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Fragrance Component Analysis for Nebulvapours of European Anchovy Oils by Using Colorimetric Printing and Electronic Nose

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Abstract: Analysis of odor components about biochemicals find the wide space in the evaluation of flavor parameters and anchovies as biological materials. Food dye solutions as printer's inks were sprayed on to the fabric throughout the printing operation and skin oil vapors of anchovy were simultaneously sent to the paper hopper of printer intensely via a nebulizer device. Before and after dyeing process, images of tela fabric were taken by smartphone and analyzed by software in the smartphone for the purpose of determination of colorimetric fragrance component concentrations and visual odor profile in range of visible region. The ten major ingredient contents (with relative percentages) (aldehyde compounds intensely such as 2,4-heptadienal (23%), (E,E)-2,4-nonadienal (17%)) of anchovy skin essential oils were determined. For colorimetric printing analysis via smartphone, LOD and LOQ were 1 ppm and 3 ppm, respectively. Methodology can be used in the analysis of toxic components that interact with foods.

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1. INTRODUCTION

Analysis of odor components in foods and chemicals are important in the evaluation of flavor parameters. As biological materials, anchovies stand out with their pungent odor. Anchovies process is located in the top position in Turkey's seafood industry [1]. According to the Turkish statistical data in 2018, while the most produced sea fish in Turkey by fishing was anchovy with 27%, the rate of anchovies used in oil production was 16% [2]. Microbial deterioration, due to the growth of microorganisms naturally present in fish, can occur. As microorganisms grow, they produce nutrients and metabolites such as sulfuric compounds, aldehydes, ketones, esters, trimethylamine and the total volatile base nitrogen [3]. Previous studies reported an increase in protein and relative RNA content of anchovy, a decrease in carbohydrate and relative lipid content of anchovy as the anchovy larvae was growing [4, 5]. In another study, silver ion high-performance liquid chromatography was used to examine the diversity and variations of the molecular triglyceride species derived from fish oils extracted from the laboratory [6]. The selected fish oils were separated and the fatty acid distribution of the fractions was determined by gas-liquid chromatography. Trace amounts of wax esters, free fatty acids or sterols were available in fish oils. The South African anchovy oils contained

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very small amounts of monoenoic acids. The lipids were visualized by spraying with copper (II) sulfate [6].

Smartphone and printer technology stands out in many studies. For example; in immunohistochemical breast cancer study, the differentiation of positive (brown color) and negative (blue color) stained cells by manual counting was evaluated by 3D printed adapter and smartphone used to obtain microscopic images of stained tissue slides [7]. Smartphone biosensors are cost-effective, portable devices that can be used in colorimetric analysis. Smartphone colorimetric readers allow the analysis of photographs of liquid samples taken using a camera by means of software programs [8]. With the combination of digital camera, printer, scanner, desktop or portable computer and proprietary software, the way of captured, identified and dimensioned of image can be changed naturally [9].

Azo group paints have been widely used in cosmetic, tattoo, food and beverages, pharmaceuticals, printing inks, plastics, leather and paper industries [10]. Colorimetric indicators (eg, labels, prints) could interact with target compounds such as CO_2, O_2, ammonia gas and aldehydes to exhibit visible color changes [11, 12]. Synthetic food dyes such as Tartrazine, Allura Red, Brilliant Blue were classified according to the US Food and Drug Administration (FDA) test criteria [13]. Certified food dyes (such as Tartrazine, Allura Red, Brilliant Blue) have been commonly used synthetic compounds because of their color and shelf life stability, so as not to alter food tasting and color [14]. Synthetic paints, which are less expensive and more stable than their natural counterparts, have been used in many commercial products [15]. Food dye mixtures (Tartrazine, Allura Red, Brilliant Blue) were applied to plates such as SiO_2, TiO_2 by inkjet printers used as office chromatography concept, and the separation performance was measured using a smartphone [16]. Enzyme ratios for enzyme systems were obtained by a two-dimensional code design color distribution by a method which uses pressure on inkjet paper [17]. A new colorimetric method based on polyaniline film for the development of intelligent packaging was defined as a chemical sensor for color change of various essential volatile amines, which were microbial degradation products on the surface of the fish [18]. In other study, colorimetric indicators have been created with ink-jet printing technology to develop a CO_2 sensitive colorimetric indicator [19].

Odor is one of the most important parameters when evaluating the freshness of food. Each product has a characteristic profile of volatile compounds and, therefore, its own characteristic odor [20]. The odor caused by fish degradation was attributed to amines (eg; trimethylamine, dimethylamine, ammonia, histamine, putresine, cadaverine), short chain carbonyls, sulfur compounds, aldehydes [21]. DNA-based oligo deoxy floroside was measured using the digital values of red, blue, green, luma signal channels under epifluorescence microscopy by staining for fluorescence via inkjet printing of various dyes on cellulose paper for detection of volatiles resulting from food spoilage [22]. Shima company has improved the print quality for knitwear and knitted fabrics with a new inkjet printer [23]. Aerosols produced from a variety of solutions with high quality programmed print modes using an inkjet printer were characterized by an aerodynamic particle sizer for determining of particle sizes [24]. In patients with cystic fibrosis, inhalation of natural oils by nebulizers was attempted to treat chronic lung infections [25]. Five different formulation containing *Breu* essential oils were evaluated using an inhalation chamber and GC-FID combined with nebulizer for sedative and antinociceptive activities in mice [26].

This study aimed to develop a new method that is practical, fast and cheap and that can be applied accurately and sensitivity in terms of detection limits alternative to expensive GC-MS application. Furthermore, the method will be used in the analysis of foreign volatile components like formaldehyde that can interact with foods. In this respect, nebulvapor safety risk parameters will be minimized at the environment in terms of analysis. Odor components will be measured qualitatively and quantitatively. Without mass detection, density of volatile

components, which have low and high molecular weights, will be visualized with smartphone cumulatively by food staining technique via a printer. The study can shed light on the evaluation of the waste fish skin oil profile and can be applied with 3-D printer technology combined with different technologies like confocal laser scanning microscope in the future.

2. MATERIAL and METHODS

2.1. Chemicals and Materials

Hydrochloric acid (reagent grade, 37%, 25 mL) (CAS No: 7647-01-0) was purchased from Sigma- Aldrich for acidic hydrolysis of anchovy skins for the purpose of obtaining essential oils. Tartrazine (≥85%, 100 g, CAS No:1934-21-0), which is an anionic, hydrophilic azo dye with an orange-yellow color used in fabrics, foods and cosmetics, was purchased from Sigma- Aldrich. Among the other azo food dyes, Allura red (100 g, 80%, E129, CAS No:25956-17-6), which is an effective scatterer in luminescence spectroscopy, was purchased from Merck. Brilliant Blue food dye (E133, blue powder, 85%, 100 g, CAS No: 3844-45-9) was purchased from Sigma- Aldrich. 100% Polyester tela fabric (1 m^2) was purchased from Manufacturer, Trading Company (Guangdong, China (Mainland)) for the purpose of printing paper. Dried anchovy skins (500 g) were purchased from Shandong Kingsun Foods Co., Ltd. (Shandong, China (Mainland)) for essential oil analysis because of the thought with higher oil yield from Turkey anchovies. All dye reagents were solved with ultrapure water produced by using Milli-Q® IQ 7003/7005 Ultrapure Lab Water System (Merck).

2.2. Instruments and Analysis

EPSON A4 size flatbed printer machine (with heat function, print speed:20 ppm, printing size: 300 mm x 210 mm, 90 pieces x 6 colours Nozzle, high quality USB data line and software settings) that is one kind of inkjet printer which can print on almost materials, such as plastic, metal, glass, wood, stone, leather, and fabric, was purchased from Shenzhen Bettsens Industrial Co. Ltd. (Guangdong, China (Mainland)) for printing on tela fabric by using the food dyes as inks. Vivocare Steamy Compressor Jet Nebulizer Device (particle size <4 μm, Bayer company, Germany) was used for vaporizing of European anchovy skin oils (120 mL). Colorful printing distribution with food dyes of European anchovy volatiling oil components on tela fabric captured by smartphone (Samsung Galaxy S5 (Seoul, South Korea)).

The images of color distribution of volatiling components on tela fabric were further analyzed by ImageJ combined with application of Zoner Photo Studio 17 PRO (Brno; Czech Republic) software for color intensity acquiring using for all three channels (R, G and B) in a region on the photograph responding to diameter of the dots on tela fabric matrix [27, 28]. Dried anchovy fish skins (500 g) were powdered via a blender (Model SHB 3062; Sinbo, Istanbul, Turkey). Powders of dried European anchovy fish skins (500 g) were hydrodistilled via a Clevenger-type device (Sesim Kimya Laboratuvar, Ankara, Turkey) by using 980 mL distilled water and 20 mL hydrochloric acid (37%) for 4 h according to modified method of Tural and Turhan [5].

Essential oils of anchovies extracted with acidic hydrolysis were collected to amber coloured vials (Merck, volume 4 mL, glass, 15 mm × 45 mm) and stored in the refrigerated incubator (FOC 215I, Velp Scientifica, TempSoft™ dedicated software for times and temperatures) at 4 °C until used. With electronic nose (PERES foodsniffer (Swiss Technology) uses four sensing nozzle including temperature, humidity, ammonia and volatile organic compound sensors), which measures the levels of volatile organic compounds and works in conjunction with a smartphone, vaporizing chemical compositions of European anchovy essential oils were analyzed safely [29].

2.3. Method Validation

Validation parameters (linear range, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision) were applied for determination of visualizing concentration limits and tela fabric matrix effects about food dye printing applications for analyzing of European anchovy essential oils. The linear regressions (slopes, correlation coefficient (r^2)) of drawn calibration curves by using ImageJ program were used to assess the linear range for concentration measurements based on the color intensity of the painted essential oils on tela fabric with trying triplicate smartphone applications.

The LOD and LOQ (detection limits) were determined by the average of standard deviation of the calibration curves by created via Zoner Photo Studio 17 program in terms of 3 times and 10 times respectively in which the smallest point zones of pictures painted with food dyes taken with the help of smartphone for vaporizing European anchovy essential oil test levels.

To determine the accuracy and precision, in intra-day and inter-day conditions, replicated applications for assessment of visual color intensity with smartphone, were evaluated. In addition, matrix effects caused by dye solvent or tela fabric were eliminated by creating stable zone areas. Color intensity processing data depending on concentration with captured of light signals via smartphone was correctly analyzed by removing dark blind spot signals and colorless zone areas. For validation, databases of painted zone photographs on tela fabric of volatiling components of European anchovy skins were processed by the algorithm in smartphone [30].

2.4. Statistical Analysis

Statistical analysis was carried out by using Microsoft Excel (Microsoft Office Corporation, 2010, Redmond, Washington) and SPSS Version 21.0 software program. All statistical analyses were reported significantly (p<0.05) with standard deviation. Data were processed by ANOVA test.

Table 1. Detection limits and linear working range based on colorimetric color intensity of total dyed zone areas (average particule size: 3 A°) containing essential oil components on tela fabric.

For Total Pointed Picture Areas of Dyed Vaporizing Odor Components Including of Anchovy Oils					
Detection Limits (± SD, n:3)		Linear Working		Color Intensity Recovery (%)	
LOD (ppm)	LOQ (ppm)	Range (n:3)	Equation (r^2: 0.9996)	Intra Day	Inner Day
1+-0.01	3+-0.03	1 ppm-5 ppm	y = 2.08x	96	90

SD: Average Standart Deviation, n: analysis repetition, ppm: parts per million, 95 % confidence interval, critical ratio: p<0.05

3. RESULTS and DISCUSSION

Optimized loaded liposomes were more delivered effectively by an air-jet nebulizer, than a vibrating- nebulizer during 10 min period via the abbreviated impactor in a study [31]. Similar to this conclusion, in this study, with applying in parallel of jet nebulizer, anchovy oil vapors were sent on tela fabric surface in effective rate successfully. Schneider et al. and Sunoj et al. reported that the method based on visualizing phenological comparisons of plants was developed by using a standard ColorChecker chart with six different color schemes coded ImageJ plugin named ‘ColorCal’ (version 1.52d, with [3 × 3] color calibration matrix). The use of three basic color corrections (red, yellow and blue) was sufficient for quality color calibration [32, 33].

In this study, painted colored zone areas of anchovy essential oils were formed on the tela fabric by using tartrazine, allura red and brilliant blue food dyes (250 mg /mL, aquatic form each other) separately via a printer. Pictures of these spot painted fragments were taken with the smartphone. The visible region (400-700 nm) color scale (a standard ColorChecker chart color schemes) generated by 3 basic color patches (red, yellow, and blue) in the smartphone was used as a calibrator. The black and bright light signals and small zone areas (zone radius < 2 A°) were eliminated with the help of the algorithm in the smartphone. Calibration graphs of the color intensities of the zone fields in the captured images were created (Figure 1).

Figure 1. Systematic analysis of nebulous vapors of anchovy essential oils by fabric dye printing combined with electronic nose and smartphone applications.

Detection limits (LOD: 1 ppm, LOQ: 3 ppm) were respectively determined as 3 times and 10 times of the average ratios (slope / standard deviation) obtained from the calibration graphs with triplicate smartphone applications. Linear range was found between 1 ppm-5 ppm (Table 1). For intra-day and inter-day conditions, the differences in color intensity measurements between the wet or dried dyed images of volatile European anchovy oils on tela fabric constituted a standard deviation of approximately 0.03, so it was statistically significant ($p< 0.05$). The light signaling sent from the device by connected to the smartphone device calibration was stable. The method was found to be accurate and precise because recovery of color intensity was high compared with optimal values of color stability in the studies about colorimetric determination of streptomycin with the help of anthocyanin dye and iron (III) in bioethanol fuel via smartphone [34, 35]. A previous study investigated the desirable odor healing of chicken oil after lipoxygenase treatment by the techniques of gas chromatography and sensory evaluation. The amounts of volatile compounds (ethyl acetate, pentanal, 2-pentyl furan, E-2- heptenal and nonanal) found in modified chicken oil was higher than in the original chicken oil [36].

Also, in this study, after essential oil vapor components of dried European anchovy skin powders (500 g) had been painted with food dyes via printer, electronic nose (PERES foodsniffer (Swiss Technology), with combined smartphone odor component library program) was used for odor components analysis of oil vapors, simultaneously.

The data acquisition time was 120 s. 13 odorant components (methane-thiobis, thiophene, toluene + butanoic acid ethyl ester, hexanal, 1-hexanol, 1-octen-3-one, 1-octen-3-ol, dimethyl-

trisulfide, octanal, 1-nonen-3-ol, (E)- 2-nonenal and 2 unknown compounds) of 144 volatile compounds were detected in European seabass flesh via GC-MS in another study [37].

Also, in this study, electronic nose (PERES foodsniffer) is an artificial effective detection equipment simulates the olfactory function of the mammalian nose and is effectively used for food analysis, adsorbed the odor molecules by the sensors and generated signals from sensors were evaluated by connecting to volatile molecules for principal component analysis via smartphone signal processing system and component library (containing volatile molecules database). Electronic nose working principle was explained in a lot of studies [38, 39]. Especially, in a study, electronic nose was applied for analysis of the content of nebulizer vapors in wood vinegar extract of black garlics successfully [40].

Fabric images obtained from the high resolution digital camera image acquisition system which are defined at each thread transition point with a multi-zone fuzzy segmentation-based approach [41]. A microfluidic chemistry analyzer which was consisting of a fan-shaped microchip for simultaneous measurements of glucose, triglyceride and total cholesterol from serum samples has been developed with accurate, reliable and reproducible results via smartphone with specific LED light source and camera [42]. Immediately, a thermal inkjet printer heated the liquid in a microfluidic chamber and was successfully applied onto the print cells exiting the nozzle head by removing the droplets to high velocity and providing the necessary pressure drop [43]. For hypothetical testing of drugs seized in forensic cases, a microfluidic device has been developed which permits multiplex detection of various compounds including cocaine, opiates, ketamine and various phenethyl amines by colorimetric reaction by forming hydrophilic channels on chromatographic paper using wax press and thermal lamination [44].

In this study, as different from the others, nebulizer instead of headspace unit was used for evaporation of European anchovy essential oils in inside the printer tela fabric reservoir. Detected volatiling components with PERES foodsniffer were determined as the percentage of concentration with the help of the smartphone analyzing program which uses molecular library database with network by making signal processing.

The ten major ingredient contents (with relative percentages) of European anchovy skin essential oils were 2,4-heptadienal (23%), 2-pentyl furan (8.5%), pentanal (3.5%), (E,E)-2,4-nonadienal (17%), 1,3-octadiene (5%), 2-ethyl-1-hexanol (3%), p-xylene (2.5%), 2-butanone (15%), 2-methyl-1-pentene (7%), (E)-3-undecene (15.5%) (Table 2).

Table 2. Nebulous steam odor components on tela fabric detected by electronic nose.

Number	Detection Time (tsecond)	Component Names	% Relative Quantity
1	15	2,4-heptadienal	23
2	25.5	2- pentyl furan	8.5
3	33.5	pentanal	3.5
4	45	(E,E)-2,4-nonadienal	17
5	54.5	1,3-octadiene	5
6	61.5	2-butanone	15
7	88.5	p-xylene	2.5
8	98	2-ethyl-1-hexanol	3
9	110.5	2-methyl-1-pentene	7
10	120	(E)-3-undecene	15.5
		Total:	100

Blue colored Brilliant Blue dye interacted with aldehyde compounds, found in the highest concentration of these volatile components which are stained with three different color food dyes via molecular dyeing printing technology. Tartrazine and allura red were also azo food dyes with different colors (yellow and red, respectively) tied with high affinity to other group volatile components especially hydrocarbons on tela fabric. The diagram of multi distribution points including inner and outer zone areas of tela fabric visualized information of dying volatile components (Figure 2).

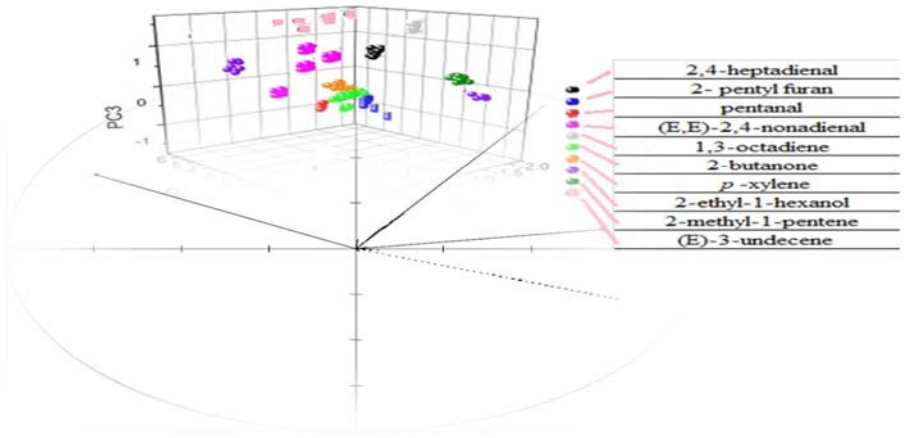

Figure 2. Dyed multi-odor component distribution points including inner and outer zone areas of tela fabric.

4. CONCLUSION

For odor components analyzing of nebulizer vapors of anchovy essential oils, colorimetric printing application and electronic nose detection have been performed firstly and successfully by coordinated with smartphone. Colorimetric printing application method combined with smartphone was validated to determine the colorimetric detection limits of the total volatile components. 10 different compounds (high aldehyde amounts) were detected as relative percentages of concentrations by making multi component analyzes of European anchovy skin essential oil vapors with electronic nose connected smartphone network component library. This methodological study can be used in the analysis of toxic components that interact with foods in the future.

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Effects of Different Growing Media on Plant Growth and Nutrient Contents of Petunia (*Petunia hybrida*)

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Abstract: The objective of this study was to investigate the effects of different growing media on plant growth and nutrient contents of petunia (*Petunia hybrida*). The experiment was conducted in a chamber room under controlled conditions at the laboratory. Seven different media (soil, 3:1 soil: peat (P1), 2:1 soil: peat (P2), 3:1 soil: barnyard manure (BYM1), 2:1 soil: barnyard manure (BYM2), 3:1 soil: sugar beet pulp (SBP1), 2:1 soil: sugar beet pulp (SBP2)) were used as plant growing media. The experiment was ended after three months following transplanting of seedlings. Plant growth and flower parameters and macro-micro nutrient contents were determined in harvested plants. The highest stem diameter, branch number, flower diameter, flower stalk diameter, plant fresh weight and plant dry weight were in 2:1 ratio of soil: peat mixture as 7.00 mm, 6.33, 8.91 cm, 3.59 mm, 48.47 g and 4.52 g while the highest plant lenght, lateral branch number and flower number means were found as 27.43 cm, 24.67 and 24.67 in ratio of 3:1 soil: peat growing media respectively. The highest potassium, magnesium and zinc means of plants were obtained as 6.6%, 2.2% and 32.50 mg kg^{-1} in soil: barnyard manure (2:1) growing media while the highest phosphorus and calcium means of plants were found as 0.27% and 4.5% in soil and peat growing media respectively. The highest iron and copper means of plants were determined as 231 mg kg^{-1} and 32.50 mg kg^{-1} in ratios of 2:1 and 3:1 of soil: sugarbeet pulp growing media respectively.

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1. INTRODUCTION

In the last decades ornamental plants are grown for decorative purposes in garden and landscape design projects, as house plant, for cut flowers. The commercial production of ornamental plants is growing and the related market is developing fast. Petunia (*Petunia hybrida*) belong to the Solanaceae family [1]. Petunias are perennials in warm climates and are used mainly as annual bedding and container plants in temperate regions [2]. Petunias are among the most popular bedding plants in the world because of their adaptability, variety and flower color range. Petunias are quantitative long-day plants flowering under any photoperiod but flowering faster under long days. Short photoperiods (8-10 hours) delay flowering, retard elongation of the main stem and support lateral branching. The average of daily temperature

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degree changes range of in 10-25 ºC for flowering time, plant height and lateral branching [3]. Hybrid petunias are garden standbys from several South American petunia species. These sun and heat-loving annuals or tender perennials were among the first ornamentals grown in the bedding plant market since the 1950's. Petunia cultivated in flower beds and pots requires full sunlight to produce plants and flowers with bright attractive colors. They need abundant sun and grow best in rich soil with good drainage. They bloom best with regular fertilization and will continue to flower all seasons [4].

Increased flower production, quality of flower and perfection in the form of plants are recognised as important issues in bedding and flower production. According to Boodley [5], quality of flowers are considered as function of nutrient level. Nitrogen, phosphorus and potassium enormously influence the production and quality of flowers. Whereas using of synthetic fertilizers and chemicals in flower breeding increase cost of production. In addition these materials can lead environmental pollution problem with potantial hazards to flora, fauna and human.

Under this circumstances, using of organic manures such as compost, vermicompost, barnyard manures and biofertilizers is ecofriendly, easily avaliable and cost effective. It is reported that organic materials provide nutrient conversion of organic fertilizer and mineral fertilizer combination, protection of soil moisture, increasing of cation exchange capacity, improving soil physical and biological properties and providing control of erosion [6-10]. Among the organic materials peat often enchances aeration and water retention [11]. Ko et al. [12] reported that cattle manure compost can be used as a source of soil amendment and organic matter in agriculture which improves the quality of the crop and the environment.

The aim of this study was determine the effects of peat, barnyard manure and sugar beet pulp applications on growth and flowering parameters and nutrient contents of petunia.

2. MATERIAL and METHODS

This experiment was conducted in a randomized experimental design with three replication in a chamber room under controlled conditions at Department of Soil Science and Plant Nutrient Laboratory. Petunia (*Petunia hybrida*) were used as experiment plant in this study. Seven different media (soil, 3:1 soil: peat (P1), 2:1 soil: peat (P2), 3:1 soil: barnyard manure (BYM1), 2:1 soil: barnyard manure (BYM2), 3:1 soil: sugar beet pulp (SBP1), 2:1 soil: sugar beet pulp (SBP2)) were used as plant growing media. Soil and organic materials were mixed according to volume basis. The experiment was ended after three months following transplanting of seedlings. Plant length, steam diameter, branch number, lateral branch number, flower number, flower diameter, flower stalk diameter, plant fresh weight, plant dry weight and macro-micro nutrient contents were measued harvested plants. The nutrient contents of the harvested plant samples were analyzed in dried and grinded plant samples according to following methods reported by Kacar [13]. The P level was analyzed by the spectrophotometric method, and K, Ca, Mg, Fe, Mn, Zn, and Cu levels were determined by using an atomic absorption spectrophotometer (Thermo ICE 3000 series). Soil properties and some properties of growing medias were determined using the standart analyses methods [14]. Physical and chemical properties of the growth media had loamy texture, non saline, slightly alkaline, low in organic matter, insufficient in phosphorus and zinc contents sufficient in calcium, magnesium, manganese and copper contents (Table 1).

Table 1. Properties of the experiment soil

Texture	pH	Salinity	Lime	OM	P	K	Ca	Mg	Fe	Mn	Zn	Cu
		($\mu S\ cm^{-1}$)	(%)	(%)		(mg kg^{-1})						
Loamy	7.81	360.7	3.86	1.32	5.50	298	3034	405	5.58	29.84	0.58	0.81

Some properties of the organic materials used in this study were given in Table 2.

Table 2. Some properties of organic materials used in different growth media

Organic material	pH	EC (mS cm^{-1})	Moisture (%)
Peat	6.52	0.16	47.79
Barnyard manure	8.66	8.55	6.78
Sugar beet pulp	4.02	0.55	89.57

pH levels of organic materials were determined as close to neutral, alkaline and acide for peat, barnyard manure and sugar beet pulp respectively. When the salinity of organic materials were evaluated barnyard manure was found saline while peat and sugar beet pulp were non saline. Sugar beet pulp had the highest water content according to other organic materials. The lowest water content were determined in barnyard manure. Variance analyses of the experimental data were done by SPSS statistical program [15].

3. RESULTS and DISCUSSION

The variance analyses of results and the effects of different growth media on plant growth in petunia are given in Table 3 and 4 respectively. According to the variance analyses different growing media significantly ($p<0.01$) influenced all of the plant growth parameters (Table 3).

Table 3. Variance analyses of the results for plant growth in petunia

V. Source	DF	Plant length	Stem diameter	Branch number	Lateral branch number	Flower number	Flower diameter	Flower stalk diameter	Plant fresh weight	Plant dry weight
Application	6	21.34**	6.692**	12.77**	41.02**	27.40**	17.88**	5.26**	74.624**	53.31**

**significant at 0.01

When it was noticed that Table 4, the highest means belong plant growth parameters were shown with peat applications. The highest plant length, lateral branch number, flower number were obtained as 27.45 cm, 24.67 and 24.67 in 3:1 ratio of soil: peat mixture (P1) while the highest stem diameter, branch number, flower diameter, flower stalk diameter, plant fresh weight and plant dry weight were in 2:1 ratio of soil: peat mixture as 7.00 mm, 6.33, 8.91 cm, 3.59 mm, 48.47 g and 4.52 g respectively. Generally plant growth parameters and flower parameters means obtained in barnyard manure and sugar beet pulp applications were lower according to those obtained in control and peat applications (Figure 1 and 2). The lowest means of plant length, stem diameter, lateral branch number, plant fresh weight and plant dry weight were found as 8.33 cm, 2.67 mm, 2.00, 0.93 g and 0.12 g in 2:1 ratio of soil: barnyard manure mixture (BYM2). In this study the means of plant growth parameters and flower parameter means obtained by different organic metarials exhibit the following decreasing trend: peat>sugar beet pulp>barn yard manure. Generally the decreases in plant growth and flower growth were determined by increasing amounts of barnyard manure and sugar beet pulp in their mixtures by soil. Although increasing amounts of peat in its mixtures by soil increased plant and flower growth, the means of plant and flower growth parameters indifferent peat ratios were in same group according to Duncan's multiple comparison test. The high pH level and salinity in barnyard manure lead to decreases plant and flower growth. Similarly, sugar beet pulp having low pH level may have been caused decreasing in plant and flower growth. It was known that soil properties of plant growth media have very importance for nutrient uptake, grow and yield. It was reported that sugar beet pulp and cattle manure addition to soil improve

soil quality, soil organic matter, soil aggregat stability, water holding capacity and nutrient content (especially N, P and K) [12,16,17].

Table 4. Effects of different growth media on plant growth in petunia

Application	Plant length (cm)	Stem diameter (mm)	Branch number	Lateral branch number	Flower number	Flower diameter (mm)	Flower stalk diameter (mm)	Plant fresh weight (g)	Plant dry weight (g)
Control	20.67b	3.17b	1.00b	7.00b	7.00b	6.60b	2.72bc	9.03b	0.85b
P1	27.43a	6.83a	4.67a	24.67a	24.67a	8.83a	3.27ab	45.10a	4.20a
P2	26.40a	7.00a	6.33a	23.33a	23.00a	8.91a	3.59a	48.47a	4.52a
BYM1	9.17cd	3.25b	1.00b	3.33b	3.33b	2.83d	2.50bc	1.93b	0.26b
BYM2	8.33d	2.67b	1.00b	2.00b	2.50b	3.10cd	2.25c	0.93b	0.12b
SBP1	14.50c	4.67b	2.67b	6.00b	3.67b	4.78c	2.08c	4.37b	0.50b
SBP2	10.50cd	3.50b	1.67b	3.67b	3.50b	4.63cd	2.22c	2.60b	0.34b

(P: Peat; BYM: Barnyard manure; SBP: Sugar beet pulp)

Effects of different growth media on plant growth parameters and flower parameters are also given in Figure 1 and 2.

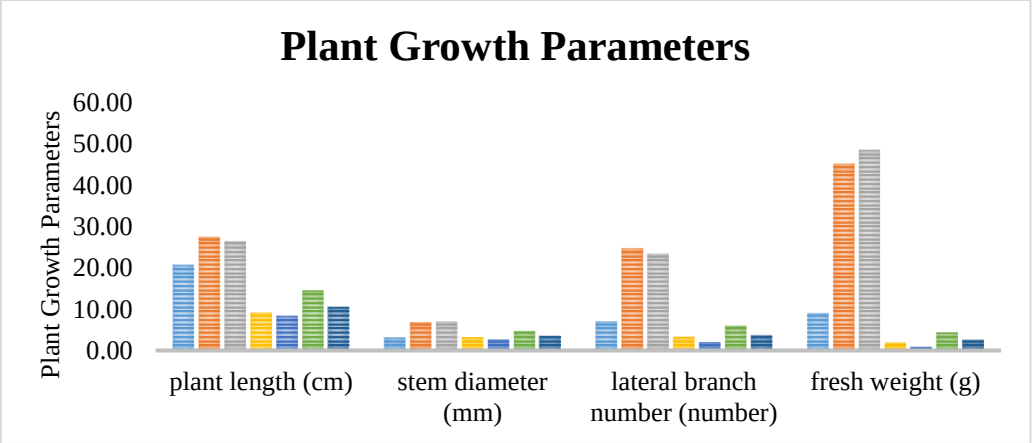

Figure 1. Effects of different growth media on growth parmeters in petunia

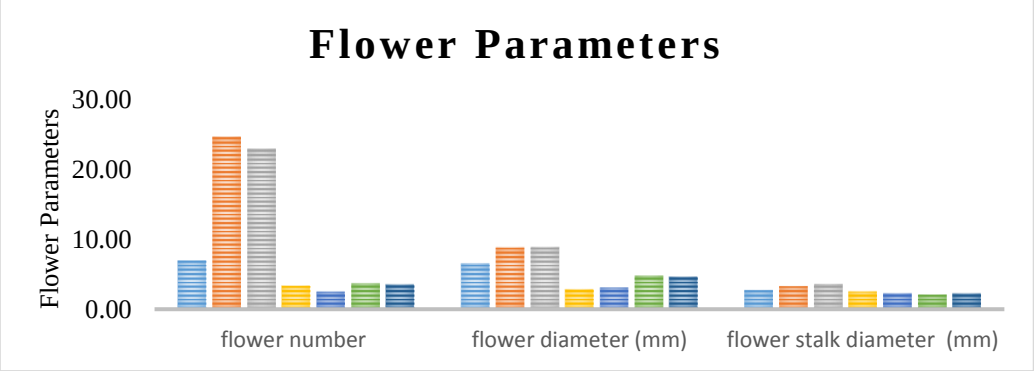

Figure 2. Effects of different growth media on quality parameters in petunia

Our results were correspond with the referred literature knowledges about sugar beet pulp and cattle manure. Nkongolo et al. [18] and Gülser [19] reported that peat addition to soil improved plant growth in *Tagetes*. Chamani et al. [20] reported that flower number, leaf length, plant fresh and dry weight increased by vermicompost and peat applications according to control application. Increasing flower production, flower quality and perfection in the forms of plant are the important goals in bedding and flower production. The flower quality was considered as a function of nutrient level [5]. Although nitrogen, phosphorus and potassium considerably influence flower quality and production they lead directly on cost of production. In addition, application of synthetic fertilizers and chemicals in high level lead environmental problem and damage to flora, fauna, and human. In recently, depletion of non renewable resources and environmental deterioration together with their high prices lead utilization of alternative materials as peat, zeolite, perlite animal manure, compost and various organic wastes. In this study, addition of peat to growth media increased plant and flower growth while sugar beet pulp and barnyard manure had non ameliorative effect according to control. It was thought that changes in plant and flower growth in different growth media caused by properties of organic materials. James [21] reported that petunia and begonia can grow in growth media having EC levels in range of 1.7- 6.1 and 2.1- 5.4 dS m^{-1} respectively. It was thought that high EC level of baryard manure and low level of sugar beet pulp influenced negatively plant and flower growth of petunia. The variance analyses of results and the effects of different growth media on nutrient contents in petunia are given in Table 5 and 6 respectively.

Table 5. Variance analyses of the results for nutrient contents in petunia

V. Source	DF	P	K	Mg	Ca	Fe	Mn	Zn	Cu
Application	6	6.325**	38.45**	6.21**	8.07**	6.22**	7.02**	14.63**	1.80**

**significant at 0.01

Table 6. Effects of different growth media on nutrient contents in petunia

Application	P (%)	K (%)	Ca (%)	Mg (%)	Fe (mg kg^{-1})	Cu (mg kg^{-1})	Zn (mg kg^{-1})	Mn (mg kg^{-1})
Control	0.27a	4.55cd	4.47a	1.90ab	174.00ab	25.00a	28.26ab	158.91bc
P1	0.19c	4.56cd	4.42a	1.41c	59.60c	17.58ab	17.80c	135.82c
P2	0.20bc	4.84c	4.47a	1.38c	44.65c	16.79ab	18.96c	131.30c
BM1	0.25ab	5.74b	4.42a	2.13a	113.63bc	13.94b	31.28ab	163.73bc
BM2	0.27a	6.63a	4.41a	2.25a	100.19bc	17.71ab	32.50a	140.77c
SBP1	0.21bc	4.10de	3.68b	1.47bc	160.57ab	32.50a	27.37b	181.77b
SBP2	0.17c	3.82e	3.40b	1.46bc	230.78a	21.29ab	28.31ab	232.93a

(P:Peat; BM: Barnyard manure; SBP: Sugarbeet pulp)

According to the variance analyses different growing media significantly ($p<0.01$) influenced P, K, Ca, Mg, Fe, Mn, Zn and Cu contents. The highest macro nutrient contents means were obtained in BM growth media except Ca. While the highest micro nutrient contents means in SBP growth media except zinc. The highest Ca and Zn contents means were found as 4.47% and 32.50 mg kg^{-1} in P2 and BM2 growth media (Figure 3 and 4). In this study obtained nutrient contents were correspond with the nutrient contents reported for the other plants belong Solanaceae family such as tomato, potato, eggplant [22]. When nutrient contents levels were evaluated according to reported limit values for eggplant (*Solanum melongena*) were not found deficiencies except iron (< 50 mg kg^{-1}) and zinc (< 20 mg kg^{-1}) contents obtained in P2 and P1

growth media respectively (Figure 4). The pH has a major role in the availability of nutrient ions. It was known that Marschner [23] macronutrients and micronutrients are more available at levels of ≥ 7 pH and ≤ 7 pH respectively. Sugar beet pulp has lower pH level than those in the other materials. So micronutrients contents were obtained in high amounts in this growth media compaire to the other materials.

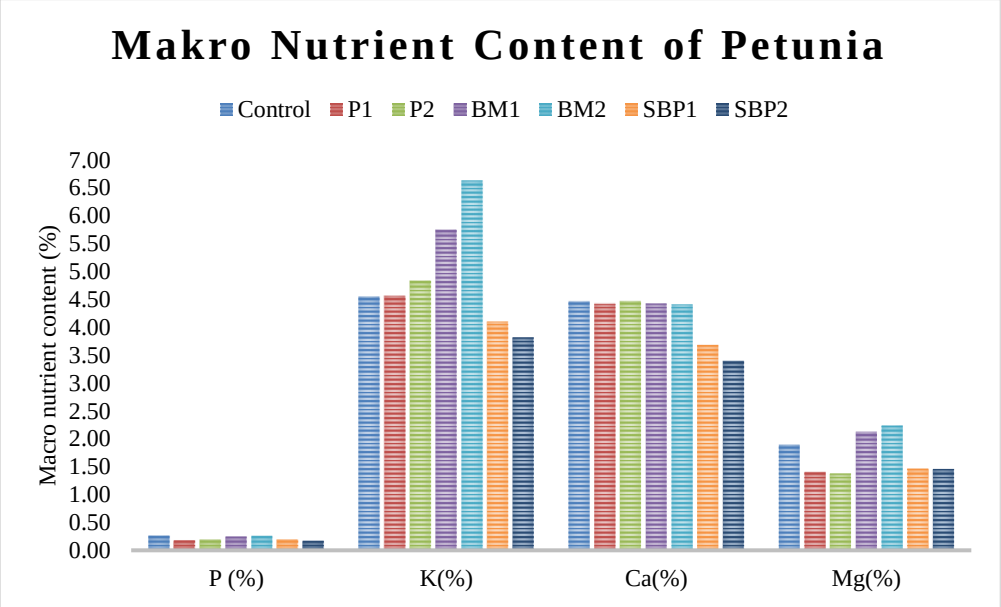

Figure 3. Effects of different growth media on macro nutrient contents in petunia

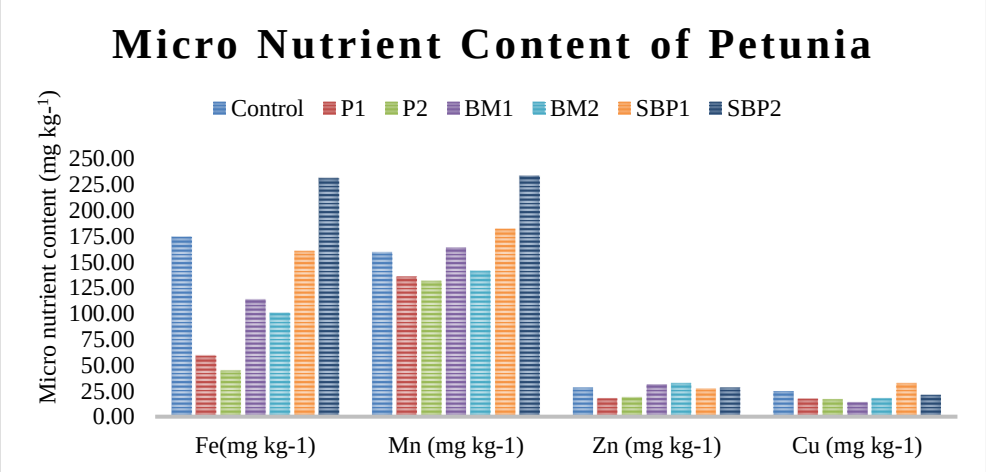

Figure 4. Effects of different growth media on micro nutrient contents in petunia

Our results were correspond with the reffered literature knowledges. [24] reported that addition of sugar beet pulp to soil increased organic matter level. Cattle manure compost addition to soil improve soil quality, soil organic matter, soil aggregat stabilty, water holding capacity and nutrient content (especially N, P and K) [16,17,25]. Raviv et al. [26] reported that peat moss serves as the main component of transplant growth media, mainly due to the following physical and chemical properties: adequate Free Air Space (FAS) at 0-10 cm water suction; high water content at low tension at 10-100 cm water suction; high Cation Exchange Capacity (CEC) which minimizes loss of nutrients and facilitates adequate mineral nutrition. Inbar et al. [27] reported that during last two decades due to improved popularity for protected agriculture, evolution of plant growth techniques has increased demand for container substrates such as peat, zeolite and perlite but supply have been decreasing. Depletion of non

renewable resources and environmental deterioration with high price of those substrates have favored the utilization of alternative materials as growth substrates [28].

4. CONCLUSION

As a result, using of peat can be useful for petunia growth in bedding and flower production. In addition, it can be suggested investigates about separeted mixtures of barnyard manure and sugar beet pulp with soil in different ratios in growth media for petunia breeding. Additionally, it was thought that peat may be beneficial in the stuation of application as combined with barnyard manure and sugar beet pulp for optimum nutrition in petunia.

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Conflicts of Interests

Authors declare that there is no conflict of interests.

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In vitro and *in silico* Evaluation of Some Natural Molecules as Potent Glutathione Reductase Inhibitors

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Abstract: Glutathione reductase inhibitors are very popular antimalarial and anticancer agents. In this study, *in vitro* inhibition effects of *β*-sitosterol, stigmasterol, diosgenin and jervine which containing steroidal structure were determined against glutathione reductase enzyme. *β*-sitosterol, diosgenin and jervine were isolated from *Veratrum album* and stigmasterol was isolated from *Artemisia dracunculus* L. by chromatographic methods. According to the results obtained, IC_{50} values of *β*-sitosterol, stigmasterol, diosgenin and jervine were found as 1.2580, 5.2116, 0.1916 and 0.7701 µM, respectively. Among test compounds, diosgenin showed the strongest inhibitory effect against glutathione reductase with Swissdock docking figure. In current study first time, *β*-sitosterol, stigmasterol, diosgenin and jervine were found to be much more glutathione reductase inhibitors.

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1. INTRODUCTION

Glutathione reductase (GR, EC 1.8.1.7), found in most organism is an important homodimeric flavoprotein and an antioxidant enzyme in protection a critical intracellular reducing environment against oxidative stress. It catalyzes the reduced glutathione (GSH) generation from oxidized glutathione using nicotinamide ademine dinucleotide phosphate (NADPH). Oxidized glutathione is generated via the oxidation of GSH by oxidants such as reactive oxygen species (ROS) that occur during oxidative stres. The cell is protected against oxidative stress through discontinuation of oxidants by GSH. GSH prevents the removal of oxygen radicals and lipid intracellular peroxidation. It is a reaction partner for the detoxification of endobiotics and xenobiotics, and is the mode of storage and transport of cysteine. It has important functions in the protection of the cell against oxidative stress, in the maintenance of thiol redox potential in the cell and in the production of deoxyribonucleotides. The lack of GR and GSH leads to oxidative damage in the cell, which can cause many diseases such as malaria and cancer [1-5].

Malaria is an essential worldwide most dangerous disease and seen especially in tropical regions. The most fatal malaria parasite is *Plasmodium falciparum*. The antimalarial drug developing is very difficult due to this parasite has high mutation rating [6]. Because, when the drugs destroy sensitive parasites, the resistant mutant parasites reproduce and infect other host.

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It has been reported in the literature, *P. falciparum* developed resistance to chlorokine, which is commonly used as an antimalarial drug [7]. Antimalarial drugs target GR enzyme inhibition and host cells in the malaria parasites. GSH plays a fundamental role in antioxidant defense in both malaria parasite and host cells [6].

The steroidal compounds *β*-sitosterol (1), stigmasterol (2), diosgenin (3) and jervine (4) are important bioactive natural products that are quite common in plants and animals (Figure 1) [8, 9]. Jervine is the major active steroidal alkaloid compound found in *Veratum* species belonging to the *Liliaceae* family. It was reported that jervine is a teratogenic ingredient responsible from the malformation in birth after *Veratrum* species were consumed by pregnant animals [10]. It has been determined to inhibit the hedgehog signaling pathway, which is directly associated with cancer cell proliferation [11]. *β*-sitosterol, a steroid found in almost all plant species, has been reported to display a wide variety of biological activities, such as estrogenic, immunomodulatory, anti-arthritic, antioxidant, and anti-ulcer [9, 12-14].

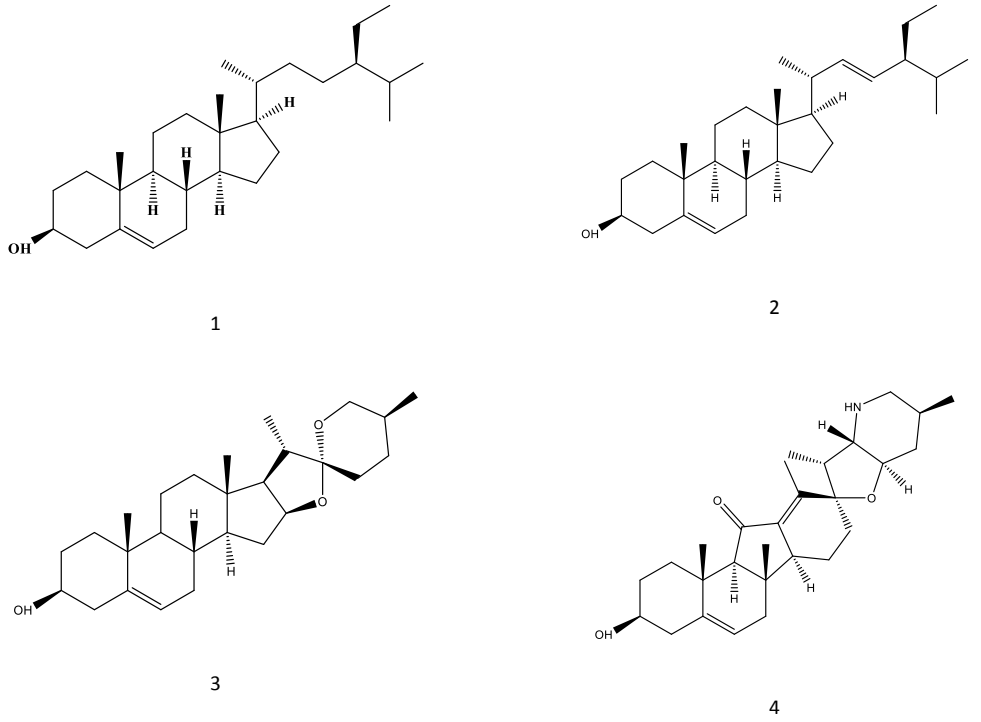

Figure 1. The chemical structures of test compounds 1-4. *β*-sitosterol (1); stigmasterol (2); diosgenin (3); jervine (4).

Diosgenin, which is used as a starting material for the synthesis of hormones cortisone and progesterone, is a very important natural steroidal sapogenin using in synthesize of new synthetic drugs. It has proapoptotic and anticancer properties [15]. In the previous study by our team, the stigmasterol was isolated from tarragon (*Artemisia dracunculus* L.) [16]. Stigmasterol is a common plant steroid and has anti-inflammatory and anti-angiogenic activities [17].

In this study, we have tried to find out that *β*-sitosterol, stigmasterol, diosgenin and jervine can enter the enzyme active site more easily compared to bulky molecules such as NADPH and GSH, which are the substrates of GR. Thus, it was considered to evaluate these substances as GR inhibitors and to obtain important pharmacological data. Compounds 1-4 are important steroidal bioactive natural compounds, and no studies have been conducted related to the inhibitory effect of these metabolites on GR enzyme. In study first time, the inhibitory effects of *β*-sitosterol, stigmasterol, diosgenin and jervine were investigated on the GR enzyme.

2. MATERIAL and METHODS

2.1. Plant Materials and Chemicals

As in our previous study, *β*-sitosterol, diosgenin and jervine compounds were isolated from rhizomes of *Veratrum album* and stigmasterol was isolated from *Artemisia dracunculus* L. [9, 16]. GR enzyme was purchased from Sigma-Aldrich. All other chemicals used in the study were purchased commercially from Tekkim and Sigma-Aldrich.

2.2. Extraction and Isolation

β-sitosterol, diosgenin and jervine compounds were purified from the acetone extract of *Veratrum album* according to the procedure in our 2014 study [9]. The stigmasterol was purified from the dichloromethane extract of *Artemisia dracunculus* L. according to the procedure in our previous study [16]. The spectroscopic findings of the compounds were showed as in the our previous study [9, 16].

2.3. Inhibition of GR

GR enzyme activity was measured by Beutler's method [18]. An enzyme unit was defined as 1 µM NADPH oxidation per minute under test conditions (25 °C, pH: 8.0). Using a spectrophotometer, a time of 3 minutes was determined at 340 nm absorbance. Different inhibitor concentrations were used and compounds were tested in triplicate at same process. Control cuvette activity was accapted as 100% in the absence of inhibitor. An Activity %−[Inhibitor] graph was drawn for each inhibitor [19 ,20].

2.4. *In silico* Docking Studies

In silico docking studies were performed in order to investigate the interactions between structure containing molecules and amino acid residues within the GR active site. *In silico* docking figures were taken from Swissdock.

3. RESULTS and DISCUSSION

GR is a primary enzyme which maintains the reduced state of cell. Therefore lack of GR is be directly associated with various disease related to oxidative damage [21]. In this study, we report *in vitro* inhibitory effects of the steroidal natural compounds *β*-sitosterol, stigmasterol, diosgenin and jervine on GR enzyme. These compounds were found to be highly potent inhibitors of GR enzyme at micromolar level as shown in the Table 1. The IC_{50} (µM) values of the compounds was determined as 1.2580, 5.2116, 0.1916, 0.7701 for *β*-sitosterol, stigmasterol, diosgenin and jervine respectively. The IC_{50} value of the *N*,*N*-bis(2-chloroethyl)-*N*-nitro used as reference substance was obtained from the literature as 647 µM [22].

Table 1. IC_{50} values, FullFitness and Estimated ΔG scores of test compounds on GR enzyme.

Inhibitor	IC_{50}, µM	FullFitness, kcal/mol	Estimated ΔG, kcal/mol
β-sitosterol	1.2580	-2327.41	-7.30
Stigmasterol	5.2116	-2334.39	-6.76
Diosgenin	0.1916	-2347.70	-8.24
Jervine	0.7701	-2347.71	-7.75
N,*N*-Bis(2-chloroethyl)-*N*-nitrosourea	647	-	-

As we were unable for the moment to crystallize a GR in complex with one of the natural steroidal structure containing molecules, we performed *in silico* docking studies in order to investigate the interactions between natural steroidal structure containing molecules and amino acid residues within the GR active site. To this end, fully flexible docking methodology for

both receptor residues (1GRA pdb file was used for the target structure) and docked ligands was used by FullFitness and Estimated ΔG which was implemented with the Swissdock. *β*-sitosterol, stigmasterol, diosgenin and jervine were docked at the binding site of the target (hGR). FullFitness and Estimated ΔG scores of docked inhibitors at hGR targets and corresponding binding interactions were shown in Table 1. Also diosgenin docking figure of GR enzyme was shown in Figure 2.

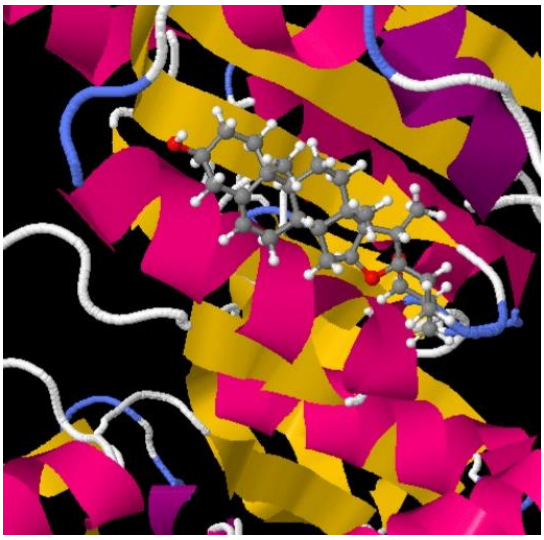

Figure 2. GR enzyme with diosgenin docking figure.

Among the test compounds, the diosgenin showed the strongest effect and stigmasterol showed the weakest effect. When we compare these results with the docking results, docking supports the results that diosgenin is the most potent GR inhibitor. Besides, it appears that the diosgenin is a stronger GR inhibitor than the reference compound *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea.

In literature, IC_{50} values of 1,4-naphthoquinone, 4-nitrobenzothiadiazole and methylene blue which are inhibitors of *P. falciparum* GR, have been reported as 2.71, 8.38 and 19.23 µM respectively. These inhibitors were greatly reduced GSH formation [4]. In a study of some *N*-methyl pyrrole derivatives, 0.104 to 4.942 µM results were obtained for GR enzyme [23]. Similar results were obtained in other studies on GR enzyme of natural substances such as thiamine, tyrosine, dopamine, lysine and glutamic acid [24, 25]. These values are similar to our results.

In terms of structure and activation relationship, steroidal molecules with little electron density have shown better GR inhibitor effect. The only difference in the chemical structure of 1 and 2 compounds is the double bond in the stigmasterol. According to results, *β*-sitosterol with IC_{50} value 1.2580 µM is a more potent GR inhibitor than stigmasterol. This result can be interpreted as stigmasterol has higher IC_{50} value because its double bonds is surrounded by hydrophobic amino acids. GR has a better inhibitory effect on structures with less electron density. Jervine has more electronegative atoms. Therefore, it has less interaction with the active site of GR enzyme. In the current study, IC_{50} results support this.

The results of our study showed that diosgenin was a very potent GR inhibitor with an IC_{50} value, 0.1916 µM (3376 times more effective than the reference molecule). So we may suggest to diosgenin as potent GR inhibitor. Diosgenin is a raw material in the drug industrial that using in the synthesis of steroidal agents such as progesterone, testosterone, norethisterone and glucocorticoids. It has cardioprotective, anticancer, antiaging and contraceptive properties and high economic value. Progesterone which produced by many pharmaceutical companies is synthesized from diosgenin [26].

4. CONCLUSION

In the current study, it has been presented *β*-sitosterol, stigmasterol, diosgenin and jervine as novel GR inhibitors that have IC_{50} values at micromolar level. The most widely used anticancer drug *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea in the literature has an 647 µM IC_{50} value against the GR enzyme. However, this drug which is used as a GR inhibitor, leads to toxicity and inhibition of DNA synthesis [22]. We report natural steroidal compounds as novel natural GR inhibitors. The IC_{50} values of the substances used in the study were found to be significantly lower than the positive control so these compounds were evaluated as strong inhibitors. Strong inhibitors may exhibit inhibitory effects at low concentrations, therefore it is thought that their side effects will be less. In this study, the natural steroidal molecules were exhibited much potent inhibitory activities against GR with IC_{50} values ranging between 0.1916 and 5.2116 µM. Inhibition of GR leads to death of malaria parasite and cancer cell due to an abnormal increase in ROS levels. Therefore strong GR inhibitors are very important for antimalarial and anticancer drug research and development.

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Antioxidant, Antibacterial and Antifungal Activities of Different Extracts of *Silybum marianum* Collected from Duhok (Iraq)

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Abstract: In this study, antioxidant, antibacterial and antifungal activities of *Silybum marianum* (L.) Gaertn. collected from Duhok (Iraq) were determined. Ethanol, methanol and dichloromethane extracts of the fruit part of plant were obtained. Antioxidant potential was determined with TAS and TOS kits using ethanol extracts. Antibacterial and antifungal activity were determined using agar dilution method. Antibacterial activity was determined against 6 bacterial strains (*Staphylococcus aureus*, *S. aureus* MRSA, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*). Antifungal activity was determined against *Candida albicans*, *C. krusei* and *C. glabrata*. As a result of the studies, it was determined that plant extracts have high antioxidant activity. It was also found to be effective against bacteria at 25-400 µg/mL concentrations. Plant extracts were found to be more effective against gram negative bacteria. It was found to be effective against *Candida* species at 400-800 µg/mL concentrations. As a result, it was determined that the fruit parts of plant could be a natural antioxidant and antibacterial source.

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Antifungal,
Medicinal Plants,
Silybum marianum

1. INTRODUCTION

Traditional medicine practices are common in many parts of the world. It is particularly common in China, India, Japan, Pakistan, Sri Lanka and Thailand. In China, about 40% of medical consumption comes from traditional medicines [1]. Iraq, where the study material is collected, hosts many natural plants due to its geographical diversity and climatic conditions. Iraq is located north and east of the mountainous Turkey and Iran regions and has a similar geography. In Iraq, 363 medicinal plant species belonging to approximately 98 families and 270 genera have a natural distribution [2]. In this study, *S. marianum* plant collected from Duhok region of Iraq was selected as the material. *S. marianum*, a member of Asteraceae, is a one-year herbaceous plant. The plant spreads naturally in Europe, Africa and Asia [3]. In previous studies, antioxidant, hepatoprotective, protective effect against DNA damage, anti-aging, anti-aflatoxin, antibacterial and immunomodulatory activities of *S. marianum* were

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reported [4-8]. In this study, it was aimed to determine the antioxidant, oxidant, antibacterial and antifungal activities of *S. marianum* fruit parts. *S. marianum* is consumed extensively by local people. Therefore, it was aimed to determine the antioxidant activity and activity of the plant. In addition, by determining oxidant level and oxidative stress index, it was aimed to determine the oxidative risk status in consumption.

2. MATERIAL and METHODS

Samples of *S. marianum* were collected from Iraq's Duhok-Bamarne region. Flora of Iraq was used to identify the plant [9]. Herbarium specimens were collected at Zakho University, Faculty of Arts and Sciences, Department of Biology herbarium.

2.1. Laboratory studies

Plant samples collected from the field were brought to the laboratory under appropriate conditions. The samples were allowed to dry in a shade and breathing environment. Dried marianum samples were cut and pulverized. 30 g of the fruit parts of the plant were extracted with soxhlet extractor with ethanol (EtOH), methanol (MeOH) and dichloromethane (DCM) at 50 ° C for about 6 hours. The extracts obtained were concentrated by rotary evaporator.

2.2. Antioxidant, Oxidant and Oxidative stress Tests

The antioxidant and oxidant values of EtOH extracts of *S. marianum* were determined using Rel Assay TAS and TOS kits [10,11]. Trolox was used as a calibrator for TAS tests. Hydrogen peroxide was used as a calibrator for TOS tests. Oxidative stress index (OSI) (Arbitrary Unit = AU) value was determined according to the following formula.,

$$\text{OSI (AU)} = \frac{\text{TOS, } \mu\text{mol } H_2O_2 \text{ equiv./L}}{\text{TAS, mmol Trolox equiv./L X 10}}$$

2.3. Antibacterial and Antifungal Activity Tests

Antibacterial and antifungal activity capacities of EtOH, MeOH and DCM extracts of *S. marianum* were determined using agar dilution method. Minimal inhibitor concentrations (MICs) of the extracts were tested against standard bacterial and fungus strains. *Staphylococcus aureus* ATCC 29213, *S. aureus* MRSA ATCC 43300, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606 were used for antibacterial activity. *Candida albicans* ATCC 10231, *C. krusei* ATCC 34135 ATCC 13803 and *C. glabrata* ATCC 90030 strains were used for antifungal activity. Bacterial strains were pre-cultured on Muller Hinton Broth medium. *Candida* strains were pre-cultured on RPMI 1640 Broth medium. Turbidity of bacteria and fungi was prepared according to McFarland 0.5 scale to obtain a standard inoculum. Plant extracts were tested at 12.5-800 µg/mL concentrations. Dilutions were made with distilled water. Fluconazole and Amphotericin B were used as reference drugs for *Candida* strains. Amikacin, Ampicillin and Ciprofloxacin were used as reference drugs for bacterial strains. The lowest concentration that prevents the growth of bacteria and fungi were determined as MIC [12-17].

3. RESULTS and DISCUSSION

3.1. Antioxidant and Oxidant Potential

Antioxidant-based drug formulations are used in the prevention and treatment of serious health problems such as atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer [18,19] Therefore, it is very important to determine the natural materials that can be used in

antioxidant drug formulations. In this context, antioxidant and oxidant potentials of *S. marianum* were determined. In addition, oxidative stress status was determined due to TAS and TOS values. The values obtained are shown in Table 1.

Table 1. Antioxidant, Oxidant status and Oxidative stress index of *S. marianum*

	TAS (mmol/L)	TOS (µmol/L)	OSI (TOS/(TASx10))
S. marianum	5.767±0.128	12.144±0.060	0.211±0.003

Values are presented as mean±SD; Experiments were made in 5 parallels

Different researchers in different parts of the world have reported that *S. marianum* has antioxidant activity. In a study conducted in Egypt, ethanol extracts of the air-dried plant material of *S. marianum* were used and reported to have high antioxidant activity [20]. Methanol and hexane extracts of *S. marianum* seeds were used in the study conducted in Greece. Seed extracts of *S. marianum* collected from 30 different regions of Greece have been reported to have antioxidant potential [21]. It was reported that ethanol and petroleum ether extract of the seeds of *S. marianum* collected from Turkey were the antioxidant potential [22]. In China, ethanol extracts of leaf, man stem, root, fruit receptacle and pappus of *S. marianum* were used. Main stem showed the highest antioxidant activity. The lowest antioxidant activity was determined in extracts of root and pappus parts. In addition, *S. marianum* has been reported to have strong antioxidant activity [23]. In a study conducted in Russia, ethanol extracts of fruit parts of *S. marianum* were reported to have antioxidant activity [24]. In our study, fruit parts of *S. marianum* were used and it was found to have antioxidant potential. TAS, TOS and OSI values of *S. marianum* were determined for the first time. In studies on different plant species, TAS value of *Rosa canina* collected from Turkey was reported 4.602 mmol/L, TOS value was 6.294 µmol/L and OSI value 0.138 [25]. TAS value of *Salvia multicaulis* collected from Turkey was reported 6.434 mmol/L, TOS value was 22.441 µmol/L and OSI value 0.349 [26]. TAS value of *Rhus coriaria* var. *zebaria* collected from Iraq (Duhok) was reported 7.342 mmol/L, TOS value was 5.170 µmol/L and OSI value 0.071 [27]. TAS value of *Mentha longifolia subsp. longifolia* collected from Turkey was reported 3.628 mmol/L, TOS value was 4.046 µmol/L and OSI value 0.112 [28]. Also, TAS values of *Thermopsis turcica, Brassica rapa* and *Calendula officinalis* were reported as 2.06, 1.25 and 5.55 mmol/L, respectively [29-31]. The TAS value indicates the level of antioxidant compounds produced in the plant. The TOS value indicates the levels of oxidant compounds produced in the plant. OSI value indicates how much antioxidant compounds produced in plants suppress oxidant compounds. Compared to these studies, TAS value of *S. marianum* used in our study was higher than *M. longifolia* subsp. *longifolia, R. canina, T. turcica, B. rapa* and *C. officinalis*. It is lower than *R. coriaria* var. *zebaria* and *S. multicaulis*. TOS and OSI values of *S. marianum* was lower than *S. multicaulis, R. canina* and *R. coriaria* var. *zebaria*. Also TOS and OSI values of *S. marianum* was higher than *M. longifolia subsp. longifolia*. As a result, *S. marianum* has a high TAS value. In this context, it was determined that the fruit parts of *S. marianum* could be used as a natural antioxidant source.

3.2. Antibacterial and Antifungal Activity

Since ancient times, many plant species have been a source of medicine for humans. Evidence has been found that the Neanderthals who lived in Iraq 60.000 years ago used plants such as hollyhock. To date, plant species containing a lot of information on ethnobotany have been widely used. Today, almost half of the plant species used as medicines in the United States are used for antimicrobial purposes. Unconscious use of antibiotics in humans has led to the formation of resistant forms of microorganisms. For this reason, the antibiotics used in the market are insufficient in the fight against microorganisms [32,33]. In this context,

determination of antimicrobial potential of plant species is very important. In this study, the lowest extract concentrations of *S. marianum* that prevent the growth of bacteria and fungi were determined. The findings are shown in Table 2.

Table 2. Antibacterial and Antifungal Activity of *S. marianum*

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*
EtOH	100	100	400	25	50	100	400	400	400
MeOH	100	200	400	50	50	400	800	800	800
DCM	200	200	400	50	50	400	800	800	800
Ampicillin	1.56	3.12	1.56	3.12	3.12	-	-	-	-
Amikacin	-	-	-	1.56	3.12	3.12	-	-	-
Ciprofloksasin	1.56	3.12	1.56	1.56	3.12	3.12	-	-	-
Flukanazol	-	-	-	-	-	-	3.12	3.12	-
Amfoterisin B	-	-	-	-	-	-	3.12	3.12	3.12

*(A) *S. aureus*, (B) *S. aureus* MRSA, (C) *E. faecalis*, (D) *E. coli*, (E) *P. aeruginosa*, (F) *A. baumannii*, (G) *C. glabrata*, (H) *C. albicans*, (J) *C. krusei*
*800, 400, 200, 100, 50 and 25 µg/mL extract concentrations

Previously, n-hexane extract of seeds of *S. marianum* collected from Turkey was reported to be effective against *P. aeruginosa, E. coli, S. aureus, Salmonella typhi, Aspergillus niger* and *C. albicans* at different concentrations [34]. Methanol, n-hexane and chloroform extracts of *S. marianum* collected from 10 different regions of Pakistan were reported to be effective against *E. coli, Salmonella* spp., *Shigella* spp., *S. aureus* and *V. cholerae* at different concentrations [35]. Aqueous and methanol extracts of *S. marianum* collected from Algeria were reported to be effective against *S. aureus, E. coli, Klebsiella pneumoniae, P. aeruginosa, Enterobacter aerogenes* and *C. albicans* at different concentrations [36]. In our study, EtOH, MeOH and DCM extracts of fruit parts of *S. marianum* were used. EtOH extracts of plant showed the highest activity. Plant extracts were generally found to be more effective against gram negative bacteria (*E. coli, P. aeruginosa* and *A. baumannii*). Antifungal activity of plant extracts was found to be low. Plant extracts were found to be effective against bacterial species at 25-400 µg/mL concentrations. It was found to be effective against *Candida* species at 400-800 µg/mL concentrations. As a result, it was determined that the fruit parts of *S. marianum* could be a natural antibacterial agent against the tested microorganisms.

4. CONCLUSION

In this study, antioxidant, oxidant, antibacterial and antifungal activities of fruits parts of *S. marianum* collected from Duhok (Iraq) region were determined. Fruit parts of the plant have been found to have high antioxidant activity. In addition, plant extracts were found to be more effective against gram negative bacteria. The antifungal activity of the plant extracts were found to be low. As a result, it was determined that the fruit parts of *S. marianum* could be natural antioxidant and antibacterial agent.

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Soil Properties and Mineral Nutrients of Clementine Mandarine (*Citrus reticulata* Blanco) Grown in the Koycegiz Region of Mugla Province

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Abstract: Soil properties of the samples from orchards and the nutrients (macro- and microelements) in the clementine mandarin (*Citrus reticulata* Blanco), widely grown in the Köyceğiz region of Muğla Province Turkey, were studied. Mandarin tree leaves and soil samples were collected from 10 different orchards. The soil samples were analyzed for its pH, $CaCO_3$, EC, sand, organic matter, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu) and boron (B) while leaves were analyzed for its macro- and microelements. The results obtained from soil analysis showed suitable amount of calcium carbonate and EC. Analysis of the soil showed that organic matter, N, K and Mn were insufficient in all orchards, while Fe was higher in amount. Slight alkaline, strong alkaline and neutral pHs were determined in the orchards. Besides, the leaf samples collected from the orchards reflected deficient amount of N and Ca while higher amount of Mg and Fe.

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1. INTRODUCTION

Plants need to be balanced with the necessary nutrients for their growth and increase in yield. The deficiency of one or more nutrients in the available form significantly affects soil fertility and plant development [1]. As in all plants, the effects of fertilizers, applied to especially perennial plants such as fruit trees, on yield and quality have been proved by many studies. Since fruit trees are perennial plants, compared to single-year plants, it is much more important to determine the correct amount of nutrients to be applied for their fertilization and to confirm the effects of fertilization on product quantity and quality [2].

Citrus trees are comprised of a group of plants including citrus fruit tree species with high economic value such as orange (*Citrus sinensis*), mandarin orange (*Citrus reticulata*), lemon (*Citrus lemon*), grapefruit (*Citrus paradisi*), bitter orange (*Citrus aurantium*) and bergamot (*Citrus bergamia*). Citrus fruits are the most produced fruit species in the world with a production of approximately 136 million tons. 52.63% of the world citrus production is orange, 21.13% is mandarin, 11.19% is lemon, 6.22% is grapefruit and the rest is other citrus fruits [3].

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Total citrus production in our country is 3.783,263 tons. Nearly 47.04% of Turkey's citrus production is orange, 27.67% is mandarin, 19.16% is lemon, 6.06% is grapefruit and 0.07% is citrus fruits [4].

Due to its temperate climate as well as its 1124-kilometer coastline, Mugla city has important agricultural potential and suitable conditions for the cultivation of almost all agricultural products. In Mugla, citrus is the largest fruit crop in terms of production amount [5]. Agricultural production, especially citrus production, stands out in 3 districts of Mugla, which are Koycegiz, Dalaman, and Ortaca, and 90% of citrus fruits are cultivated in these districts [6]. Hamitkoy, Zaferler, Dogusbelen, Toparlar, Beyobasi, Kavakarasi, Yesilkoy, Koycegiz village, and Koycegiz county center are important citrus production areas. The majority of citrus fruits in Koycegiz are Washington oranges. However, due to its higher economic returns, it is observed that there has been some transition to Valencia-type orange in recent years [7].

This study aims to determine the mineral nutritional status and soil properties of Clementine mandarin (*Citrus reticulata* Blanco) cultivated in Koycegiz, Muğla. As a result of this study, stimulating/ directive contributions have been made in terms of fertilization programs via informative meetings held with the producers.

2. MATERIAL and METHODS

The locations of the Clementine mandarin (*Citrus reticulata* Blanco) where mandarin samples were obtained are shown in Table 1. Samples for this study were collected from 10 different Clementine mandarin orchards.

Table 1. The locations of the Clementine mandarin (*Citrus reticulata* Blanco) and name of the producers where leaf and soil samples were obtained.

Orchard Number	Name of the owner	Acreage (decare)	Number of trees	Tree age (year)	Latitude	Longitude	Location
1	Cemil Ölemez	4	80	30	36°57'28.87"N	28°36'41.69"E	Mugla Koycegiz
2	H. İbrahim Kaya	2	80	20	36°58'1.34"N	28°36'14.13"E	Mugla Koycegiz
3	Mehmet Ölemez	5	200	42	36°59'1.28"N	28°36'12.55"E	Mugla Koycegiz
4	H. İbrahim Kaya	2	90	20	36°58'54.94"N	28°36'10.12"E	Mugla Koycegiz
5	Mehmet Ölemez	4	140	42	36°59'22.82"N	28°36'41.93"E	Mugla Koycegiz
6	Elif Sertel	7	180	20	36°57'48.55"N	28°39'36.68"E	Mugla Koycegiz
7	Niyazi Çetinkaya	5	175	50	36°57'53.52"N	28°39'17.51"E	Mugla Koycegiz
8	Hüseyin Demirkol	4	200	40	36°58'26.59"N	28°40'16.49"E	Mugla Koycegiz
9	Niyazi Çetinkaya	4	120	60	36°58'51.01"N	28°40'7.93"E	Mugla Koycegiz
10	Yusuf Çatak	6	150	20	36°58'45.53"N	28°40'44.82"E	Mugla Koycegiz

2.1. Soil and leaf sampling and analysis

Representative soil samples were collected at a 0–30 cm depth, sampling in a "W" pattern, using a 5 cm diameter auger, after removing the aboveground biomass in early September. Sand, silt and clay fractions were measured by hydrometer method according to Bouyoucos [10], soil pH and the amount of salt by 1:2.5 soil-water mixture method according to Jackson [11], soil organic matter content by Walkley and Black [12] wet oxidation method, lime content according to Allison and Moodie [13] in calcimetry, nitrogen in soil by Keeney and Bremner [14] theoretical method, phosphorus spectrophotometrically according to Olsen et al. [15], K, Ca and Mg by ICP-OES method according to Thomas [16], Fe, Zn, Mn, Cu and B were measured by ICP-OES method according to Lindsay and Norvell [17].

The method proposed by Chapman [8] was taken into consideration when leave samples were collected. In the designated orchards, the middle leaves of 6-7 month spring growths which are human height were taken as samples from all over the trees by drawing zig zags. Leaf samples were prepared in the laboratory for analysis as reported by Kacar and Inal [9]. Determination of plant nutrient contents to eliminate possible contamination in leaf samples, they were dried to constant weight in the oven at 65-70 ^{o}C after being washed with tap water and pure water. The samples were homogenized by grinding to a particle size of less than 0,5 mm. The total nitrogen content of leaf samples burned by the Kjeldahl method was measured by steam distillation [18]. To determine the amount of other nutrients, samples were dissolved by dry combustion method [19] and then, phosphorus, potassium, calcium, magnesium, iron, zinc, manganese, copper and boron concentrations of the filtrated leaves were determined in ICP-OES device [9].

3. RESULTS and DISCUSSION

3.1. Analysis Results of Orchard Soils

As a result of the analyzes, some physical and chemical properties of the soils belonging to the orchards were given in Table 2. Basic reference values at the evaluation of the results were given in Table 3 and Table 4. The highest pH value of orchard soils was determined as the highest (8.56) in the 5th orchard whereas the lowest (7.46) was in the 8th orchard. The average pH value of all orchard soils was observed as 8.08 (Table 2) and basic properties (Table 4). Mendilcioglu [20] reported that the pH limit values for citrus fruits should be between 5 - 8.5. It was seen that 80% of the research orchards were between the limit values. The EC values of the orchard soils were observed as the lowest in the 10th orchard (0.08 mS/cm) and as the highest in the 7th orchard (0.27 mS/cm) and the average EC value of the orchard soils was measured as 0.16 mS/cm (Table 2). As a result of their study, Waters et al. [21] reported that the limit values for EC should be between 1.51–2.25 mS/cm. When the limit values are taken into consideration, it is seen that all the research orchards can be classified as salt-free and there is no problem with salinity (Table 3).

The organic matter content of the soils belonging to these orchards was found to be the lowest in the orchard 2 (0.54%) and the highest in the 4th orchard (2.77%). The average organic matter content of the orchards was 1.37% (Table 2). According to Anonymous [22], the limit value of organic substances in mandarin-cultivated soils should be between 2.01 and 3.0%. It is possible to say for the research orchards that 40% of them were very low, 40% of them were low and 20% of them were moderate (Table 3).

3.1.1. Macro and Microelement Status of Orchard Soils

The total nitrogen content of the soils belonging to the research orchards was measured as the lowest in the 2nd orchard (0.03%) and the highest in the orchard 4 (0.14%) while the average total amount of N was observed to be 0.06% (Table 2). As a result of Chapman's study

[8], it was reported that N limit values for mandarin should be between 0.11–0.15%. When the amount of N in the orchards is examined, it is possible to say that 40% of them have very low, 50% of them have low and 10% of them have a moderate amount of N (Table 3). In their study, Saatci and Mur [23] looked at the N contents of the orchards cultivated by Satsuma mandarin plants and stated that the N contents in soils varied between 0.12-0.47%. When the available Phosphorus concentration of orchard soils was examined, it was measured that it varied between 5.51-24.2 ppm and the average value was 13.10 ppm (Table 2). It has been revealed by the analyzes that 20% of these orchards have a low-level and 80% of them have a sufficient level of phosphorus (Table 3). In their study, Hakerlerler et al. [24] stated that the P concentrations of the orchards varied between 5.6-80 ppm. In this respect, our study was found to be consistent with the literature. When the changeable Potassium concentration of orchard soils was examined, the lowest level was observed in the 10th orchard (36.88 ppm), the highest level in number 4 orchard (105 ppm) and the average changeable K values of the orchards were determined as 65.6 ppm (Table 2). When the amount of K in the orchards was examined, it was seen that 30% of them were too little and 70% of them were less (Table 3). Li et al. [25] found that the amount of K in the soil of grapefruit orchards in the Fujian region of China varied between 35-645 ppm. This literature knowledge supports our study.

The changeable Calcium concentration of horticultural soils was measured as low as 557 ppm and as high as 3143 ppm. The average Ca value was determined to be 1875.2 ppm (Table 2). When the limit values were taken into consideration and the Ca values of the orchards were analyzed, it was measured that 30% of them were low and 70% of them were sufficient (Table 3). Kilic [26] suggested that the concentration of Ca in the orchards he investigated in the Gumuldur region ranged from 2850 to 4740 ppm. Saatci and Mur [23] found in their study that the concentration of Ca in the orchards was between 2900–4500 ppm values. When these studies are evaluated in general terms, it is possible to say that they support our study. The changeable Magnesium concentration in the soils belonging to these orchards varied between 461-2704 ppm, and the average value was revealed by the analyzes as 1138.5 ppm (Table 2). Considering the limit values, 10% of the orchards in which the research was conducted were sufficient, 60% of them were more than sufficient and 30% of them had too much magnesium. (Table 3). Hakerlerler et al. [24] measured the concentration of Mg in the soil as 116-480 ppm in their Satsuma mandarin research conducted in Gumuldur and Balcova. Our study is consistent with the literature. While the iron (Fe) concentration of the soils belonging to these orchards was 11.49 ppm, the highest value was found to be 24.79 ppm and the average value was measured as 17.84 ppm (Table 2). The iron concentration in all of these orchards was revealed to be a high amount by analyzes (Table 3). As a result of the study that Surwase et al. [27] carried out in India, the Fe values of orange orchards were measured to be between 8.64–18.6 ppm and these results show similarity with our study.

The Manganese concentration of the orchard soils was measured at 5.33 ppm as the lowest and at 12.98 ppm as the highest, the average was observed as 9.08 ppm (Table 2). It was seen that all of these orchards were low in Manganese (Table 3). In a study conducted in Izmir, it was measured that the Mn composition of the orchards was between 1.45-3.29 ppm [23]. When this literature review is taken into account, it is observed that it is supportive of our research findings even if it is different in terms of the limit values. The available Manganese which is deficient according to the limit values should be increased to the required level for yield and quality [28]. The available Zinc (Zn) composition in these subject soils was found to vary between 0.69-6.52 ppm and the average value was measured as 1.71 ppm (Table 2). According to these limit values, it can be said that 10% of the available Zinc value was less, 80% of it was sufficient and 10% of it was more (Table 3). In Kilic's [26] study conducted in Gumuldur, the Zn composition of soils cultivated by citrus was determined (0.59-9.13 ppm).

Table 2. Physical and chemical properties of orchard soils

Orchard Number	N (%)	P	K	Ca	Mg	Fe (ppm)	Mn	Zn	Cu	B	pH	EC (mS/cm)	Lime (%)	Organic Matter (%)	Texture
1	0.04	10.5	48.25	2530	498	15.91	6.28	0.69	1.52	0.51	8.35	0.14	6	0.73	Loamy
2	0.03	14.73	40.34	1743	461	15.19	5.33	0.8	0.8	0.35	8.04	0.13	3.7	0.54	Loamy
3	0.1	5.98	91.12	2930	2704	24.16	12.98	1.17	2.48	0.49	8.39	0.24	11	1.98	Silty-Clay
4	0.14	24.2	105	3143	1669	11.49	9.1	2.41	2.32	1.06	8.08	0.23	14.8	2.77	Silty-Clay
5	0.1	17.76	68.87	2104	1394	14.77	7.95	6.52	2.45	0.59	8.56	0.19	11.4	2.04	Clay-Loamy
6	0.06	11.43	54.06	2229	999	16.1	8.71	0.86	1.72	0.38	8.24	0.19	5.5	1.25	Loamy
7	0.06	5.51	67.44	2354	1516	14.07	10.53	0.97	1.86	0.63	8.52	0.27	5.5	1.25	Clay-Loamy
8	0.05	9.71	68.44	570	785	24.79	8.16	1.09	2.87	0.44	7.46	0.1	2.1	0.92	Loamy
9	0.05	19.52	75.6	557	766	22.42	9.84	1.24	1.95	0.38	7.73	0.09	2.1	0.95	Sandy-Clay-Loamy
10	0.06	11.71	36.88	592	593	19.56	11.92	1.37	1.96	0.41	7.5	0.08	1.4	1.27	Loamy
Average	0.06	13.10	65.6	1875.2	1138.5	17.84	9.08	1.71	1.99	0.52	8.08	0.16	6.35	1.37	

Table 3. Reference values are taken as basis in the evaluation of the analysis results of soil samples

Soil Properties	Symbol	Unit	Low	Medium-Low	Suited	High	Very High	References
Total Nitrogen	N	(%)	< 0.05	0.06–0.10	0.11–0.15	0.16–0.20	> 20	Chapman [8]
Available Phosphorus	P	(ppm)	< 3.0	3.0–7.0	7.1–25.0	> 25		Anonymous [22]
Exchangeable Potassium	K	(ppm)	< 50	50-200	201-250	251-320	> 320	Anonymous [37]
Exchangeable Magnesium	Mg	(ppm)	< 55	55-115	116-475	476-1500	> 1500	Anonymous [37]
Exchangeable Calcium	Ca	(ppm)	< 714	714-1438	1439-3862	3863-6108	> 6108	Anonymous [37]
Available Iron	Fe	(ppm)	< 2.5	2.5-5	6-10	11-25	> 25	Lindsay ve Norvell [17]
Available Zinc	Zn	(ppm)	< 0.2	0.2–0.7	0.8–2.5	2.6-8	> 8	Lindsay ve Norvell [17]
Available Manganese	Mn	(ppm)	< 4	4-14	15-50	51-170	> 170	Lindsay ve Norvell [17]
Available Copper	Cu	(ppm)		< 0.2	> 0.2			Lindsay ve Norvell [17]
Extractable Boron	B	(ppm)	< 0.4	0.4–1.0	> 1.0			Lindsay ve Norvell [17]
Organic Matter		(%)	< 1.0	1.0–2.0	2.01–3.0	3.01–4.0	> 4,0	Anonymous [22]
Lime		(%)	< 1.0	1.0–5.0	5.1–15.0	15.1–25.0	> 25	Allison ve Moodie [13]
Salt		mS/cm	< 0.50	0.50–1.50	1.51–2.25	> 2.25		Waters vd. [21]

Table 4. Reference values based on soil pH assessment

Soil Properties	Strong acid	Mid-Acid	Low acid	Neutral	Alkaline	Strong alkaline	Reference
pH	< 4.5	4.5–5.5	5.6–6.5	6.6–7.5	7.6–8.5	> 8.5	Jackson [11]

When the available Copper (Cu) concentration was considered in these orchards, the lowest value (0.8 ppm) was measured in the 2nd orchard, the highest one (2.87 ppm) was in the 8th orchard and the average amount of available Cu in these soils was 1.99 ppm (Table 2). When the soils analyzed were compared with the determined limit values, it was proved by the data that there was no deficiency in Cu (Table 3). Li et al. [25] in their study suggested that the Cu content ranged between 0.01-29.62 ppm in the soils of grapefruit orchards in the Fujian region of China. Similar values were obtained in our study.

The extractable Boron (B) content in these soils was measured to be between 0.35-1.06 ppm analysis (Table 2). When the limit values were examined, it was found that 30% of the orchards were less, 60% of them were little and 10% of them were sufficient in extractable Boron (Table 3). Saatci and Mur [23] measured that the B compositions of the orchards in Izmir where Satsuma mandarins were grown were between 0.30-0.80 ppm. Similarly, Papadakis et al. [29] claimed that the amount of B in the soils in Greece where mandarins were grown varied between 0.53-0.62 ppm. The results of both studies support our study. According to the B limit values in these soils, 90% of the orchards were low in Boron.

3.1.2. Nutrient content of leaf samples

Macro (N, P, K, Mg, Ca) and micro (Fe, Zn, Mn, Cu, B) nutrient amounts of the Clementine mandarin leaves were given in Table 5. References used in the evaluation were given in Table 6.

Table 5. Nutrient contents of leaves of Clementine mandarin orchards.

Orchard	%					ppm				
Number	N	K	Mg	P	Ca	Fe	Mn	Zn	Cu	B
1	2.41	1.09	0.6	0.13	2.91	277	10.45	17.44	3.99	43.43
2	2.84	0.93	0.62	0.14	2.41	248	218	248	7.09	17.68
3	2.73	0.75	0.71	0.16	2.42	209	15.05	16.92	23.04	30.1
4	2.43	0.92	0.7	0.14	2.64	250	193	281	11.06	29.69
5	1.97	0.91	1.11	0.17	2.21	237	41.69	60.01	15.97	45.31
6	2.52	0.97	0.67	0.19	1.93	180	14.71	17.61	14.77	28.48
7	2.29	1.18	0.75	0.19	2.04	262	17.88	18.96	13.32	26.7
8	2.3	1.03	0.71	0.14	1.61	294	12.58	18.99	13.19	236
9	2.46	1.03	0.7	0.14	2	220	14.25	21.23	12.85	169
10	2.23	0.73	0.79	0.16	2.53	247	12.5	20.02	22.22	177
Average	2.41	0.95	0.73	0.15	2.27	242.4	55.01	72.01	15.75	80.33

The lowest N content of the leaves was found at 1.97% and the highest at 2.84%. The average value of N% in the plant leaf samples taken from all orchards was determined to be 2.41% (Table 5). Considering the limit values, it was observed that the N content of Clementine mandarin plant leaves of all the orchards subjected to the research was deficient (Table 6). Cakmak et al. [30] reported that the average value of N% of mandarin leaves in the Cukurova region was 2.58%. In this literature study, similar results to our study were found.

According to the analysis results, K % content in leaves was measured between 1.18-0.73% and the average K value of leaf samples taken from all orchards was determined to be 0.95% (Table 5). When the limit values were examined, it was determined that 20% of Clementine mandarin leaves were deficient, 70% of them were sufficient and 10% of them were more in K content (Table 6). In a study conducted in Dortyol, Hatay, the amount of K% in citrus leaves in 2004 was determined as 1.15% [31]. This study is consistent with our research. When the amount of P% in the leaves was examined, the lowest was measured at 0.13%, the highest at 0.19% and the average P-value was measured as 0.15% (Table 5). When these values were examined, it was determined that 50% of the orchards were deficient at a level that could be regarded insignificant in terms of P content of Clementine mandarin leaves, but the leaf P content in the rest of the orchards was sufficient (Table 6). In Kilic [26] 's study in which he evaluated the nutritional status of citrus leaves in Gumuldur, it was reported that the P content was between 0.12-0.18%. This research supports our study. When the analysis results of our research were examined, it was determined that the Mg% content in the leaves ranged from 0.6% to 1.11% and the average value was measured to be 0.73% (Table 5). According to the limit values, it was determined that the Mg content of all the Clementine mandarin leaves was high (Table 6). Jian et al. [32] found that the Mg content of citrus leaves in the Fujian, China varied between 0.20-0.22%. Erdal et al. [33] argued that even the same species of plants grown in different ecological conditions might have different leaf analysis results. According to the results of our study, the Ca% content in the leaves was measured at the lowest in the 8th orchard (1.61%) and the highest in the 1st orchard (2.91%) while the average value was measured as 2.27% (Table 5). When we compared our results with the limit values, it was observed that all the Clementine mandarin leaves were deficient in the Ca content (Table 6). Ranjha et al. [34] reported that the Ca content of citrus leaves in the Sahiwal region of Pakistan was between 5-22%. The Ca deficiency in leaves was thought to be caused by the antagonistic effect between Fe and Ca.

According to the results of the analysis, the Fe% content in the leaves was between 180-294 ppm and the measured average value was 242.4 ppm (Table 5). When the limit values were considered, it was observed that the Fe content of 100% of Clementine mandarin leaves was high-level (Table 6). Kilic [26] found in his study in Gümüldür that the Fe content was between 54-115 ppm. This research; however, is in contradiction to our study. The lowest Mn% content in leaves 10.45 ppm and the highest value was determined as 55.01 ppm while the average value was 218 ppm (Table 5). According to the limit values, 70% of the Mn content of Clementine mandarin leaves was deficient, 10% of them were sufficient and 20% of them were found to be high in concentration (Table 6). Kaplankıran et al. [35] found that the average value of Mn of Valencia orange leaves grafted on local citrus fruits was 91.52 ppm. This result supports our study. The highest Zn% content in leaves was observed in the 4th orchard (281 ppm) while the lowest content was in 3rd orchard (16.92 ppm) and the average value was measured as 72.01 ppm (Table 5). According to these values, while 70% of the Zn content of the Clementine mandarin leaves were determined to be sufficient, 30% were determined to be high in concentration (Table 6). Toplu et al. [31] found in their study in Hatay the average Zn amount of citrus plant leaves was 28 ppm. Similar results were found also in our study. According to the analysis results, the Cu% content in the leaves ranged between 7.09-23.99 ppm and the average value was 15.75 ppm (Table 5). When the limit values were considered, it was found that 60% of Clemantine mandarin leaves were sufficient and 40% were more in Cu concentration (Table 6). In the study conducted in Gumuldur, the Cu content of the leaves was found to vary between 4 and 62 ppm [26].

Table 6. Reference values of nutrients for the Clementine mandarin plant [9].

Element	Low	Medium	High
N %	< 3.00	3.00–3.40	> 3.40
P %	< 0.15	0.15–0.25	> 0.25
K %	< 0.90	0.90–1.10	> 1.10
Ca %	< 3.00	3.00–5.00	> 5.00
Mg %	< 0.17	0.17–0.40	> 0.40
Fe (ppm)	< 60	60–150	>150
Mn (ppm)	<25	25–100	>100
Zn (ppm)	< 5	5–29	> 29
Cu (ppm)	< 6	6–15	>15
B (ppm)	< 30	31 – 100	> 100

According to the results of the analysis, the lowest B concentration was measured in the 2nd orchard as 17.68 ppm and the highest was measured as 236 ppm. The average B value was observed at 80.33 ppm (Table 5). According to the limit values, it was determined that 50% of the Clementine mandarin leaves were deficient, 20% of them were sufficient and 30% of them were high in B concentration (Table 6). Jian et al. [32] suggested that the B amounts of citrus leaves in the Fujian, China ranged between 20-150 ppm. The result of this research is similar to our study. The use of foliar fertilizer to remove Boron deficiency in leaves was suggested to be a helpful method [36].

4. CONCLUSION

Plant analysis is complementary to soil analysis. It does not indicate the amount of nutrients present in the soil, but how much the plant can benefit from the nutrients in the soil. As a result of this thesis, It was determined that there were nutrition problems related to significant plant nutrients in both soil and leaf samples. To eliminate these problems, it is necessary to increase the amount of organic matter in the soil and, through the soil, mandarin orchards should be given the nutrients they need the most. For this purpose, periodical soil and plant leaf analyzes should be done, a general nutritional status should be revealed and fertilization programs recommended by experts should be followed.

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Characterization of Secondary Metabolites in Two *Cousinia* species

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Abstract: *Cousinia* is one of the widespread genera of Asteraceae family. According to previous studies on some *Cousinia* species it was found that these species are rich of triterpenes, sesquiterpenes, flavonoids, acetylenes and steroids. According to our knowledge, there are no published reports on the chemical composition of *C. iconica* Hub. - Mor. and *C. aintabensis* Boiss. & Hausskn., thus we aimed to investigate secondary metabolites of these species. In this study, the phytochemical constituents of these species were evaluated. Seven of identified compounds were quantified. The quantitative and qualitative determination of compounds within the extracts was carried out by LC-MS/MS. Phytochemical analyses revealed the presence of flavonoids, saponins, terpenes and steroids. Preliminary examination of the mass spectrums revealed the presence of phenolic acids and derivatives and flavonoid compounds in extracts. According to quantitative analyses the main compound of *C. iconica* (CI) and *C. aintabensis* (CA) extracts was rutin with the highest contents (169.779 µg/mg$_{extract}$ and 161.638 µg/mg$_{extract}$). Moreover, qualitative and quantitative study combined with different biological activities will shed new lights to the advanced studies.

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1. INTRODUCTION

Cousinia Cass. is one of the widespread genera of Asteraceae family with 600-700 species distributed in Central and South-West Asia. There are 39 species and 6 sections of *Cousinia* genus in Turkey [1]. In the literature, taxonomic and systematic studies are generally performed on the genus of *Cousinia*, but phytochemical and activity studies are rarely seen. Numerous studies have shown that plants of the genus are rich in triterpenes, sesquiterpenes, flavonoids, acetylenes and steroids [2-8]. In phytochemical studies it was reported isolation of guianolide type sesquiterpenes from *Cousina picheriana* Bornm. ex Rech.f., *C. piptocephala* Bunge. and *C. canescens* DC. [2], oxygenated bisabolene derivatives from *C. canescens* DC., phenolic and triterpenic compounds from *C. adenostica* Bornm., *C. aitchisonii* Boiss. [5, 9-12] and fatty acids from *C. aurea* C.Winkl., *C. seversovii* Regel, *C. umbrosa* Bunge. [13, 14]. In a study, ethanol extracts from different *Cousinia* species were subjected to cytotoxic screening on the fibrocarcinoma cell line. The highest activity was observed in *C. verbascifolia* Bunge. (IC_{50} =

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18.4 ± 0.59 µg / mL) [15]. According to Iranshahy et al. sesquiterpene compounds namely desoxyjanerin and raserolit obtained from the dichloromethane extract of *C. aitchisonii* were subjected cytotoxic screening on five different cell lines. As a result, both compounds showed significant cytotoxic effect on breast cancer MCF-7 cell line (IC_{50} = 4.5 µg / mL and 4.6 µg / mL, respectively) [10]. In another study MMP inhibitor effect of *C. shulabadensis* Attar & Ghahr. was investigated and reported to have a considerable inhibitory effect (IC_{50} = 49.2 ± 0.51 µg / mL) [16]. In a study by Shahverdi et al. the antibacterial effects of ethanol extracts of seven different *Cousinia* species was investigated by disk diffusion method in different Gram (+) and Gram (-) strains and the highest effect was determined in *C. phyllocephala* Bornm. & Gauba extract against *Staphylococcus aereus* and *Bacillus subtilis* (MIC = 4 mg / disc) [15]. *Cousinia iconica* Hub.- Mor. from Cousinia section is endemic to Turkey and distributed in open areas, scrublands and stony slopes. *Cousinia aintabesis* Boiss. & Hausskn. is a perennial herb with purple flowers from Cynaroidae Bunge. section [1]. Because a few studies were reported on these species we aimed to investigate the phytochemical properties of these species. In this study, phytochemical profile of methanol extracts and quantitative analyses of determined compounds were quantified.

2. MATERIAL and METHODS

2.1. Chemicals

All chemicals, standarts and reagents were analytical or HPLC grade and purchased from Sigma-Aldrich.

2.2. Plant material and preparation of extracts

The flowering aerial parts of *C. iconica* was collected from Konya and *C. aintabensis* from Mardin in July 2013. The voucher specimens were deposited at the Herbarium unit of the Science Faculty, Selcuk University, Konya, Turkey (Voucher No. 1, KNYA 11.040; Voucher No. 2, KNYA 77.81 respectively). Air dried aerial parts of *C. iconica* (500 g) and *C. aintabensis* (500g) were powdered and extracted three times with methanol by maceration, at room temperature. Combined macerates filtered and evaporated to dryness under reduced pressure at 37°C using a rotary evaporator. The crude extracts were stored in a dark at -20°C. Yields of methanol extracts of CA and CI were %10 and %15 respectively.

2.3. Preliminary phytochemical analysis

The secondary metobilites of CA and CI extracts were evaluated by following standard methods [17-19].

2.3.1. Test for carbohydrates

Fehling's test: 2 mL of Fehling A and 2 mL of Fehling B was added to 1 mL of test solution in a test tube and carefully heated in a water bath. Precipitation of red Cu_2O indicated the presence of reducing sugars.

Benedict's test: 1 mL of test solution was taken in a test tube and 2mL of Benedict's reagent was added to test solution. The mixture was boiled, and a reddish-brown precipitate was occurred. This result indicated the presence of the carbohydrates.

2.3.2. Test for flavonoids (Shinoda test)

The crude extract was taken in a capsule and 5 mL of a mixture of ethanolic hydrochloric acid (ethanol-HCl-water 1: 1: 1 v / v) was added. Finally, 5-6 magnesium ribbon was added in this mixture. Appeared Pink scarlet color indicated the presence of flavonoids.

2.3.3. Test for saponins

Crude extract was shaked with 5mL of distilled water in a test tube. The formation of stable foam was indicated the presence of saponins. Liebermann's test: Crude extract which mixed about 2 mL of chloroform is evaporated to dryness on a water bath in a porcelain capsule. Then the residue was dissolved by the addition of 1 mL of glacial acetic acid. About1-2 mL of concentrated H_2SO_4 carefully added. A color change from violet to blue to green represented the presence of steroidal saponins.

Salkowski's test: For preparation test solution crude extract was mixed with 2mL of chloroform. Then about 2 mL of concentrated H_2SO_4 was added and shaken gently. A reddish brown colour remarked the presence of steroidal ring.

2.3.4. Cardiac glycosides

Keller-Killiani test: Crude extract was dissolved in 2mL of glacial acetic acid (containing 1-2 drops of 2% $FeCl_3$ solution. Then 2mL of concentrated H_2SO_4 was added. a brown ring at the interphase indicated the presence of cardiac glycosides. Baljet test: Crude extract was dissolved with chloroform ethanol mixture (4:1). Following this sodium picrate reagent and 2 drops of 20% NaOH was added to mixture. If cardiac aglycon is present yellow to orange color will be seen.

Kedde test: Crude extract is treated with a small amount of Kedde reagent (Mix equal volumes of a 2% solution of 3,5-dinitrobenzoic acid in menthol and a 7.5% aqueous solution of KOH) and 2 drops of 20% NaOH solution. Development of a blue or violet color showed presence of cardiac aglycon.

2.3.5. Test for alkaloids

Crude extract was mixed with 2 mL of 1% HCl and heated gently. Then reagents of Mayer and Wagner were added to the test solution. Turbidity of the resulting precipitate was showed the presence of alkaloids.

2.3.6. Test for tannins

Crude extract was boiled with 20 mL distilled water for 5 min and filtered while hot. Then 1 ml of cool filtrate was diluted to 5 mL with distilled water and a few drops (2–3) of 10% ferric chloride were added and observed for the formation of precipitates and any color change. A bluish-black or brownish-green precipitate indicated the presence of tannins.

2.3.7. Test for combined anthraquinones

Powdered sample (1 g) was boiled with 2 mL of 10% hydrochloric acid for 5 min. Then the mixture was filtered while hot, cooled and partitioned with the equal volume of chloroform. The chloroform layer was taken into test tube and an equal volume of 10% ammonia solution was added, shaken and allowed to separate. Rose pink color in separated aqueous layer indicated the presence of anthraquinones.

2.4. Qualitative and Quantitative LC–MS/MS Assay

Compounds in CA and CI extracts were determined qualitative and quantitative by using liquid chromatography-electrospray ionization–mass spectrometry/ mass spectrometry (LC-ESI-MS/MS, Shimadzu 8040). The liquid chromatograph was a Shimadzu (Kyoto, Japan) Nexera XR system with an SIL-20AC autosampler, an LC-20AD high-pressure gradient pump system (20-µL mixer), a DGU-20A3R vacuum degasser, and a CTO-10AS VP column oven. Mass spectrometry was conducted using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface in the negative-ion mode.

The following instrument settings were used for analysis: column Restek (150 x 4.6 mm x 3 µm); column heat, 40°C; heat block temperature, 400 °C; DL temperature, 250 °C; nebulizing gas (N_2), 3 L/min; drying gas (N2), 15 L/min; collision energy, 25.0, 12.0, 9; dwell time, 100 msec. A mixture of methanol: formic acid (99:1 v/v) (A) and water: formic acid (99:1, v/v) (B) was selected as the mobile phase. The mobile phase consisted of 50% solvent A and 50% solvent B at a flow rate of 0,4 mL/min, and injection volume was 1 µL.

3. RESULTS and DISCUSSION

3.1. Preliminary phytochemical analysis

The phytochemical characteristics of two extracts were summarized in the Table 1. From the results, it was found that, carbohydrates, flavonoids, steroids and saponins were present, but alkaloids, anthraquinones and cardiac glycosides were absent in the plant extracts. Although, tannins were not detected in CI extract, but CA extract showed positive result for this secondary metabolite. The preliminary phytochemical tests are helpful in finding chemical constituents in the plant material that may lead to their quantitative estimation and also in locating the source of pharmacologically active chemical compound.

Table 1. Phytochemical constituents of extracts

Phytochemical	Type of test	*C. iconiensis*	*C. aintabensis*
Carbohydrates	Fehling	+	+
Alkaloids	Benedict	+	+
Cardiac glycosides	Mayer	-	-
Saponins	Wagner	-	-
Flavonoids	Keller-Kiliani	-	-
Tannins	Baljet	+	+
Anthraquinones	Kedde	+	+

"- " the result of the test is negative, "+" the result of the test is positive

3.2. Qualitative analysis of chemical compounds

The identification of chemical compounds in methanol extracts was evaluated on the basis of the accurate mass, the registered mass spectra fragmentation patterns and literature data. The mass spectrometric behavior of the compounds was studied using both positive-ion, and negative-ion mode. But negative-ion mode provided a better sensitivity than the other for these compounds due to more efficient ionization, simpler fragmentation, and lower baseline noise. Total ion chromatograms (TIC) of extracts were shown in Figure 1. The mass spectrums of extracts revealed the presence of 3 phenolic acids (vanilic acid, chlorogenic acid and caffeic acid), 2 organic acids (quinic acid and malic acid) and 2 flavonoid (rutin and isorhamnetin 3-*O*-rutinoside) compounds in methanol extract of *C. iconica* and *C. aintabensis* (Table 2). The mass spectra of extracts were shown in Figure 2.

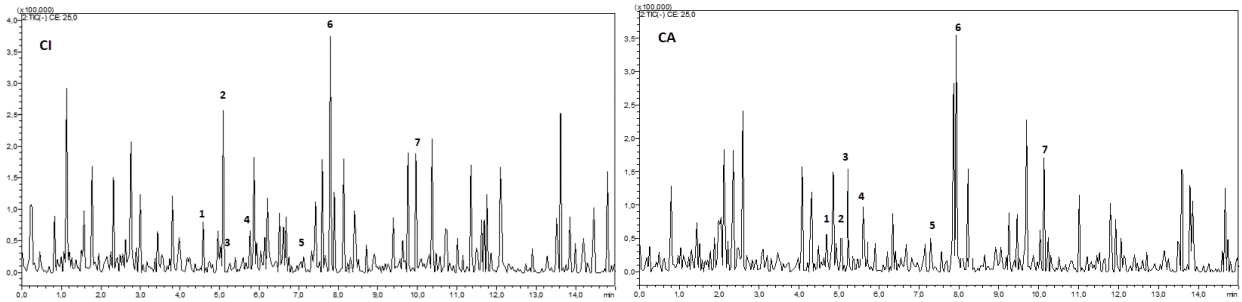

Figure 1. TIC profile of extracts. CI- methanol extract of *C. iconiensis*, CA- methanol extract of *C. aintabensis.*

Table 2. Phytochemical constituents of extracts

Peak No	RT (min)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Compounds
1	4.6	167	123, 152	Vanilic acid [31]
2	5.1	353	191, 179, 173, 135	Chlorogenic acid [32]
3	5.2	191	191,108, 93	Quinic acid [33]
4	5.6	179	135, 179, 87	Caffeic acid [34]
5	7.2	133	133,115,71	Malic acid [35]
6	8	609	300, 301	Rutin [36]
7	10.2	623	285, 300, 315	Isorhamnetin 3-*O*-rutinoside [32]

RT: Retention Time

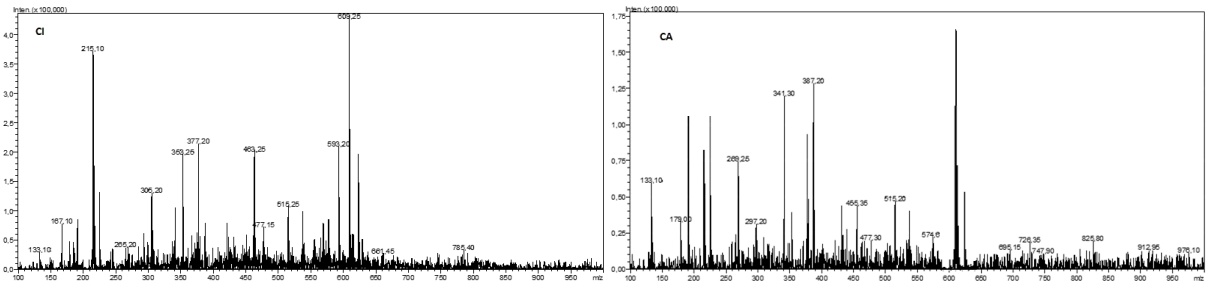

Figure 2. TIC profile of extracts. CI- methanol extract of *C. iconiensis*, CA- methanol extract of *C. aintabensis*

3.3. Quantitative Analyses of Compounds

3.3.1. Optimisation of LC-MS/MS Condition

The mass spectrometric behavior of compounds was studied using both positive-ion and negative-ion mode. Negative-ion mode provided a better sensitivity for these compounds due to more efficient ionization, simpler fragmentation, and lower baseline noise. These compounds were subsequently analyzed in Q1Scan (Product Ion Scan) mode, using $[M-H]^-$ ions as precursors. Obtained MS^2 spectras were used to select the optimal product ions. The MRM parameters, such as the precursor ion m/z, collision energy, and product ion m/z for compounds were optimized by an automatic MRM optimization function. For malic acid, due to the loss of water $[M-H-H_2O]^-$ providing an ion at m/z 115 and with the loss of CO_2 an intense ion at m/z 71 [20]. The peak identified as a chlorogenic acid (m/z 353), produced to the loss of one of the caffeoyl moieties [M-H-caffeoyl]$^-$, and subsequent fragmentation of ion yielded the fragments at m/z 191 (deprotonated quinic acid), 179 [caffeic acid-H]$^-$, 135 and the peak of the ion at m/z 173 (the absence of a C4 substituent) [21]. Fragmentation of $[M-H]^-$ ion (m/z 609) of rutin resulted in two major ions at m/z 300 and 301, showing the loss of rhamnose–glucose unit. The other flavonol diglycoside isorhamnetin 3-*O*-rutinoside is a 3'-methoxylated derivative of rutin. Fragmentation of this molecule $[M-H]^-$ ion (m/z 623) resulted ions m/z 285, 300 and 315. Isorhamnetin represents specific fragmentation with the loss of CH_3 radical from the deprotonated aglycone, thus giving m/z 315→ m/z 300 and the m/z 285 pattern as a result of fragmentation in C-ring [22]. With the loss of CO_2 providing an intense ion at m/z 123 for vanilic acid [23]. The obtained LC-MS/MS chromatogram and mass spectrum of compounds are presented in Figure 3.

3.3.2. Preparation of Standard and Sample Solutions

Stock solutions of compounds were prepared in methanol at 8 µg/ mL concentrations. The extracts solutions were prepared in methanol at 10 µg/mL.

3.3.3. Calibration Curve

Linearity of the methods was established by triplicate injections of each concentration of standard solutions. Response function of the standards calibration curve was y = 10074x + 994.36 for malic acid, y = 33716x – 2152.2 for chlorogenic acid, y = 16535x + 275.47 for quinic acid, y = 181197x + 9999 for caffeic acid, y = 511143x - 4056 for rutin and y = 18006x + 928.47 for isorhamnetin 3-*O*-rutinoside and y = 8656.4x + 184.21 for vanilic acid. The correlation coefficient (r^2) of the calibration curves was 0.9988, 0.9995, 0.9994, 0.9991, 0.9997, 0.9996 and 0.9991 respectively. The quantitative results of compounds are given in Table 3. As shown in table, the main compounds in CI extracts were rutin (169.779 µg/$mg_{extract}$) and chlorogenic acid (26.051µg/$mg_{extract}$). But, the main compounds in CA were rutin (161.638 µg/$mg_{extract}$), quinic acid (37.715 µg/$mg_{extract}$), and isorhamnetin 3-*O*-rutinoside (37.273 µg/$mg_{extract}$).

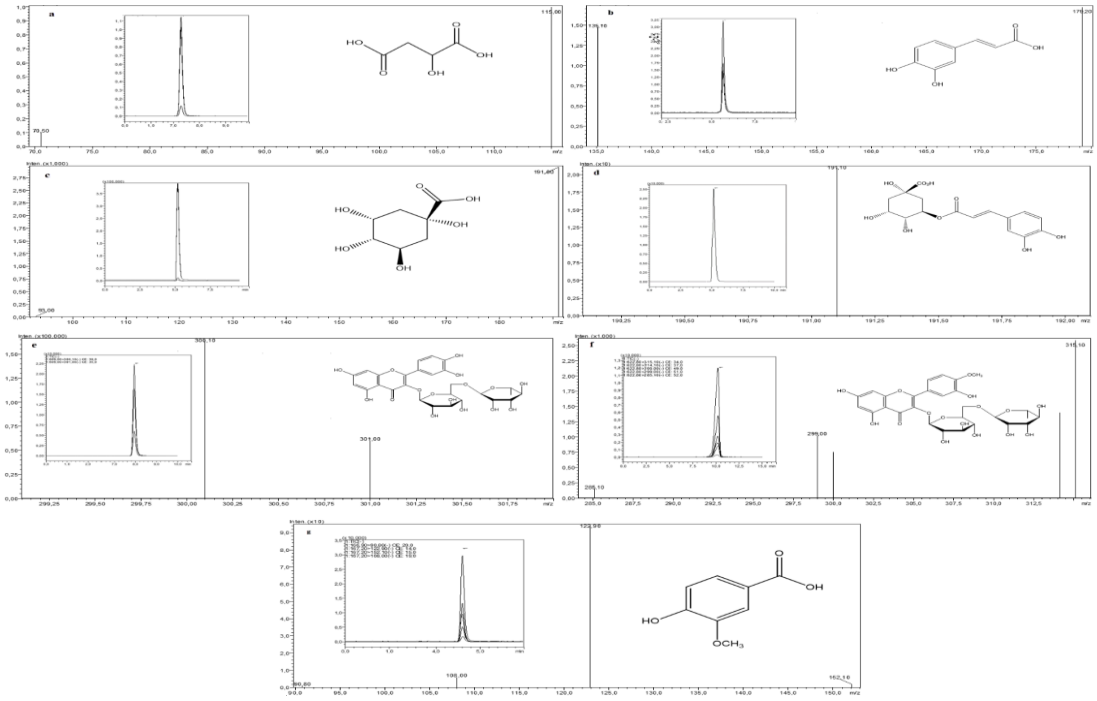

Figure 3. LC-MS/MS chromatogram and mass spectra of malic acid (a), caffeic acid (b), quinic acid (c), chlorogenic acid (d), rutin (e), isorhamnetin 3-O-rutinoside (f) and vanilic acid (g)

Table 3. Contents of compounds in extracts (µg/$g_{extract}$ ± SD).

Constituent	RT (min)	Content[a] (*µg/mg* $_{extract}$)	
		CI	CA
Vanilic acid	4.6	5.111±0.066	5.261±0.633
Chlorogenic acid	5.1	26.051±0.066	12.028±0.136
Quinic acid	5.2	3.958±0.614	37.715±0.044
Caffeic acid	5.6	0.260±0.066	0.961±0.038
Malic acid	7.2	1.670±0.250	25.328±0.933
Rutin	8	169.779±0.453	161.638±0.203
Isorhamnetin 3-*O*-rutinoside	10.2	8.606±0.398	37.273±0.914

RT-retention time. [a]Mean ± SD (*n*=3). CI- methanol extract of *C. iconiensis*, CA- methanol extract of *C. aintabensis*

To date, sesquiterpene lactones (*C. picheriana*, *C. piptocephala*, *C. canescens*), triterpenes (*C. adenostica*), steroids (*C. canescens*) and flavonoids (*C. verbascifolia*) have been isolated from *Cousinia* genus [2-8, 24]. In the present study the preliminary qualitative analysis of secondery metobilities in *C. iconica* and *C. aintabensis* revealed the presence of carbohydrates, flavonoids, tannins, saponins and steroids which have a wide range of cytotoxic and antitumor effects. Moreover, phenolic acids, organic acids and flavonoid compounds were identified in these species by LC-MS/MS. For the first time vanilic acid, chlorogenic acid, quinic acid, caffeic acid, malic acid, rutin and isorhamnetin 3-*O*-rutinoside were detected and quantified in these two species. In a result, rutin was found to be the most abundant among the compounds and about 10% of two methanol extract. Some of these detected chemical compounds display a remarkable spectrum of biological activities including antidiabetic [25], antiulcer and antioxidant [26], anti-inflammatory [27], cytotoxic, anticarcinogenic [28], antispasmodic [29] and antidepressant [30]. Moreover, qualitative and quantitative study combined with this activities evaluation will shed new lights to the advanced studies.

4. CONCLUSION

This is the first report on the phytochemical characterization of these species from *Cousinia* genus. Moreover, it was thought that chemical compounds identified in this genus could represent a chemical marker of the *Cousinia* genus as contributing to the chemotaxonomy.

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Morphological Changes of Salicylic Acid Application on Pepper (*Capsicum annuum* L.) Seedling under Cold Condition

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Abstract: This project was developed to investigate the contribution of salicylic acid (SA) to the development of pepper seedlings grown in low temperature (0 ^{0}C) conditions. The research was carried out in the controlled plant growing cabinet in the research- investigation area of the Department of Horticulture, Faculty of Agriculture, Siirt University. As a vegetable material, Urartu F1 pepper type (capia) which is used in greenhouse cultivation has been used. As a dose of different salicylic acid; 0.01 and 0.05 mmol doses were applied. The dose of 0 mmol salicylic acid was used as a control group. Application frequency; It was applied 1 time, 2 times and 3 times.3 different cold application times were also investigated; 24 hours, 48 hours and 72 hours. The experiment was designed in randomized plots and 3 replications. In the pepper seedlings Rate of Lost Seedling Weight (ROLSW) and Rate of Lost Seedling Length (ROLSL) were investigated. At the end of the research; Both SA applications increased the ROLSW rate according to the control. The application of 0.01 ppm was the SA application with the highest ROLSW rate. The application of 0.01 ppm SA also increased the ROLSL rate compared to the control. The highest ROLSW and ROLSL rates were obtained from 24-hour cold application. There was no statistically significant difference between the frequencies of application.

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1. INTRODUCTION

Temperature is a major factor in abiotic stress and to determine agricultural productivity and crop productivity. The rate is reduced and the amount of absorption of water and nutrients from cold stress, leading to cell drying and starvation and called extreme forms of cold stress stresses frozen and cause the formation of ice in the cell fluid, which leads to dehydration and death in plants. The low temperature (LT) is the environmental stress that affects crop production and quality. Regulates the expression of several proteins, metabolites and many genes [1].

Pepper plants are initially from tropic areas and require high-temperature conditions for their advancement. Subsequently, the ideal development temperature is in the vicinity of 25 and 30 °C, such that temperature changes influence an assortment of physiological capacities and

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morphological improvement. At the point when temperature diminishes underneath 15 °C, pepper development is decreased, and sprout and organic product generation stop [2].

Salicylic acid's activities incorporate practicing a thermogenic impact [3], expanding thermotolerance [4], empowering extrinsic root arrangement [5], demonstrating herbicides impact [6], lessening leaf shed [7], giving protection against pathogens [8], manages ethylene biosynthesis [9-10] and changing the quality and amount of proteins [11]. It has been asserted [12] that SA and comparative phenolic mixes practice their impact of giving protection against various anxiety factors in plants corrosive [13] and cytokinins [12]. These perceptions and reports on numerous other physiological impacts achieved by SA conjured in a few scientists this substance may be another plant development controller [14]. The experiment was designed to study the effect of different concentrations of salicylic acid on resistance to low temperature. The aim of this study to find out the answer to these questions; which morphological changes occur at different low-temperature periods. And just how do doses of salicylic acid effect at cold condition on pepper seedlings' morphological features?

2. MATERIAL and METHODS

This study was carried out in the horticulture department, agriculture faculty of Siirt University in 2018. Siirt is a province and located between the 41˚- 57˚ East longitude and 37˚ -55˚ northern latitudes in as its geographical location. In Siirt province, it dominates the continental climate which is the most important feature of the four seasons, continental climate prevails. The summers are hot and arid, with no precipitation in June and October [15].

2.1. Plant Materials

A capia pepper variety (Urartu F1) was used for plant material. This pepper variety could be grown at the cool climate in the greenhouse. Pepper seedlings were grown at a growing chamber.

2.2. Methods

0.01 mmol, 0.05 mmol, and 0 mmol doses of salicylic acid were applicated before applying cold stress. For control groups only distilled water was applicated each time. After 0 ^{0}C temperature application; did cold application create a non-irreversible wilting to seedlings, do all seedlings' have cold damage on their leaves and stems? Are there differences in seedlings weight and lengths? Also, salicylic acid was 1, 2 and 3 times applicated for each dose. Polysorbate [16] (Twin 20) was also used enough quantity for adhesive. For easier penetration of salicylic acid thin holes in the leaves were made by small needles.

2.2.1. Seedling Weight (gr)

Before and after cold application, 5 randomly seedlings per application were selected and then measured by scale sensitive to 0.01 g (Figure 1).

Figure 1. Seedling weight (before and after) application

2.2.2. Seedling length (cm)

5 seedlings per applications were randomly selected. The distance from the soil surface to the seedling's top was measured for each seedling by a ruler. All measurements were performed before and after the cold application (Figure 2).

Figure 2. Seedling length (before and after) application

2.2.3. Experimental Design

The experimental design used was a Randomized Complete Parcel Design (RCPD) with factorial. The treatment in each experiment had three replicates. Where was statistically analyze the data and compared means using "LSD"s Multiple Range Test at 0.05 and 0.01 levels.

3. RESULTS and DISCUSSION

The differences between the doses to Least Square (Sq.) Mean was significant. Least Sq. Mean of ROLSW ranged between -46.0 – - 63.1 percent. The average Least Sq. Mean of doses was -52.0 %. The highest Least Sq. Mean value of ROLSW was obtained from 0.01mmol dose as -46.0 %, and the lowest ROLSW's Mean value was obtained from the control concentration of salicylic acid as -63.1 %. The highest Least Sq. Mean value was obtained from frequency 1 as -47.8 %, and the lowest Least Sq. Mean value was obtained from frequency 2 as -54.3 %. The highest Mean of doses value of ROLSL was determined from 0.01 mmol dose as -52.0 % (Table 1).

Table 1. Effect of Doses, Time and Application Frequency of Salicylic Acid

	Doses (ppm)			Mean	Time (hour)			Mean	Frequency (times)			Mean
	0.01	0.05	Con.		24	48	72		1	2	3	
ROLSW	-46.0 a	-47.0 a	-63.1 b	-52.0	-13.8 a	-67.6 b	-74.6 b	-52.0	-47.8 a	-54.3 a	-54.1 a	-52.0
ROLSL	-52.0 a	-59.0 ab	-65.4 b	-58.8	-28.6 a	-60.2 b	-87.6 c	-58.8	-58.4 a	-57.9 a	-60.2 a	-58.8

ROLSW: Rate of Lost Seedling Weight, ROLSL: Rate of Lost Seedling length

The best value (-28.6 %) belong to 24-hour application in the ROLSL mean values. Three and two time frequency values have the same statistical group as the best frequency application (respectively -60.2 % and -57.9 %) for ROLSL (Table 1).

According to the Interaction of Time, doses and Frequency of salicylic acid; the highest Least Sq. Mean of ROLWS values were obtained from frequency 2 and time 24 h as -10.9 %, and the lowest Least Sq. Mean values was obtained from frequency 2 and time 72 h as -80.5 %. When the first three results are observed, 24 h applications was the best result and very different from the other applications. The highest Least Sq. Mean of ROLWS values was obtained from frequency 1 and dose 0.01mmol as -41.4 %, and the lowest Least Sq. Mean values was obtained from frequency 3 and control dose as -67.2 %. The differences between the frequencies, doses and times for Least Sq. Mean was significant. Least Sq. Mean ranged between -9.84 – -96.50 percent at Time x Doses x Frequency interaction of ROLWS. The average Least Sq. Mean of frequencies and doses and times was -52.02 %. The highest Least Sq. Mean values was obtained from frequency 2 and control dose and time 24 h as -9.84 %, and the lowest Least Sq. Mean values was obtained from frequency 3 and control doses and time 48 h as -96.50 %. In the Time x Doses interaction of ROLWS; the highest value was obtained from 24 h and dose 0.05 mmol interaction as -12.8 %, and the lowest Least Sq. Mean values was obtained from time 48 h and control dose as -92.80 %. (Table 2).

At the frequency x Time interaction of ROLSL; the differences between the frequencies and times to Least Sq. Mean were significant. The average Least Sq. Mean of frequencies and times was -58.8 %. The highest Least Sq. Mean values was obtained from frequency 2 and time 24 h as -24.6 %, and the lowest Least Sq. Mean values was obtained from frequency 2 and time 72 h as -89.1 %. It has been clearly observed that the first three results, 24 h applications was the best result and very different from the other applications, while the last three results have the worst results. Least Sq. Mean of the frequencies and the doses was significant; least Sq. Mean ranged between -43.8 – -68.8 percent. The average Least Sq. Mean of frequencies and doses were -58.8 %. The highest Least Sq. Mean values was obtained from frequencies 1 and dose 0.01 mmol as -43.80 %, and the lowest Least Sq. Mean values was obtained from frequency 2 and control dose as -68.8 %. At the point when watched the initial three outcomes, and two of the concentration at 0.01 mmol and one of the other at 0.05 mmol applications were the best outcome and altogether different from other applications. While the last three outcomes have fewer outcomes. The average Least Sq. Mean of frequencies and doses and times of ROLSL was -58.79 %. The highest Least Sq. Mean values was obtained from frequency 1 and dose 0.01 mmol and time 48 h as -19.31 %, and the lowest Least Sq. Mean values was obtained from frequency 2 and control dose and time 72 h -95.65. The highest Least Sq. Mean value of ROLSL was obtained from time 24 h and control dose as -21.2 %, and the lowest Least Sq. Mean values was obtained from time 72 h and control dose as -92.6 %.

Processing the pepper seedlings with salicylic acid for 16 hours, the results obtained, it was the negative effect on fresh weight increase, and dry weight increase in the application 5- and 10-mM SA. The result is different from this study because the times used are different from each other's (24, 48 and 72 hours in the test) [17]. In winter wheat leaves grow at low temperatures. It is sprayed with salicylic acid, the influence of external factors decreased and also the decreased freezing injury [18]. Salicylic acid sprinkled on coriander plant with at concentrations of 20 and 35 mg/L. got results on the significant increase in the soft weight of the vegetative group, and the number of flowers inflorescences, and the number of seeds per inflorescence, and weight 100 seed and production of the plant seeds [19]. It could be recommended that foliar spraying with salicylic acid at 100 ppm, to increase the final yield and fruit quality of sweet pepper plant during the low temperatures of autumn plantations [20]. The researcher found [21] the effect of salicylic acid on macrophomina and the evolution of fever disease on the Sun Flower plant. Results experience recommended that all pots concentrations may be affected significantly in reducing the percentage of injury. As for the concentrations of 200 and 250 mg /L salicylic acid effect of the dry weight increase total of vegetables and also an increase in the dry weight total of the roots. The study indicated that high concentrations of

Table 2. Effect of Interaction of Time, Doses and Frequency of Salicylic Acid

	Interaction	ROLWS	ROLSL		Interaction	ROLWS	ROLSL
Time x Doses	24 x 0,01	-14.9 a	-24.3 a	Time x Doses x Frequency	1 x 0,01 x 24	-21.61 a	-27.05 ab
	24 x 0,05	-12.8 a	-40.4 b		1 x 0,05 x 24	-14.16 a	-40.33 a-d
	24 x Con.	-13.7 a	-21.2 a		1 x Con. X 24	-16.20 a	-21.86 ab
	48 x 0,01	-51.5 b	-41.4 b		2 x 0,01 x 24	-12.40 a	-23.28 ab
	48 x 0,05	-58.5 bc	-56.8 c		2 x 0,05 x 24	-10.71 a	-28.21 a-c
	48 x Con.	-92.8 e	-82.4 d		2 x Con. x 24	-9.84 a	-22.27 ab
	72 x 0,01	-71.6 cd	-90.2 d		3 x 0,01 x 24	-10.57 a	-22.46 ab
	72 x 0,05	-69.7 cd	-79.9 d		3 x 0,05 x 24	-13.56 a	-52.53 c-g
	72 x Con.	-82.8 de	-92.6 d		3 x Con. x 24	-15.18 a	-19.43 a
	Mean	-52.0	-58.8		1 x 0,01 x 48	-23.01 a	-19.31 a
Frequency x Time	1 x 24	-17.3 a	-29.8 a		1 x 0,05 x 48	-66.60 b-e	-68.71 e-i
	1x 48	-58.8 b	-58.8 b		1 x Con. x 48	-86.87 d-f	-88.43 i-k
	1 x 72	-66.9 bc	-86.5 c		2 x 0,01 x 48	-65.86b-e	-44.75 b-e
	2 x 24	-10.9 a	-24.6 a		2 x 0,05 x 48	-53.43 b	-46.46 b-f
	2 x 48	-71.4 bc	-59.9 b		2 x Con. x 48	-95.04 f	-88.46 i-k
	2 x 72	-80.5 c	-89.1 c		3 x 0,01 x 48	-65.69 b-e	-60.26 d-h
	3 x 24	-13.1 a	-31.5 a		3 x 0,05 x 48	-55.32 bc	-55.28 d-g
	3 x 48	-72.5 bc	-61.9 b		3 x Con. x 48	-96.50 f	-70.19 f-j
	3 x 72	-76.5 c	-87.1 c		1 x 0,01 x 72	-79.44 c-f	-85.03 h-k
	Mean	-52.0	-58.8		1 x 0,05 x 72	-58.60 bc	-83.64 h-k
Frequency x Doses	1 x 0,01	-41.4 a	-43.8 a		1 x Con. X 72	-62.75 b-d	-90.83 i-k
	1 x 0,05	-46.5 a	-64.2 bc		2 x 0,01 x 72	-67.84 b-e	-91.50 i-k
	1 x Con.	-55.3 ab	-67.0 cd		2 x 0,05 x 72	-77.97 b-f	-80.08 h-k
	2 x 0,01	-48.7 a	-53.2 a-c		2 x Con. x 72	-95.71 f	-95.65k
	2 x 0,05	-47.4 a	-51.6 ab		3 x 0,01 x 72	-67.37 b-e	-93.91 jk
	2 x Con.	-66.9 b	-68.8 d		3 x 0,05 x 72	-72.56 b-f	-75.94 g-k
	3 x 0,01	-47.9 a	-58.9 bc		3 x Con. X 72	-89.83 ef	-91.37 i-k
	3 x 0,05	-47.2 a	-61.3 bc		Mean	52.02	-58.79
	3 x Con.	-67.2 b	-60.3 bc				
	Mean	-52.0	-58.8				

salicylic acid have been reduced by the number of stone bodies (sclerotia). While low concentrations of salicylic acid have significant differences with control treatment. The application of SA is different from this study, while application of SA was sprayed on the pepper plant at the low temperature. The researcher concluded [22] that spraying plants with low concentrations of salicylic acid can stimulate endure of vital and abiotic stresses such as cold and tolerance high-temperature. This is what we applied to our effects of the salicylic acid application on cold tolerance and gene expression in pepper seedling in the test. The researchers [23]'s study showed that plants did not show a saturated seed high concentration of 1 mM at the SA any change in tolerance iced, while the low concentrations of 0.1-0.5 mM at the SA encouraged tolerance to sedative stress in bean and tomato.

The results of the researcher [24] were that in the low temperatures showed cultivated plants decreased by 50–70 % in the number of leaves and the length and dry weight compared to the high-temperature system. It was also shown in the cold system of plants grown an increased number of shoots in the armpits. Also, the content of proteins and chlorophyll decreased in both temperature treatments. The total nitrogen content was slightly higher at low temperatures, but nitrate was lower. These results, are like the result that we obtained from the test, and an action the researchers' studies in the field, but we used the laboratory in the test. The researchers [25] concluded that Salicylic acid increases plant's resistance to inappropriate conditions especially against the stress that is the plant is exposed to stress (saline and drought), it works on the organization of some physiological processes of plant photosynthesis and transpiration. And the results of this conformity our results in the test while we used to tolerate pepper plants for low temperatures. The found in an experiment [26] results showed the superiority of cultivated plants in the first date in plant height, the leaf area, the dry weight of vegetative total and the dry weight of root total. The superiority of plants salicylic acid treatment with 100 mg/L concentration in the leaf area, the plant height, dry weight of vegetative growth and dry weight of roots, as well as the content of roots of *Arctium lappa* L. plant dry weight. Aziz et al. [27] found about the response of *narcissus* plants to spraying with plant growth regulators salicylic acid the characteristics of the vegetative growth and syphilis and bulbs characteristics of plant daffodils. The results increased at the 80 mg/L SA in the plant height, the number of branches/plant, and the ratio of chlorophyll and wet weight and flower diameter, weight wet and dry. The effect of overlap between the two studied factors is significant in most studied traits. The effect of water stress and the external application of SA on the growth and production of eggplant were investigated by Hameed et al. [28]. Results showed a significant increase for the first irrigation level W1 (water stress1) compared with level W2 (water stress2) transactions W3 (water stress3) for all indicators. Interacted treatment W1A2 (water stress1 x Applcation2) showed a significant increase compared with other treatments.

4. CONCLUSION

Three different doses of salicylic acid (SA) (0 mM, 0.01 and 0.05 mM) were given from the leaf. Each dose was administered in three different frequencies (1 time, 2 times and 3 times spraying). Each application was exposed to cold (0 C^0) in three different times (24 h, 48 h and 72 h). The best dose of SA was 0.01 mM in the Rate of Last Seedling Weight measurements, the worst result was obtained from the control group without SA treatment. Two (1 and 2 times) frequency applications applied to the seedlings exposed to low temperatures were the highest value in the Rate of Seedling Weight. Rate of Lost Seedling Lenght data, the best dose of 0.01 mM dose, the most appropriate frequency of applications 1 and 2 times were found to be.

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