and seeds showed antioxidant activity base on scavenging. When the activity scores are analyzed, higher scores were found in the methanol extract of lavender seed. The antioxidant activity is lower in the extracts with hexane. Total phenolic substance amounts were calculated according to Folin-Ciocalteu method, substance concentrations in mg/GAE g, equivalent to gallic acid based on mg/ml gallic acid equivalent (GAE). The phenolic substance amounts in the leaf extracts (jojoba leaf: 313 mg/g GAE, lavender leaf: 314.4 mg/g GAE) were found more compared to seed extracts. When DPPH radical clearance activity results were analyzed, it was seen that the highest activity was exhibited by jojoba leaf extract (43.20%, 0.5 mg/ml concentration).

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Assessment of Volatile Oil Composition, Phenolics and Antioxidant Activity of Bay (*Laurus nobilis*) Leaf and Usage in Cosmetic Applications

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Abstract: In this study, the components of the volatile oil obtained from *Laurus nobilis* leaves by steam distillation were determined using Agilent 6890 Gas Chromatography (GC) - 5975 Mass Spectrometry (MS). The antioxidant activities of different extracts of *L. nobilis* leaves were determined by using DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity, β-carotene-linoleic acid bleaching assay and ABTS$^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical decolorization assay. Determination of the total phenolic contents of *L. nobilis* leaf extracts were performed using the Folin-Ciocalteau procedure and total flavonoid contents were measured using a spectrophotometric assay. According to the GC/MS results, 1,8-cineole (46.16%), *alpha*-terpinyl acetate (10.62%), *alpha*-pinene (6.27%), terpinen-4-ol (5.07%) and sabinene (4.99%) were found to be the major compounds in volatile oil. The obtained volatile oil was used to make skin care lotion. Stability tests and organoleptic analyses of final product were performed after 1, 5, 30 and 90 days of production. The highest amounts of total flavonoid content were found to be 5.48 ± 0.65 and 8.60 ± 0.12 µg QEs/mg in ethyl acetate and ethanol extracts, respectively. The highest amounts of total phenolic compounds were found to be 54.42 ± 0.14 and 25.32 ± 0.10 µg PEs/mg in ethyl acetate and ethanol extracts, respectively. According to the results of ABTS$^{\bullet+}$, DPPH$^{\bullet}$ and β-carotene linoleic acid assays, ethyl acetate extract was found to be the most active extract (24.98±0.87 µg mL^{-1}, 75.65±0.77 µg mL^{-1} and 19.32±1.04 µg mL^{-1}).

Keywords: *Laurus nobilis*, Volatile oil, Antioxidant, Cosmetic, GC/MS

1. Introduction

Volatile oils are secondary plant metabolites, which are found in different parts of plants including flowers, roots, bark, leaves, seeds, peel, fruit and wood produced in cytoplasm and plastids of plant cells [1]. These oils, also known as essential oil, etheric oil by people, can contain terpenic hydrocarbons and their oxygenated derivatives as well as organic acids, alcohols, phenols and ketones [2]. The main components of volatile oils are usually mono and sesquiterpenes. In some cases their main derivatives are hydrocarbons (e.g. turpentine, formed

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by *alpha* and *beta*-pinene), while in others the main constituents are oxygenated (e.g. cloves formed by eugenol) [3, 4]. Some of the aromatic plants have a volatile oil formed mainly of aromatic compounds derived from allyl or isoallyl phenol. The plants containing those compounds, although they are less frequent than plants containing terpenes, only allow such compounds to be selective.

There has always been a great deal of interest in volatile oils throughout history. Although many of the intended uses of volatile oils have disappeared over time, it has generally been accepted that since the beginning of mankind, humans have obtained these oils from aromatic plants. Volatile oils have various applications for different purposes. Volatile oils are not only used for cooking in order to improve the taste and health of the food, but also for the manufacture of perfumes and cosmetic products [5].

The ancient Egyptians have used volatile oils in perfumery, medicine and even in the preparation of bodies and organs for mummification. In ancient Asia, Vedas coded intended uses of perfume and aromatics for therapeutic purposes. Indeed, throughout history, many civilizations have used volatile oils and fragrances for a variety of purposes, including religious rituals, perfumes and therapeutic against infectious diseases. During the Renaissance period, the use of volatile oils in perfumery and cosmetic products has been spread to the world [6].

Volatile oils can be obtained by water distillation, water and steam distillation, or steam distillation alone, which are the most commonly used methods. The part of the plant where the volatile oil is to be obtained may be fresh, partially or completely dried, but if the volatile oil is to be obtained from the flower part, the flowers should be fresh [7].

Different methods have been used to control and analyze essential oils [8]. Currently, however, the identification of essential oil components is usually carried out with the aid of gas chromatography–mass spectrometry (GC/MS) equipped with flame ionization detector (FID) and mass spectrometer (MS) detectors, a capillary column and a split. Test conditions may vary depending on the column and the sample [9].

The main sources of volatile oils are medicinal and aromatic plants, which are widely used since ancient times in medicine, cosmetics and preserving and improving the flavor of foods. Especially in recent years, there are numerous artificial chemical-free productions, mainly cosmetics, due to the increased interest in natural products [10]. As a natural ingredient, volatile oils are a growing market trend, being used in skin care cosmetics (e.g creams, lotions), balms, shampoos, soaps and perfumes [11].

Laurus nobilis (*Lauraceae*), one of the main sources of plant volatile oils, is an evergreen shrub that can grow up to 8 meters tall. It has dark green leaves about 8-14 cm long and 3-4 cm wide [12, 13].

This plant, belonging to *Lauracea* family and unique to the southern Mediterranean region, is widely grown in Europe and USA as an ornamental plant. It is cultivated commercially for aromatic oil, found in its leaves, in Turkey, Algeria, Morocco, Portugal, Spain, Italy, France and Mexico. Turkey is the leader bay leaf exporter of the World. [14].

The volatile oils obtained from the leaves of *L. nobilis* still maintains the importance in both traditional and modern medicine with its pharmacological activities. Studies have shown that *L. nobilis* volatile oil has antioxidant [15], anticonvulsant [16], analgesic, anti-inflammatory [13], antiviral [17], anticholinergic [18], antibacterial [19] and antifungal activities [20]. *L. nobilis*, which is a powerful medicinal and aromatic plant with these pharmacological properties, has been reported in cosmetic uses. *L. nobilis* leaf volatile oil is used for the preparation of hair lotion due to its antidandruff activity and for the treatment of psoriasis [21].

The usage of volatile oils in the production of cosmetics and similar products can both increase the dermo-cosmetic effects of these products and marketing trend for the final product. A great number of usages of plant materials such as volatile oils in cosmetics products provide extra benefits to the skin more than ordinary products. [22].

In this study, the chemical composition of *L. nobilis* leaf volatile oil was examined using GC/MS. The antioxidant activities of different extracts of *L. nobilis* leaves were determined by using DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity, β-carotene-linoleic acid bleaching assay and ABTS$^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical decolorization assay. Determination of the total phenolic contents of *L. nobilis* leaf extracts performed using the Folin-Ciocalteau procedure and total flavonoid contents were measured using a spectrophotometric assay. In addition, we prepared skin lotion using the volatile oil obtained with steam distillation. After preparation of lotion, we performed stability tests and organoleptic tests on the final product.

2. MATERIALS AND METHODS

2.1. Standards and Reagents

Ethanol, n-hexane, methanol, ethyl acetate and chloroform were of analytical grade purity were supplied by Merck (Darmstadt, Germany). Essential oil standards were supplied from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Water was HPLC grade (18.2 MΩ), purified by a Milipore Milli-Q (Molsheim, France) system that includes reverse osmosis, ion exchange and filtration steps. β-carotene, tween-40, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS$^{\bullet+}$), potassium persulfate ($K_2S_2O_8$), linoleic acid, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), poly(acrylic acid sodium salt), alpha-tocopherol were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals and solvents were of analytical grade and purchased from usual suppliers.

2.2. Plant Material and Isolation of Volatile Oil

Laurus nobilis leaves were collected in June 2016 from the trees in Fethiye region of Muğla (Turkey). Authentication of the plant was performed by Dr. Ergun KAYA from Department of Molecular Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Muğla (Turkey). Collected leaves were dried under ambient temperature (25^{o}C), shadow and airy place. Dry leaves were separated from the vimen.

Air-dried leaves of *L. nobilis* subjected to steam distillation for 120 min to obtain its volatile oil. The resulting volatile oil was dried over anhydrous sodium sulphate, filtered and stored in a dark glass bottle at -21°C until analyze by Gas Chromatography-Mass Spectrometry (GC/MS).

2.3. Determination of Chemical Composition of Volatile Oil Using GC/MS

GC/MS analyses were carried out using an Agilent 6890N Gas Chromatograph equipped with a Multi Mode Inlet (MMI) (280^{o}C), a DB-1 capillary column (30m × 0.25mm; film thickness 0.25μm) and coupled with an Agilent 5975C MS Detector (MSD), operating in the electron impact (EI) mode at 70 eV. Transfer line temperature was set at 250^{o}C. The carrier gas was He (2.1 mL/min), and the oven temperature was held at 60^{o}C for 5 min, then increased up to 220^{o}C at a rate of 2^{o}C/min and held at this temperature for 10 min. The injected volume was 2 μL and the split ratio 40:1.

In this study, the identification of the compounds was based on the comparison of their retention times (RT) and mass spectra with those from the NIST 2008, Wiley 2008 and Flavor2

libraries. Relative percentages of compounds were calculated based on the peak areas from the MS data.

2.4. Determination of Antioxidant Activities

2.4.1. Preparation of Extracts

In the determination of antioxidant activities of *L. nobilis* leaf, four different extracts, hexane, ethyl acetate, ethanol and water, were used. The sample was extracted five times for 24 hours at room temperature with hexane, ethyl acetate, ethanol, water and then the combined extracts were filtered through Whatman No 4, separately. The combined extracts of hexane, ethyl acetate, and ethanol were evaporated (rotary evaporator Heidolph, Hei-VAP Precision) to dryness in vacuum, and the combined water extracts were lyophilized (Christ Freeze Dryer, Alpha 1-4 LD plus, Germany). The extracts were stored in deepfreeze until the time of study. The results were given as 50 % inhibition concentration (IC_{50}). The sample concentration ($\mu g.mL^{-1}$) inhibiting 50 % antioxidant activity (IC_{50}) was calculated from the graph of activity percentage against sample concentration.

2.4.2. $DPPH^{\bullet}$ Free Radical Scavenging Assay

The free radical scavenging activity of *L. nobilis* leaf extracts was determined using the method, $DPPH^{\bullet}$ free radical protocol [23] with slight modifications. The extract solutions prepared in different concentrations (40 µL) and ethanolic solution (120 µL) containing $DPPH^{\bullet}$ radicals (0.4 mM) were incubated in darkness at room temperature for 30 min. Absorbance was measured at 517 nm in SpectraMax 340 PC, Molecular Device (USA). The radical-scavenging activity (RSA) was calculated as a percentage of $DPPH^{\bullet}$ decolorization using the following equation:

$$\%RSA=[(A_{DPPH}-A_S)/A_{DPPH}]\times 100$$

Where A_S is the absorbance of the solution containing the sample and A_{DPPH} is the absorbance of the $DPPH^{\bullet}$ solution.

2.4.3. β-Carotene-Linoleic Acid Bleaching Assay

The total antioxidant activity was determined using β-carotene-linoleic acid test system based on the detection of inhibition of conjugated dien hydroperoxides due to oxidation of linoleic acid [24, 25]. β-Carotene (0.5 mg), dissolved in 1mL of chloroform, was mixed with linoleic acid (25 µL) and Tween 40 emulsifier (200 mg). Chloroform was evaporated under low pressure, 50mL of distilled water was added by vigorous shaking. Aliquots (1.60 µL) of this emulsion were added to 40 µL of the extract solutions at different concentrations. As soon as the emulsion was added to each tube, the zero time absorbance was initially measured at 470 nm, and then the absorbance measurements were done for every 30 min until 120 min.

2.4.4. $ABTS^{\bullet+}$ Cation Radical Decolorization Assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ scavenging activity was determined according to the previously described method [26]. The $ABTS^{\bullet+}$ (7 mM) in water and potassium persulfate (2.45 mM) reacted to give $ABTS^{\bullet+}$, stored in the dark at room temperature for 12 h, and oxidation of $ABTS^{\bullet+}$ appeared immediately, however, the stability of absorbance was gained after 6 h. Then, the sample solution (40 µL) in ethanol at different concentrations were mixed with $ABTS^{\bullet+}$ solution (160 µL), giving the absorbance at 734 nm by using a 96-well microplate reader in 10 minute. The scavenging capability of $ABTS^{\bullet+}$ was calculated using the following equation:

$$ABTS^{\bullet+}\text{ scavenging effect }\%=[(A_{ABTS^{\bullet+}}-A_S)/A_{ABTS^{\bullet+}}]\times 100$$

Where A_S is the absorbance of remaining concentration of $ABTS^{\bullet+}$ in the presence of sample and $A_{ABTS}{}^{\bullet+}$ is the initial concentration of the $ABTS^{\bullet+}$.

2.5. Determination of Total Phenolic Concentrations

The concentrations of total phenolic content in *L. nobilis* leaf were expressed as microgrammes of pyrocatechol equivalents (PEs), determined with Folin-Ciocalteu reagent (FCR) [27]. The sample solution (1 mL) dissolved in methanol was added to distilled water (46 mL) and FCR (1 mL), and mixed thoroughly, 2% sodium carbonate (3 mL) were added to the mixture in 3 min and shaken intermittently for 2 h at room temperature. The absorbance was measured at 760 nm. The concentration of phenolic compounds was calculated according to the following equation that was obtained from standard pyrocatechol graph: Absorbance = 0.0073x - 0.1665, $r^2 = 0.9976$

2.6. Determination of Total Flavonoid Concentrations

Measurements of total flavonoid concentration of the extracts were based on the previously reported method [24], and results were expressed as quercetin equivalents (QEs). An aliquot of the sample solution (1 mL) extracts in methanol was mixed with 10% aluminum nitrate (0.1 mL), 1 M potassium acetate (0.1 mL) and 80% methanol (3.8 mL) in test tubes, and then the absorbance was measured at 415 nm in 40 min, and stayed at room temperature. The concentrations of flavonoid compounds were calculated according to following equation that was obtained from the standard quercetin graph: Absorbance = 0.0082x + 0.0073, $r^2 = 0.9998$.

2.7. Preparation of Lotion

The lotion was prepared by following steps. The ingredients; oils, purified water and poly(acrylic acid sodium salt) as emulsifier polymer blend, were weighed separately. 6 g of lilac-flavored hazelnut oil and 6 g of bay leaf volatile oil were added dropwise to 87 mL of purified water in a glass beaker at room temperature under magnetic stirring.

After the mixture was stirred with a magnetic stirrer for 10 min, the formulation is completed by addition of the homogenized emulsifier polymer blend. After lotion preparation was completely homogenized, it was poured gently (to avoid the presence of bubbles) into a container.

2.8. Stability Tests of Cosmetic Preparation

2.8.1. Determination of Viscosity

The rheology analysis of the lotion formulation containing *L. nobilis* leaf volatile oil was performed using Brookfield LVDV -I+ viscometer at 10 rpm, 25°C. Rheological analyses were repeated 1, 5, 30 and 90 days after lotion preparation was completed.

2.8.2. Centrifugation Assay

The centrifuge test, which is often used for having preliminary information in the stability tests, was performed as described in [28]. The lotion sample was centrifuged twice; each was 15 min at 3000 rpm and under ambient temperature. A 10 mL centrifuge tube was used for centrifugation and it was observed whether there was phase separation.

2.8.3. pH Measurements

pH change during storage is one of the indicator for chemical stability of cosmetic preparations. The pH of the skin care lotion was measured according to the method given in [29]. The measurement was performed at room temperature with a Sartorius pH meter after 1/10 (v/v) dilution of the sample with purified water and filtration. Before the measurement, the pH meter was calibrated with the standard buffer solution (pH = 4, 7 and 10).

2.9. Organoleptic Tests of Cosmetic Preparation

Cosmetic preparation was evaluated for appearance, color, odor and spreadability. A visual evaluation was made by adding the sample to a glass container, placed over a white background, and compared it to the previous observations. Spreadability of cosmetic preparation was evaluated according to the expressions of user. All of the organoleptic analyzes were carried out in the same light, temperature and packaging conditions to avoid variations in appearance, color, odor and spreadability parameters. All tests were repeated on 1, 5, 30 and 90 days after the product was prepared.

3. RESULT AND DISCUSSION

3.1. Chemical Composition of Volatile Oil

In this study, forty-eight components were detected in the volatile oil obtained from *L. nobilis* leaves by steam distillation (Table 1). Volatile oil analyzed by GC/MS instrument and 1,8-cineol (46.16%), *alpha*-terpinyl acetate (10.62%), *alpha*-pinene (6.27%), terpinen-4-ol (5.07%), sabinene (4.99%) and *beta*-pinene (4.47%) detected as major compounds among forty-eight components. The other compounds such as 3-hexen-1-ol (0.03%), *cis*-*beta*-ocimene (0.05%) and 2-methylprop-1-enyl-cyclohexa-1,3-diene (0.07%) were minor compounds.

The chemical composition of *L. nobilis* volatile oil from different locations has been studied by different researchers. In all studies, 1,8-cineole was the major component with percentages ranging between 26.70% and 68.48% [14, 30]. Moreover, *alpha*-terpinyl acetate with percentages ranging between 0.65-25.70% [31, 32] and terpinen-4-ol with percentages ranging between 1.50-4.56% [32, 33] were found as major components. According to the our result, we found average amount of 1,8-cineol but terpinen-4-ol was found to be more than average amounts reported by previous researches (Figure 1).

Tanriverdi *et al.* [34] found that leaves of *L. nobilis* of Turkish origin contained 1,8-cineole (62.64%), *alpha*-pinene (3.14%), and terpinen-4-ol (3.11%). Riaz *et al.* [35] established that leaf oil of *L. nobilis* contained 1,8-cineole (44.12%). Florini *et. al.* [36] from France reported that *L. nobilis* leaves volatile oil contained 1,8-cineole (39.10%), linalool (10.00%), β-caryophyllene (1.60%), *alpha*-terpinyl acetate (18.20%) and terpinene-4-ol (1.40%) while Yalçın *et. al.* [37] found 1,8-cineole (58.59%), *alpha*-terpinyl acetate (8.82%), and terpinene-4-ol (4.25%) as the main components of the essential oil isolated from the leaves of the *L. nobilis* plant (from the Northern Cyprus Mountains) by hydrodistillation.

Volatile oils have been used as antioxidants for the prevention of skin disorders such as skin cancer and wrinkles which caused by oxidative stress on skin surface [38].

Table 1. GC/MS analysis of volatile oil composition of *Laurus nobilis* leaf

No	RT (min.)	Compound	Concentration (%)	Identification method
1	3.656	3-Hexen-1-ol	0.03	b, c
2	5.707	*alpha*-Thujene	0.50	a, b, c
3	5.893	*alpha*-Pinene	6.27	a, b, c
4	6.089	2,4(10)-Thujadien	0.08	b, c
5	6.262	Camphene	0.75	a, b, c
6	7.116	Sabinene	4.99	a, b, c
7	7.209	*beta*-Pinene	4.47	a, b, c
8	7.902	*beta*-Myrcene	0.30	a, b, c
9	8.301	*alpha*-Phellandrene	0.27	a, b, c
10	8.627	3-Carene	0.08	b, c
11	8.849	*alpha*-Terpinene	0.82	a, b, c
12	8.984	o-Cymene	2.61	a, b, c
13	9.394	1,8-Cineol	46.16	a, b, c
14	9.429	*alpha*-Limonene	1.90	a, b, c
15	10.369	cis-*beta*-Ocimene	0.05	a, b, c
16	10.732	*gamma*-Terpinene	1.48	a, b, c
17	10.883	trans-Sabinene hydrate	0.16	b, c
18	11.885	Unknown	0.08	a, b, c
19	12.190	*alpha*-Terpinolene	0.33	a, b, c
20	12.349	cis-Sabinene hydrate	0.12	b, c
21	12.733	Linalool	1.43	a, b, c
22	13.598	p-Ment-2-en-1-ol	0.18	b, c
23	14.305	trans-Pinocarveol	0.41	b, c
24	15.115	(E)-Sabinen hydrate	0.26	b, c
25	16.630	Terpinen-4-ol	5.07	a, b, c
26	16.861	Myrtenal	0.26	b, c
27	17.052	*beta*-Phellandren-8-ol	0.12	b, c
28	17.174	p-Mentha-1(7),8-dien-2-ol	0.10	b, c
29	17.308	*alpha*-Terpineol	2.17	a, b, c
30	17.553	Estragole	0.06	b, c
31	17.669	Myrtenol	0.41	a, b, c
32	18.428	cis-Piperitol	0.08	b, c
33	22.663	4-Thujen-2-alpha-YL	0.26	b, c
34	23.120	Bornyl acetate	0.63	a, b, c
35	25.067	Unknown	0.67	
36	25.533	1-ethyl-3,5-dimethyl-benzene	0.09	b, c
37	26.600	2-Methylprop-1-enyl cyclohexa 1,3-diene	0.07	b, c
38	26.817	Eugenol	1.18	b, c
39	27.176	*alpha*-Terpinyl acetate	10.62	a, b, c
40	28.230	Unknown	0.09	
41	29.758	Eugenol methyl ether	3.24	b, c
42	30.106	*beta*-Elemene	0.10	b, c
43	31.395	*beta*-Caryophyllene	0.12	a, b, c
44	31.785	Cinnamyl acetate	0.28	b, c
45	35.047	Isoeugenyl methyl ether	0.17	b, c
46	39.688	Spathulenol	0.19	b, c
47	39.842	Caryophyllene oxide	0.22	a, b, c
48	42.754	*beta*-Eudesmol	0.10	b, c

[a]Comparison with standard compounds, [b]GC/MS analysis, [c]Literature comparison

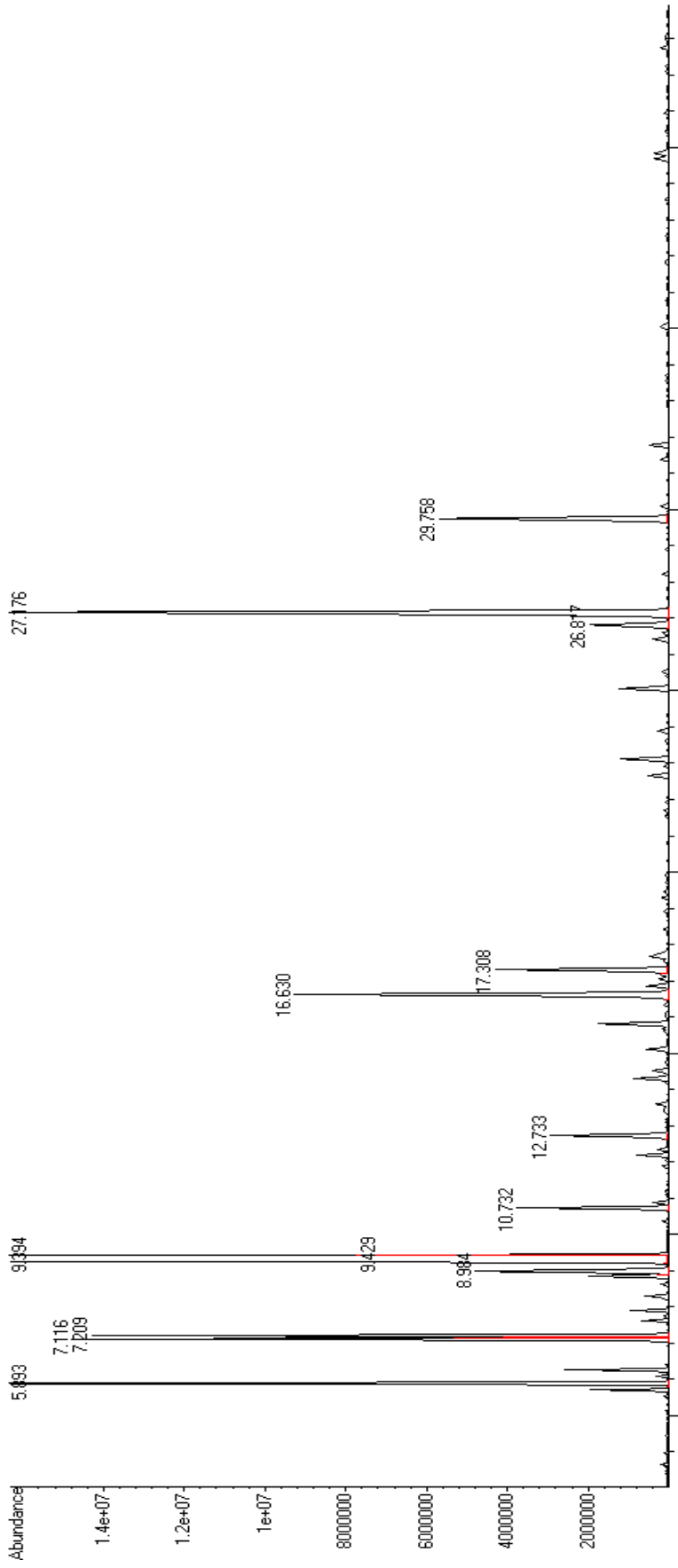

Figure 1. GC/MS chromatogram of *Laurus nobilis* volatile oil. (Retention times of the components above the concentration of 1% are given). *alpha*-pinene (5.893), sabinen (7.116), *beta*-pinene (7.209), o-cymene (8.984), 1,8-cineol (9.394), *alpha*-limonene (9.429), *gamma*-terpinene (10.732), linalool (12.733), terpinen-4-ol (16.630), *alpha*-terpinol (17.308), eugenol (26.817), *alpha*-terpinyl acetate (27.176), eugenol methyl ether (29.758)

High amount of 1,8-cineol (46.16%) and terpinen-4-ol (5.07%) makes volatile oil a potent of cosmetic ingredient due to their high antioxidant activity [39]. However, these compounds described as potent of antifungal agent by previous researches [40] and with this reason they can be considered as self-preserving ingredient for cosmetic products.

Considering the proven effects of *L. nobilis* leaf volatile oil and previous studies, the use of *L. nobilis* leaf essential oil in cosmetic formulations can result in high added value to the product and contribute to product functionality.

3.2. Antioxidant Activities

Antioxidant activities of extracts from *L. nobilis* leaves were investigated using three different methods (β-carotene-linoleic acid, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$). The antioxidant activities of *L. nobilis* leaf extracts were measured by using spectrophotometric methods after they were dissolved in appropriate solvents. When the total antioxidant activity values were examined, the 50% inhibition concentration (IC_{50}) of ethyl acetate extract was found to be 19.32±1.04 μg mL^{-1} in the β-carotene-linoleic acid assay. Compared to the other extracts and standards, ethyl acetate extract seems to be quite active.

In addition, according to the results of $ABTS^{\bullet+}$ cation radical and $DPPH^{\bullet}$ free radical scavenging assays, ethyl acetate extract was found to be the most active extract, respectively (24.98±0.87 μg mL^{-1} and 75.65±0.77 μg mL^{-1}). $DPPH^{\bullet}$ free radical inhibition IC_{50} value of ethyl acetate extract of *L. nobilis* leaves was found to be 75.65 ± 0.77 μg mL^{-1}, whereas butylated hydroxytoluene (BHT) IC_{50} value, used as standard, was 68.27 ± 0.67 μg mL^{-1} (Table 2).

Table 2. Antioxidant activity of the extracts of *L. nobilis* by β-carotene-linoleic acid, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays

		Antioxidant activity		
	Extracts	*β*-Carotene-linoleic acid assay IC_{50} (μg mL^{-1})	$ABTS^{\bullet+}$ assay IC_{50} (μg mL^{-1})	$DPPH^{\bullet}$ assay IC_{50} (μg mL^{-1})
Laurus nobilis	Hexane	47.08±0.20	55.34±1.21	201.47±0.60
	Ethyl acetate	19.32±1.04	24.98±0.87	75.65±0.77
	Ethanol	36.23±1.01	43.74±0.57	129.10±0.66
	Water	124.01±1.65	99.75±1.41	203.55±0.98
Standards	BHA	2.18±0.09	6.28±0.10	67.40±0.41
	BHT	2.05±0.11	6.37±0.08	68.27±0.67
	α-Tocopherol	3.87±0.15	6.97±0.13	10.41±0.19

IC_{50} values represent the means ± SD of three parallel measurements
BHA; Butylated hydroxyanisole, BHT; Butylated hydroxytoluene

Number of studies have been done to evaluate the antioxidant properties of different extracts of *L. nobilis* leaves. Previous researchers have reported that ethanolic extract of *L. nobilis* leaves exhibited an IC_{50} value of 22 ± 0.531 μg.mL^{-1} using by $DPPH^{\bullet}$ assay and 1 ± 0.315 μg.mL^{-1} according to β-carotene-linoleic acid assay [41]. In another study, ethyl acetate extract and water extract of *L. nobilis* leaves exhibited an IC_{50} value of 83.24 μg.mL^{-1} and 161.83 μg.mL^{-1}, respectively, using by $DPPH^{\bullet}$ assay [42]. More studies in the literature indicated that leaf extracts of *L. nobilis* provide significant antioxidant effect [43,44]. Our results are compatible with the literature with respect to antioxidant effect.

3.3. Total Phenolic and Flavonoid Concentrations

Amounts of total phenolic and flavonoid contents in extracts of *L. nobilis* leaves are given in Table 3. The highest amount of total phenolic content with the amount of 54.42±0.14 µg PEs/mg extract was determined in ethyl acetate extract of *L. nobilis* leaves and the minimum total phenolic content with the amount of 11.04±0.20 µg PEs/mg extract was measured in water extract of leaves. According to the results of total flavonoid content assessments of extracts, the highest total flavonoid content was found in ethanol extract (8.60±0.12 µg QEs/mg extract) and the lowest flavonoid content was determined in the hexane extract (1.01±0.10 µg QEs/mg extract) of *L. nobilis* leaves.

When we compared the results obtained, there is a good correlation between high antioxidant activity and high amount of phenolic compounds in extracts [45]. In previous studies, some researchers have found such positive correlations between antioxidant activity and the amount of phenolic compounds of plants [46, 47].

Table 3. Total phenolic and flavonoid concentrations of *L. nobilis* leaf extracts

	Extracts	Total Phenolic Content (µg PEs/mg extract)	Total Flavonoid Content (µg QEs/mg extract)
Laurus nobilis	Hexane	12.80±0.35	1.01±0.10
	Ethyl acetate	54.42±0.14	5.48±0.65
	Ethanol	25.32±0.10	8.60±0.12
	Water	11.04±0.20	1.07±0.10

Results expressed as mean ± standard deviation.

The ethanol and ethyl acetate extracts obtained by the subsequent extraction appear to be rich in phenolic and flavonoid content in both extracts. Ethyl acetate extracts rich in phenolic compounds were found to have highest antioxidant activity in three different methods (β-carotene-linoleic acid, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$).

Based on the results, it can be said that phenolic compounds have an important effect on the antioxidant activities of extracts. In addition to the phenolic concentrations of extracts, flavonoid concentrations of *L. nobilis* leaf extract were investigated. The total amount of flavonoid contents in the ethyl acetate and ethanol extracts with high amounts of phenolic contents were found to be 5.48 ± 0.65 and 8.60 ± 0.12 µg QEs/mg extract, respectively.

3.4. Stability and Organoleptic Tests of Cosmetic Preparation

The results obtained from the stability tests and the organoleptic analyses of the cosmetic formulation made by the cold process following the analysis are given in Table 4. No phase separation was observed in both 15 min analyzes performed on the days 1, 5, 30 and 90 in centrifugation tests. The pH measurements made on the formulation showed that there was no significant change in the pH of the lotion even after 90 days. Only very small changes due to time have been identified. The viscosity of the product has changed to negligible level due to very small pH changes and it maintains product stabilization after 90 days.

The product was homogeneous and easy to spread when it was prepared. Lotion left a soft, non-greasy feel after it was subjected. There was no change in the product's spreadability and appearance after 90 days.

Table 4. Results of stability tests and organoleptic analysis of formulation

Test / Time	1st day	5th day	30th day	90th day
Stability Tests				
pH	5.86	5.87	5.90	5.95
Viscosity(cPs)	14400	14460	14465	14502
Centrifuge	Stable	Stable	Stable	Stable
Organoleptic Tests				
Appearance	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Color	Greeny white	Greeny white	Greeny white	Greeny white
Odor	Laurel odor	Laurel odor	Laurel odor	Laurel odor
Spreadability	Easy	Easy	Easy	Easy

4. CONCLUSION

Nowadays, it is clear to see that the escape from artificial substances will further increase the importance of natural products. With this reason, investigation of composition and biological activities of natural substances is important. In this study, the volatile oil composition of *L. nobilis* leaves was accurately determined by comparative methods using GC/MS instrument. According to the our results, the amounts of 1,8-cineol, *alpha*-terpinyl acetate and *alpha*-pinene are similar to the previous studies made by different researchers. However, the amount of terpinen-4-ol in this study found to be higher than other studies.

The antioxidant potential of the extracts was determined using three complementary methods. The extracts investigated in this study have significant antioxidant activity. The highest antioxidant activity in all assays (DPPH, ABTS, β-carotene) was measured in ethyl acetate extract of *L. nobilis* leaf extract.

L. nobilis volatile oil has potency in cosmetic products due to its numerous antioxidant components. At present, the cosmetic formulation formed within the scope of the study has passed successfully the stability and organoleptic tests that were carried out. At the end of 90 days, there was no significant change in the pH and viscosity parameters of the formulation, and no change in organoleptic properties, such as product appearance, color, odor, spreadability were observed. Good stabilization of the formulation proves that the volatile oil has successfully performed its mission of acting as a preservative in the cosmetic formulation.

In the next step of the study, various dermo-cosmetic effects can be examined of the prepared formulation, and the obtained oil can be used on the formulations in different types of cosmetic products.

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