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Fungal Biodiversity of Strawberry Fields in Aydin, TURKEY

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Abstract: Strawberry is a kind of delicious and aromatic fruit, which can be consumed as fresh and also is suitable for industry. However, strawberry is exposed to many fungal diseases. The aim of this study is to determine the fungi that present in the field whether or not pathogenic. Samples were collected from different strawberry fields in Aydın in April 2015. Morphological identification was made according to the shape and color of the colonies, mycelium and spore structures. For molecular identification, ITS rDNA gene region was used. According to morphological and molecular methods, eleven different fungal genera were found on strawberries.

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Fungi, Biodiversity, Strawberry, ITS, Aydın, Turkey

1. Introduction

Biodiversity is the foundation living systems to which human success is actually associated [1]. It is one of the basic parts of nature and it ensures the survival of earth. Strawberries are known as plants belonging to the genus *Fragaria*. Taxonomically, the genus *Fragaria* is included in the family Rosaceae. Strawberry is a perennial herbaceous, short day plant. Strawberries are a consumable plant part, which also used in industry. Be that as it may, natural products are easily spoilt and as a rule have dynamic digestion amid the capacity organize [2]. The significance of organic products in human nourishment can't be overestimated as it gives basic development factors, for example, vitamins and minerals important for continuation of human life [3]. The high concentration of various sugars, minerals, vitamins, amino acids, and low pH also enhances the successful growth and survival of various forms of fungi [4]. Annual reports have shown that 20% of fruits and vegetables produced are lost to spoilage [5].

According to Food and Agriculture Organization (FAO) (2012), Turkey was in the 3rd place in strawberry production. But about 15% of the products were lost in the field before harvest due to the diseases according to farmers.

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The organic action is a fundamental factor in the physical and substance advancement of soils [6]. There are 110.000 defined fungi species were present in the World but it is estimated that 1.5 million fungi species exists [7]. The ITS region is considered to be a good candidate for accurate detection and can largely separate from all other species by this application.

It is essential to decide the decent variety of organisms, which cause infections on strawberries and their natural and hereditary impacts. In this study, fungi on strawberry fruits will be detected by morphological and molecular methods.

2. Material and Methods

2.1. Sample Collection

Samples were collected aseptically from the strawberry fields in Aydın (Yenipazar, Atça, Sultanhisar, Köşk and Umurlu) in April 2015 during harvest time. Rotten strawberry fruits were collected in sterile plastic bags and kept in the portable refrigerator until brought to the laboratory.

2.2. Isolation of Fungal Species

One gram of strawberry fruits was weighted and homogenized in 9 ml of 0.85% physiologic Saline Water (PSW). 100 µL of these homogenized samples were inoculated on Rose Bengal Choloramphenicol Agar and Potato Dextrose Agar. Samples were incubated at 27°C for 5 days. After the incubation, the differentiated fungi samples were selected and isolated from the mixed colony under the same incubation conditions.

2.3. Morphological Identification

Morphological identification of the samples was realized according to Samson [8]. Mycelium and spore structures smeared on a slide, dyed with lactophenol cotton blue and visualized under the microscope. Colonial shapes were determined and used to identify species microscopically.

2.4. Molecular Identification

Fungi samples were put in 1.5 ml eppendorf tubes using a sterile toothpick. After, samples have reduced the powder using liquid nitrogen. DNA isolation of the samples was realized with 2X CTAB isolation protocol according to Doyle and Doyle [9]. Concentration and purity of the samples were measured with a Nanodrop Spectrophotometer (Thermo). ITS rDNA gene region was used to identify the species (ITS1: 5'TCCGTAGGTGAACCTGCGG'3, ITS4: 5'TCCTCCGCTTATTGATATGC'3) [10]. PCR reaction conditions were: initial denaturation 94 ºC 5 min, denaturation 94 ºC 30 sec, annealing 60 ºC 30 sec, extension 72 ºC 60 sec with 35 cycles and a final extension at 72ºC 10 min. Reagents concentrations were: 10X Taq Buffer, 0.5M dNTP mix, 10 pM from each primer, 7.5 mM $MgCl_2$ and 1U Taq polymerase (ABM) with the final volume of 25 µl. Agarose gel electrophoresis of the samples was observed on 1.4% agarose concentration at 90 V 40 min. 100 bp DNA ladder was used for size comparison of the products. PCR products were sent to DNA sequencing (Macrogen, Holland).

2.5. Data Analysis

Sequence results were aligned with the ones in GenBank using BLASTn software to find out the species of the samples. MEGA6 was used to infer phylogenetic tree.

3. Results

3.1. Morphological Identification

Morphological methods showed eleven different fungal species (Table 1). Colony shape, mycelium and spore structures were observed to this purpose.

Table 1. Morphological identification of the species

No	Name	Location
1	*Rhizopus* sp.	Yenipazar, Atça
2	*Lichtheimia* sp.	Yenipazar
3	*Alternaria* sp.	Yenipazar, Atça, Sultanhisar
4	*Fusarium* sp.	Yenipazar, Atça, Sultanhisar, Köşk, Umurlu
5	*Syncephalastrum* sp.	Yenipazar, Atça
6	*Aspergillus* sp.	Yenipazar, Atça, Sultanhisar
7	*Cladosporium* sp.	Sultanhisar
8	*Trichoderma* sp.	Yenipazar, Atça
9	*Talaromyces* sp.	Atça
10	*Botrytis* sp.	Yenipazar, Atça, Sultanhisar, Köşk, Umurlu
11	*Syncephalastrum monosporum*	Yenipazar, Atça

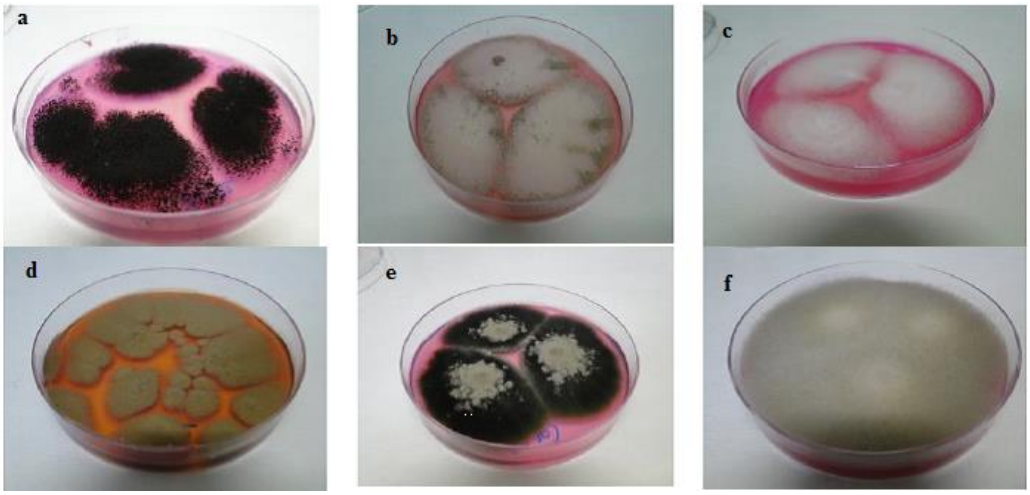

Figure 1. Colonial shapes of some fungi isolated. a: *Aspergillus niger*, b: *Rhizopus oryzae*, c: *Lichtheimia corymbifera*, d: *Cladosporium cladosporioides*, e: *Trichoderma atroviride*, f: *Botrytis cinera*

3.2. Molecular Identification

ITS rDNA gene region was used to identify fungal samples at the species level. PCR products were sent to sequencing to Macrogen (Holland). Molecular identification was made by comparing sequences with GenBank using BLASTn. Nine fungal species were found in contrast with morphological results (Table 2).

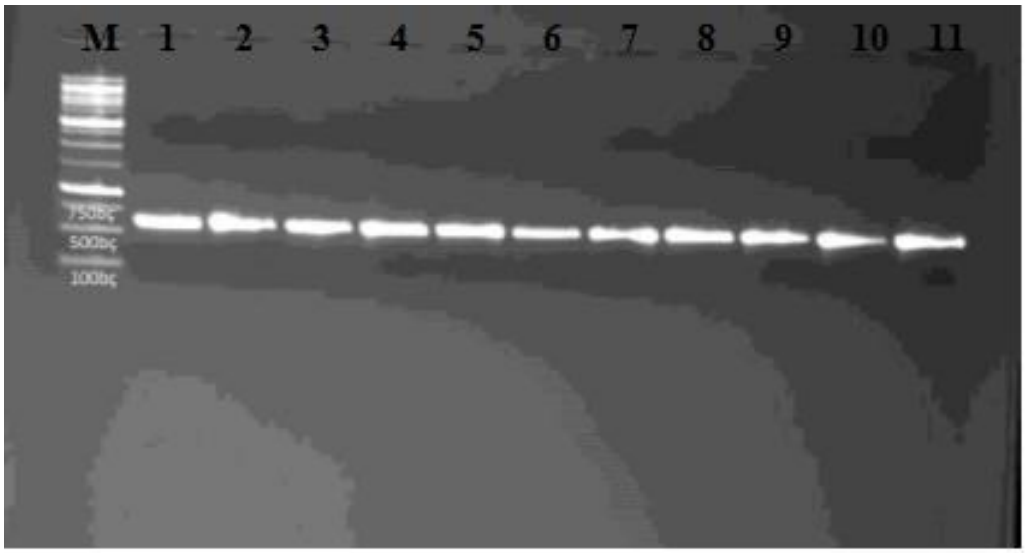

Figure 2. ITS PCR results of samples. (M: 100bp marker (ABM), 1-11: Samples)

Table 2. Molecular Identification of species

No	Name	Accession No	Location
1	*Rhizopus oryzae*	KJ417550.1, AY213685.1	Yenipazar, Atça
2	*Lichtheimia corymbifera*	LN812956.1	Yenipazar
3	*Alternaria alternata*	KP131535.1, KX463014.1, KP131533.1	Yenipazar, Atça, Sultanhisar
4	*Fusarium proliferatum*	GU074010.1, GQ856689.1, EU151490.1	Yenipazar, Atça, Sultanhisar, Umurlu, Köşk
5	*Syncephalastrum monosporum*	JQ954886.1	Yenipazar, Atça
6	*Aspergillus niger*	AF108474.1	Yenipazar, Atça, Sultanhisar, Umurlu, Köşk
7	*Cladosporium cladosporioides*	EF405864.1	Sultanhisar
8	*Trichoderma atroviride*	AF456920.1, KX538952.1	Yenipazar, Atça
9	*Bortytis cinerea*	KX766413.1, KX387891.1, KP234034.1	Yenipazar, Atça, Sultanhisar, Umurlu, Köşk

MEGA6 was used to construct the phyloenetic tree. Maximum likelihood method based on the Jukes-Cantor model was used (Figure 3). MP tree was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

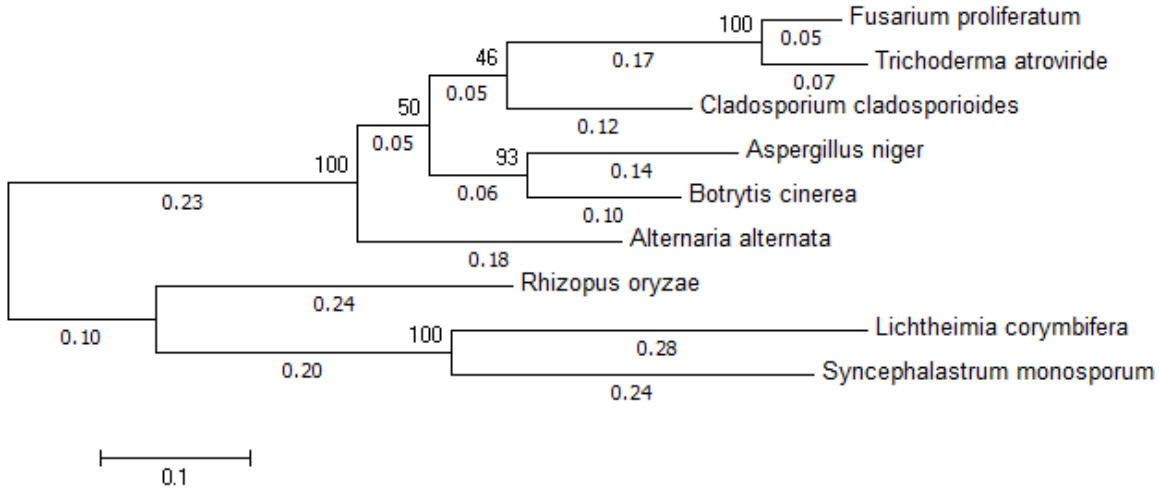

Figure 3. The evolutionary history was inferred using the Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6. Percenteces of the branches were shown on the nods.

4. Discussion

Eleven kinds of fungi differentiated from each other in morphological are identified from these strains. For molecular identification, rDNA ITS gene part of 78 examples is multiplied and as a result of the compare with GENEBANK data, 20 different kinds that belong to 9 species were determined. Literature shows that procedures, such as gathering and transporting, natural products may experience physical damage that builds post-reap misfortune and the likelihood of contagious pollution [11, 12].

Kasiamdari et al. (2002), isolated *R. solani* CFM1 isolate from cabbage, designed two primers from the ITS gene region and suggested that molecular methods would provide more accurate results than classical methods [13].

Staats et al. (2004) used the DNA sequence of 3 nuclear protein-coding genes (RPB2, G3PDH and HSP60) to classify *Botrytis* spp. They also compared them to conventional methods. The results of phylogenetic analyses were showed that *Botrytis* spp. were separated from *Sclerotiniaceae* species [14].

Khairnar et al. (2011) investigated soil-borne fungal biodiversity of some fruit crops in India and found 21 different fungal species and suggested that all twenty one fungal species can be controlled with 500 ppm Moximate, a fungicide [15].

Mailafia et al. (2017) researched fungi associated with fruit species and identified six different fungi and one yeast species [5].

Botrytis cinera is the cause of gray mold disease [16]. *Lichtheimia corymbifera* is the principle pathogen causing human and animal infections. Though only one sample was found, it wasn't widespread in the sample location [17]. *Syncephalastrum monosporum* is the endophytic fungal community of cacao and can also be found in the eyes of healthy horses, nests of laboratory reared leaf cutter ants, poultry feed, and spices [18]. *Rhizopus oryzae* is commonly found on dead organic matter and cause of disease [19]. *Alternaria alternata* is a common plant pathogen [20].

5. Conclusion

This study was made to detect fungal biodiversity on strawberries in Aydın, Turkey. As a result, nine fungal species were identified both by morphological and by molecular methods. Despite the usage of fungicides fungal diseases, such as gray mold, leaf spot disease can still be seen frequently both pre- and post-harvest. These species only were found on fruits of the plant. Investigation of soil and other plant parts can be resulted in more fungal species to be found.

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Einkorn (*Triticum monococcum* ssp. *monococcum*) *in vitro* propagation sterilization protocol

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Abstract: Einkorn is one of the oldest and important wheat species because of its increasing economic value, agronomical technical properties, and health and sanitary effects. Therefore, a faster tissue culture production protocol which may ensure a fast einkorn production is desired. The tissue culture process first requires an efficient sterilization technique of the explant to eliminate contamination factors in this hulled species. In this study, we, thus, aimed to determine the effective einkorn sterilization process for the removal of contaminating microorganisms without the loss of germination capacity and viability. We tested 22 following sterilant combinations with different concentration and exposure time: Ethanol, Commercial Bleach, Tween 20, Mercury (II) Chloride, and Sterile Distilled Water. On the other hand, obtained the data were analyzed by one-way ANOVA and Duncan test, where $p \leq 0.05$ was accepted significant. Forty percent commercial bleach which were applied fifteen minutes eliminated all microorganisms with a 92.6% seed germination. The second-best technique was one minute exposure of 70% ethanol which were followed by 10 minutes exposure of 20% commercial bleach. This assured up to 89% germination and no contamination. The worst results came from the mercury (II) chloride with 0% contamination and ≤ 25% germination rate. Mercury (II) Chloride was both highly toxic on the seeds and reduced the contamination. Commercial Bleach and Ethanol Chemicals were less toxic while providing an acceptable sterilization.

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Einkorn wheat,
Germination,
Seed sterilization,
Tissue culture

1. Introduction

Wheat (*Triticum aestivum* L.) of Poaceae family grows in the most parts of the world. It was first cultivated more than ten thousand years ago in the Fertile Crescent. Nowadays, it is the second largest annual crop produced and consumed (~700 million tons), which supplies about 20% of the world's calories [1] and a major amount of protein. Biotic (fungal, bacterial, etc.) and abiotic (salt, cold, etc.) stresses, on the other hand, cause serious yield losses in wheat production. Therefore, efforts to sustain the wheat production through the application of new approaches including tissue culture are considered to be desired alternative approaches.

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Einkorn (*Triticum monococcum* ssp. *monococcum*) is the first cultured hulled wheat species. Its domestication dates back to 9,000 B.C in Turkey. Einkorn is the most basic wheat form because of its diploid chromosome number (2n=14). Einkorn is, currently, cultivated in mountainous areas of Turkey, Greece, Bulgaria, Yugoslavia, and Italy at smaller quantities [2, 3]. Einkorn, with its low glycemic index, is a good food agent against the chronic diseases, cancer(s), obesity, diabetes, etc. In addition, it has more lutein than that of *Triticum aestivum* L., which prevents skin, eyes, and cardiovascular health problems [4, 5].

In vitro plant culture "which encompasses cell, tissue, organ, and also embryo culture", is a vital technique for mass multiplication of plants, elimination of plant diseases through meristematic tissue culture technique, plant conservation, and crop improvement through gene transfer" [6]. Fundamentally, plant culture technique consists of transferring different pieces of a plant (such as a stem tip, plantlet, scutellum, node, meristem, embryo, coleoptile or even a seed) and whole plant into a sterile nutrient medium where they multiply [7]. However, the success of plant tissue culture process strictly depends on efficient explant sterilization protocol. During plant tissue culture process, contaminations are the most serious problems which cause to losses by microorganism in explant sources. These microorganisms include viruses, bacteria, fungi, yeast, etc. Nutrient media in which the plant pieces are planted have also good source for microbial growth. These microorganisms compete hard with the plant material for nutrient in the media [7]. The presence of these microbes in the plant cultures process usually results in increased culture mortality, lowered growth, tissue necrosis, reduced shoot proliferation, and reduced rooting [8].

There are a lot of common sterilants for the surface sterilization of plant material. Popular disinfectants are sodium hypochlorite, ethanol, mercuric chloride, calcium hypochlorite, silver nitrate, hydrogen peroxide, bromine water, and Tween 20 [9]. However, many sterilants can be toxic to the plant tissues, because of their concentrations, their exposure duration to the explants or their process sequence, etc. [10]. Hence, the efficient plant tissue culture processes start use with an optimal sterilization technique. The choice of time period and chemical agents depend on the sensitivity of the explant to be sterilized. Because, the preferred procedure for sterilization eliminate all microorganisms on one hand kill the plant tissue on the other hand. The biological activity in the explant sources should be protected during the sterilizations process as well. Therefore, it is important to determine the optimal conditions for each plant species, tissues and explant types.

Up to now, there are no study reports about the surface sterilization process of einkorn wheat. Hence, the aim of this study was to determine the best sterilization protocol for *in vitro* propagation process of einkorn (*Triticum monococcum* ssp. *monococcum*) wheat at the Abant İzzet Baysal University, Biology Department, Plant Genetics & Pathology Laboratory, Bolu, Turkey in 2017.

2. Material and Methods

2.1. Seed source

The seeds of einkorn (*Triticum monococcum* ssp. *monococcum*) in this study were get from Seben (Bolu) District in the 2015/2016 at the harvest and all the experiments were conducted at the Abant Izzet Baysal University, Biology Department, Plant Genetics & Pathology Laboratory, Bolu, Turkey in the 2017.

2.2. Surface sterilization of einkorn seed

Tween 20 (Sigma), Commercial Bleach-Domestos (4.6% NaClO), Ethanol (Merck), Mercuric Chloride (Sigma) were used in this study. Used sterilants were applied at different concentrations, methods, and exposure times. Totally, 22 different sterilization methods were applied. In all methods, einkorn wheat explants were washed for one minute by distilled water

in which 5 drops of Tween 20 were poured into 100 ml at the beginning (Table 1). The variables were with or without 70% Ethanol, Domestos 20%, 30%, or 40% for 10 or 15 minutes, Mercury Chloride 0.1% or 0.3 % for 10 or 15 minutes. The labware (beakers, pens, water etc.) was sterilized in the autoclave (Nüve OT - 40L) before the experiment. And, hulls of the seeds were dehulled before the sterilization process. Then, the healthy seeds were selected before and after the surface. Used seeds were washed 3 times with sterile water and then pl anted into petri plates. All sterilizations processes were realized in the laminar airflow chamber (Nüve LN 090). All variables were repeated 3 times.

Table 1. Twenty-two diverse surface sterilization methods for einkorn wheat in this study.

Methods	Ethanol	Commercial Bleach	Mercury (II) Chloride	Sterilized Water
1	--	20% Domestos - 10 min	--	--
2	70% Ethanol - 1 min	20% Domestos - 10 min	--	--
3	--	30% Domestos - 10 min	--	--
4	70% Ethanol - 1 min	30% Domestos - 10 min	--	--
5	--	40% Domestos - 10 min	--	--
6	70% Ethanol - 1 min	40% Domestos - 10 min	--	--
7	--	--	0.1% Hg2Cl - 10 min	--
8	70% Ethanol - 1 min	--	0.1% Hg2Cl - 10 min	--
9	--	--	0.3% Hg2Cl - 10 min	--
10	70% Ethanol - 1 min	--	0.3% Hg2Cl - 10 min	--
Control	--	--	--	100 ml sdw - 10 min
12	--	20% Domestos - 15 min	--	--
13	70% Ethanol - 1 min	20% Domestos - 15 min	--	--
14	--	30% Domestos - 15 min	--	--
15	70% Ethanol - 1 min	30% Domestos - 15 min	--	--
16	--	40% Domestos - 15 min	--	--
17	70% Ethanol - 1 min	40% Domestos - 15 min	--	--
18	--	--	0.1% Hg2Cl - 15 min	--
19	70% Ethanol - 1 min	--	0.1% Hg2Cl - 15 min	--
20	--	--	0.3% Hg2Cl - 15 min	--
21	70% Ethanol - 1 min	--	0.3% Hg2Cl - 15 min	--
Control	--	--	--	100 ml sdw - 15 min

All methods were washed first with 5 drops of Tween 20 into 100 ml distilled water for one minute, Sdw; Sterilized distilled water.

2.3. Germination media and cultivation conditions

The germination of einkorn wheat seeds was induced on 4.4 g/l Murashige and Skoog (Murashige and Skoog 1962) nutrient medium (Duchefa) supplemented with 30 g/l sucrose (Merck) and 8 g/l plant Agar (Duchefa). The pH of all media was adjusted between (5.7-5.8) using 1 N HCl and 1 N NaOH before autoclaving [11]. UV light in the laminar flow was switched on 15 minutes before seeds were planted into the media. All cultures were incubated in the growth room under 16 h light 8 h dark at 23±2 ºC and 60-70% relative humidity for 10 days [12]. Germination of einkorn wheat seeds started within 4-5 days after planted, and 10 days later germination and contamination rates were calculated [10].

2.4. Statistical analysis

The germination percentage was measured in each petri after 10 days and analyzed by SPSS software version 24. Analysis of variance (ANOVA) test was performed and mean comparison was carried by Duncan's multiple range test ($p<0.05$) [13]. Contamination percentage was, on the other hand, calculated using Microsoft Office Excel Worksheet [10].

3. Findings

During this study, various surface sterilizing materials were applied by different methods at different concentrations and exposure times to determine the most efficient sterilization procedure without the loss of germination capacity in hulled einkorn wheat. Twenty-two different surface sterilization methods were evaluated for seeds of einkorn wheat and described in Table 1. Since an efficient sterilization protocol of einkorn wheat may accelerate the success in future tissue culture studies sterilization of explant in tissue culture is the first important step.

The best germination percentage was realized in 10 minutes group with the sterilization method 2 (89.00%). Sterilization method 6 had also a good germination percentage of 86.25%. Sterilization method 8, 9, and 10 had, on the other hand, ended up with the lowest germination percentage of 1.75%, 1.75%, and 0%, respectively (Figure 1). The highest contamination in 10 minutes group developed in the control group and sterilization method 6: 100% and 20%, respectively. Sterilization methods 2, 7, 8, 9, and 10 did not have in any contamination (Figure 3).

Table 2. Germination and contamination percentages of Einkorn (*Triticum monococcum* ssp. *monococcum*) wheat.

Method	Germination (%)	Contamination (%)
1	$74.62\% \pm 4.08^{bcd}$	5.5%
2	$89\% \pm 2.93^{abc}$	0
3	$61.97\% \pm 4.03^{de}$	5.8%
4	$61.62\% \pm 6.24^{de}$	17.6%
5	$58.92\% \pm 6.85^{de}$	17.6%
6	$86.25\% \pm 2.31^{abc}$	20%
7	$25\% \pm 6.45^{f}$	0
8	$1.75\% \pm 1.25^{g}$	0
9	$1.75\% \pm 0.25^{g}$	0
10	0^{g}	0
11	$61.25\% \pm 3.14^{de}$	100%
12	$52.5\% \pm 4.33^{e}$	0
13	$97.08\% \pm 1.14^{a}$	20%
14	$92.14\% \pm 3.50^{ab}$	6.6%
15	$63.66\% \pm 4.53^{de}$	11.7%
16	$92.66\% \pm 8.83^{ab}$	0
17	$72.16\% \pm 4.21^{cd}$	0
18	$16.66\% \pm 4.21^{fg}$	0
19	$10\% \pm 4.0^{fg}$	0
20	$0.25\% \pm 0.2500^{g}$	0
21	0^{g}	0
22	$61.25\% \pm 4.26^{de}$	100%

The best germination percentage in 15 minutes group was the sterilization method 13 with 97.08%. Sterilization methods 16 and 14 had almost the same germination level, 92.66% and 92.14%, respectively. These two methods were the second better techniques (Figure 2). Sterilization methods 20 and 21 had lower germination: 0.25% and 0.00%, respectively (Figure

2). Control group and the sterilization method 13 developed the highest contamination in 15 minutes group. Their percentages were 100% and 20%, respectively. Sterilization methods 12, 15,16, 17,18,19, 20, and 21 did not produce any contaminations (Figure 4).

The results of all methods showed that, 5 drops of Tween 20 for 1 minute, then 70% ethanol for 1 minute, and 20% Domestos (4,6% NaClO) for 15 minutes (sterilization method 13) were given the best for germination values. The sterilization method 16 had the highest percentages for both (Table 2), if we consider germination and contamination together in one method.

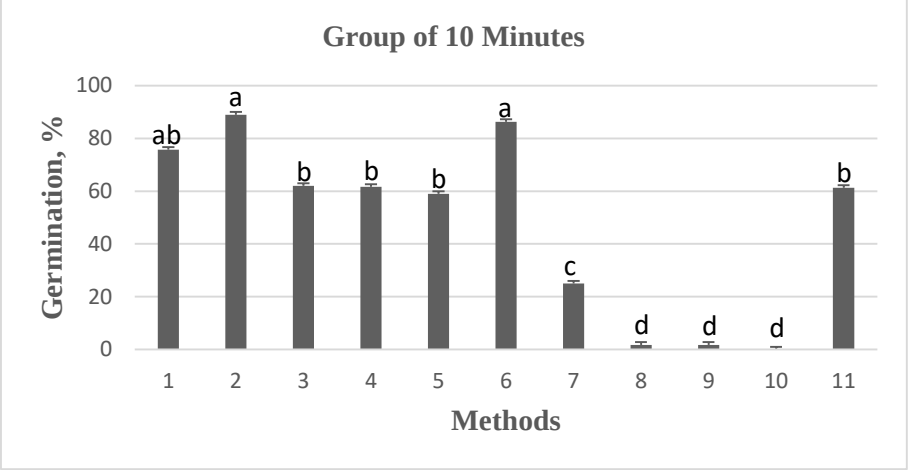

Figure 1. Germination percentage of sterilization methods. 10 minutes exposure to the sterilant. Germination rate measured after 10 days of planted date. Means indicated by different letters differ significantly at $p \leq 0.05$.

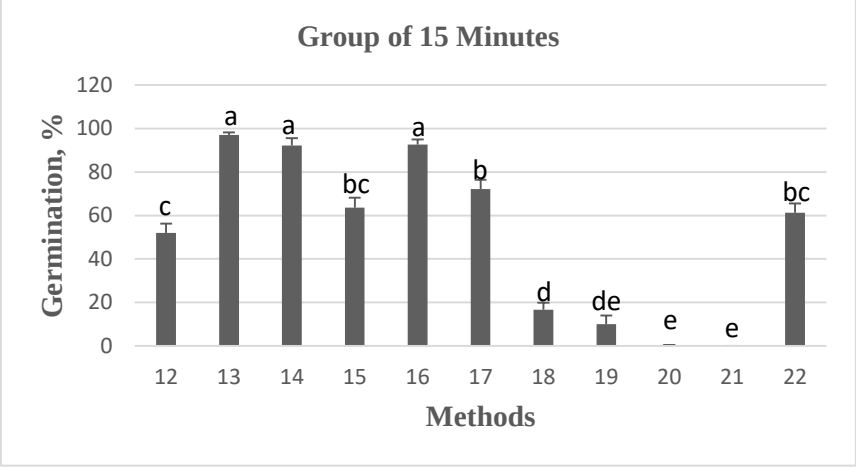

Figure 2. Germination percentage of sterilization methods: 15 minutes of exposure to the sterilant. Germination rate measured after 10 days of planted date. Means indicated by different letters differ significantly at $p \leq 0.05$.

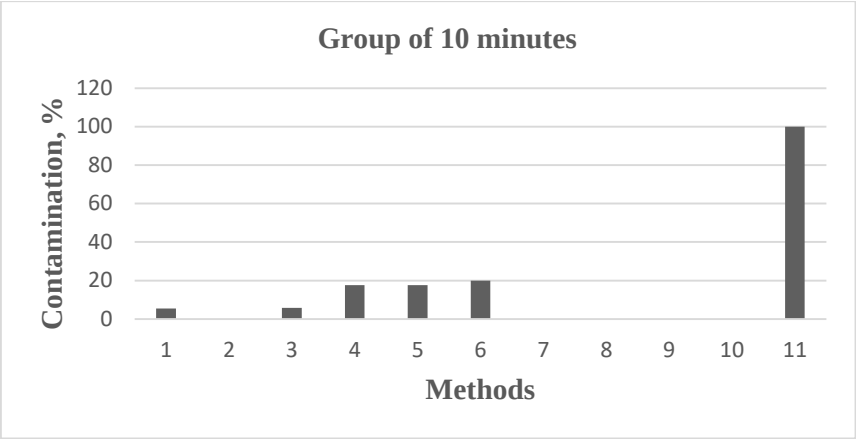

Figure 3. Contamination percentage of sterilization methods: 10 minutes exposure to the sterilant

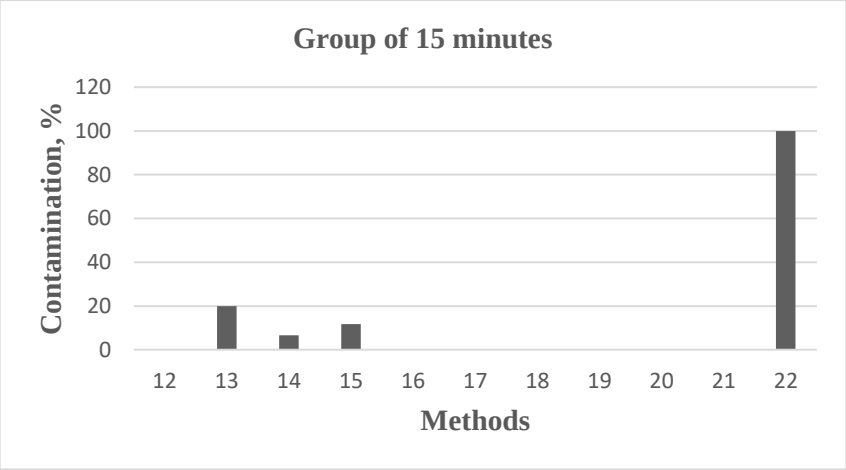

Figure 4. Contamination percentage of sterilization methods: 15 minutes exposure to the sterilant

4. Discussion and Conclusion

Tissue culture methods are successfully aided to produce many kinds of plants for commercial and research purposes [13]. During tissue culture procedure, new plants are grown from pieces of explant in a nutrient medium under sterile conditions. Several bacteria and fungi can exist and develop on these plant tissues surfaces. Therefore, it is important that the explants must be free of any microbial contaminants prior to the tissue culture since microorganisms may grow in the culture medium [14]. Thus, this study was performed to determine the best seed sterilization protocol for einkorn wheat. Many techniques in our experiment showed better germination and lower contamination rates. $HgCl_2$ was extensively applied for surface sterilization in tissue culture [15, 16]. Exposure to $HgCI_2$ decreased the survival rate of explant rate [17]. Our findings supported this statement with the lowest germination rate with the methods included $HgCl_2$. Even small amounts of $HgCI_2$ and shorter exposure time killed almost all einkorn wheat seeds (Figure 1). On the other hand, the methods with $HgCI_2$ did not allow to any contamination. That is probably because of the highly toxic properties of $HgCI_2$ to human, plant, and microorganism. Our result also showed that $HgCI_2$ was toxic to einkorn seeds and severely decreased the germination capacity (Table 2).

The use of NaClO for surface sterilization of explant sources have been reported in many previous studies as well [18-20]. NaClO is very common *in vitro* culture processes because of its simplicity, effectiveness, and cheapness. NaOCl is, also, highly effective sterilant against all kinds of bacteria, fungi, and viruses. In this study, increased NaClO amount and exposure time

amplified the germination and lessened the contamination rates (Methods 2, 14, and 16). On the other hand, Ethanol chemical is known to be powerful but also phytotoxic sterilizing agent. It is usually applied with other chemicals. Two-step sterilization process with ethanol was found to be effective in a previous study [9]. Ethanol with NaClO in our study also provided the best germination rates (Methods 2 and 13). Of these effective sterilants may provide a good start with einkorn and grain crops similar to einkorn as well and decrease the time loss when a lab starts tissue culture studies for the first time. Obtained results of the study have demonstrated that the use of simple procedure immersion in 5 drop of Tween 20 into 100 ml distilled water for 1 minute, followed by soaking in Sodium Hypochlorite 40% for 15 minutes is sufficient for the surface sterilization and of einkorn seeds without any loss of germination capacity.

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Analysis of phenolic compounds, antioxidant and antimicrobial properties of some endemic medicinal plants

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Abstract: This study was designed to evaluate the phenolic compounds and the *in vitro* antioxidant and antimicrobial activities of *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides and Ebenus macrophylla* which are medicinal plants and endemic for the flora of Turkey. The RP-HPLC-DAD (reverse phase-high performance liquid chromatography with a diode array detector) was used to evaluate the phenolic contents. The antioxidant properties were determined to use total phenolic content (TPC), ferric reducing antioxidant power (FRAP), and DPPH• radical scavenging activity assays. Antibacterial tests were performed against 11 different microorganisms by using the microwell dilution method. Each of the plant extracts were confirmed by bioactive assays which demonstrated a significant activity due to different chemical characteristics. Especially, rutin was the dominant component in *Ebenus* species with 19.434-11.808 mg phenolic/g extract. While the highest phenolic content (101.73 mg gallic acid equivalent/g extract) was observed in *O. nitida*, the highest FRAP value was in *E. laguroides* (719.09 µM $FeSO_4.7H_2O$ equivalent), and the strongest DPPH degree was in the *E. macrophylla* extract with IC_{50}: 69.45 µg/mL, respectively. Although plant extracts didn't have efficient values for antimicrobial activity, the slight effect was arisen in O. *nitida* against *B. subtilis* and *S. aureus*. The results showed that all the extracts could be used in pharmacological or dietary applications due to their valuable properties.

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1. Introduction

The traditional treatment of various plants is quite common and popular in the world. As it is widely known, medicinal value of plants comes from their secondary metabolites such as phenolic contents [1]. Phenolics are able to act as antioxidant and antimicrobial agent against oxidative stress and pathogen microorganisms [2, 3]. Antioxidants act a significant role in the attenuation of oxidative stress which is related to pathogenesis of various diseases [4]. Phenolics exert their antioxidant activities by various mechanisms such as free radical scavenging, single electron transfer and metal ion chelating [2]. Antimicrobial agents kill or inhibit the growth of

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bacteria or fungi [5]. Phenolics show potent antifungal and antibacterial activity, and through these properties they can be used for treatment of infections and diseases (such as skin diseases) [6].

Fabaceae (*Leguminosae*) contains over 18000 species widespread throughout the world, and are medicinally, economically and culturally important plants [7]. The members of this family often contain numerous chemically active constituents, such as tannins, flavonoids, and terpenes which are substances of a wide range biological activity [8, 9]. Therefore, these plants due to their therapeutic potential for the management of various conditions attract the attention of researchers. *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides and E. macrophylla* which are the members of *Fabaceae* are medicinal plants and endemic for Turkey flora [10].

O. nitida is popularly known as "fırat korungası" [10]. *Onobrychis* genus, the sainfoins, is used in the traditional treatment of bleeding, cuts, wounds in various countries [11, 12]. *Onobrychis* genus have rich content including phenolics such ferulic acid, benzoic acid, *p*-hydroxybenzoic acid, rutin, caffeic acid, quercetin, *p*-coumaric acid [13], and therefore it shows antioxidant [14] and antimicrobial activity [15].

H. cappadocicum is called as "tatlı tırfıl" in Turkey. Traditional medicine in Turkey and China uses the members of *Hedysarum* genus as a cure of the treatment for gingival and kidney diseases [16, 17]. This genus contains several physiologically active phytochemicals including kaempferol, rutin, chlorogenic acid [18]. Additionally, previous studies have reported that the members of *Hedysarum* genus or isolated compounds show antitumor [19] and antidiabetic [20] effects.

E. laguroides and *E. macrophylla*, also known as mor geven and ulu geven respectively, are two endemic plants for the flora of Turkey. Traditionally, *Ebenus* genus is used for the treatment of diseases such as skin, stomach and kidney problems [21]. These plants are rich in secondary metabolites, including methylinositol, rutin, catechin, hyperoside, picein, tannic acid, *p*-coumaric acid and have antioxidant [21, 22], anticancer [23], anticonvulsant [24] properties due to their chemical compositions.

However, to best of our knowledge there are no scientific reports on the effectiveness of combination of these plants to compare and show their properties. For this reason, the aim of the study is to evaluate the phenolic compounds and the *in vitro* antimicrobial and antioxidant activities of *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides and E. macrophylla.*

2. Material and Methods

2.1. Collection of Plant Materials

The plants were collected freshly from different locations of Erzincan during the flowering stage, in June 2015 (Table 1). Also, they were identified by Dr. Ali Kandemir. After the confirmation of taxonomic identifications of *Onobrychis nitida* Boiss., *Hedysarum cappadocicum* Boiss., *Ebenus laguroides* Boiss. var. *laguroides*, and *Ebenus macrophylla* Jaub. and Spach. were dried in sunless, their leaves and flowers were divided from the stems, and powdered, respectively and stored in tight plastic containers for further use. The voucher specimens have been collected at the Herbarium of the Espiye Vocational School, Giresu University, Giresu, Turkey.

Table 1. Collection sites and voucher number of the plant samples

Plant name	Collection site	Voucher number
Onobrychis nitida Boiss.	Iliç, north-east of Hasanova village,1391 m, Erzincan	ESPH 026
Hedysarum cappadocicum Boiss	Kemah, Kurucay roadside,1258 m, Erzincan	ESPH 027
Ebenus laguroides Boiss. var. *laguroides*	Between Kemah-Erzincan,1129 m, Erzincan	ESPH 028
Ebenus macrophylla Jaub. & Spach	Between Kemah-Erzincan,1145 m, Erzincan	ESPH 029

2.2. Plant Extract Preparation

The dried and finely powdered samples of each plant (5 g for each trial) were extracted 100 mL of methanol for 6 hours (or continued until the extract gave no coloration) using Soxhlet extractor. The extracts were then filtered by using thin paper and the homogenous solutions were evaporated until dryness by using evaporator at 40°C. Remaining extracts were divided into two parts. The first part was dissolved in distilled water for antioxidant and antimicrobial analysis. Also, the second part was prepared in pure methanol for determining of the RP-HPLC-DAD (Reverse phase-high performance liquid chromatography with a diode array detector) analyzing.

2.3. RP-HPLC-DAD Analysis

Phenolic constituents of plants were analyzed by using RP-HPLC-DAD. These analyses were achieved on Thermo Scientific Dionex Ultimate™ 3000 system (Thermo Scientific, Bremen, Germany). Chromatographic separation was performed on a Thermo Scientific™ Hypersil™ ODS C18 HPLC (250 mm × 4.6 mm x 5 µm) column (Thermo Scientific, USA) at temperature 30°C using a mobile phase (containing 2 % (v/v) acetic acid in water -A-, 70% (v/v) acetonitrile in water -B-), at a flow rate 1.2 mL/min, under gradient elution conditions. The gradient used as follows: zero-time condition was 5% B and it was increased to 60% B in 26 minutes. The eluted 10 standard phenolic acids: gallic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, rosmarinic, benzoic, and two flavonoids: rutin, quercetin were monitored by comparison at 280 and 315 nm.

The validation parameters of Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated for each standard according to the signal/noise (S/N) level of 3 and 9 respectively (Table 2).

Table 2. The standard chromatogram values of twelve individual phenolic compounds.

No	RT	Standards	R^2	RSD %(RT)	RSD %(Area)	LOD ($mg.L^{-1}$)	LOQ ($mg.L^{-1}$)
1	3.72±0.006	Gallic acid	0.999	0.168	4.315	0.070	0.213
2	6.74±0.019	Protocatechuic acid	0.998	0.291	5.973	0.495	1.499
3	10.13±0.029	*p*-hydroxybenzoic acid	0.999	0.290	4.817	0.224	0.680
4	11.46±0.027	Chlorogenic acid	0.997	0.239	6.177	0.512	1.550
5	13.49±0.023	Vanilic acid	0.994	0.168	6.794	0.171	0.518
6	13.84±0.032	Caffeic acid	0.999	0.235	6.861	0.058	0.175
7	14.79±0.012	Syringic acid	0.999	0.082	5.116	0.096	0.290
8	16.41±0.010	*p*-Coumaric acid	0.999	0.061	2.935	0.005	0.014
9	16.63±0.012	Rutin	0.999	0.075	2.855	0.311	0.942
10	18.41±0.013	Rosmarinic acid	0.999	0.069	3.388	0.162	0.492
11	18.84±0.014	Benzoic acid	0.999	0.076	2.721	0.550	1.665
12	21.71±0.019	Quercetin	0.999	0.087	2.268	0.335	1.014

2.4. Determination of Antioxidant Activity

2.4.1. Total Phenolic Content

Total phenolic content of analyzed plants was applied by the Folin-Ciocalteu method against the gallic acid calibration graph [25]. First, 680 µL distilled water, 20 µL of stock extracts, and 400 µL of 0.5 N Folin-Ciocalteu reagents were mixed in a tube and incubated for 10 minutes. Next, 400 µL Na_2CO_3 (10%) was supplied and incubated for 2 hours. The absorbance was calculated at 760 nm at the end of the incubation period. All the measurements were performed in triplicate. Total phenolic contents were represented as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

2.4.2. Ferric Reducing Antioxidant Power Assay

The FRAP method was determined according to Benzie and Strain [26]. The assay, which is based on the reduction of Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex under acidic condition, is used for the determination of cumulative antioxidant effects. The fresh FRAP reagent prepared with TPTZ, $FeCl_3$, and acetate buffer. Methodology consist of 3 mL FRAP reagent and 100 µL of the plant extracts. Absorbance was recorded at 593 nm after 4 min incubation at 25°C. Reagent and sample blanks were also tested, and the sum of these two measurements was subtracted from the final absorbance. The final absorbance was calculated with the $FeSO_4.7H_2O$ standard curve. The data were indicated as µM $FeSO_4.7H_2O$ equivalents/g extract. Higher $FeSO_4.7H_2O$ equivalent values means higher FRAP and thus higher antioxidant capacity.

2.4.3. DPPH Free Radical Scavenging Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was the main reagent on scavenging activity of compounds against 517 nm. The DPPH solution was deactivated by the antioxidants [27]. Briefly, a 50 µL portion of the extract solution was mixed with 5 mL of a freshly prepared 0.004% (w/v) DPPH radical methanol solution. Reaction volume was incubated for 30 min in the dark at 25°C. The absorbance was recorded with spectrophotometer (Mapada UV-6100PC) using a positive control blank. The inhibition percentage and extract concentration were plotted for providing 50% inhibition concentration (IC_{50}) of DPPH. The assay was performed in triplicate to give standard deviation.

2.5. Antimicrobial Activity

The antimicrobial activity of extracts were evaluated with a microwell dilution method against bacterial strains and yeast isolates [28] and minimal inhibition concentration (MIC) values were expressed as µg/mL. All test microorganisms were supplied from the Department of Biology Department at Karadeniz Technical University (Trabzon, Turkey) and were as follows: *Escherichia coli* ATCC 25922, *Bacillus subtilis* subsp. *spizizenii* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Yersinia pseudotuberculosis* ATCC 911, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* RSKK 709, *Acinetobacter baumannii* RSKK 02026, *Enterobacter cloacea* ATCC 13047, *Candida albicans* ATCC 14053.

Stock solutions of each extracts were prepared in distilled water and sterilized by filtration by 0.45 µm Millipore filters. The final concentration was adjusted with 40 mg/mL as stock solution. A dilution concentration range from 20 to 0.039 µg/mL was obtained with starting of 100 µL of stock solution in each well containing nutrient broth. Last two wells were used as a sterility control (containing culture broth plus 100 µL of stock solution, without antimicrobial substance) and a growth control. Each test and growth control well were vaccinated with 5 µL of a bacterial suspension ($5x10^5$ CFU/well). All experiments of microdilution trays were applied in triplicate and after incubation at 30 ºC (*E. cloacea*), 28 ºC (*C. albicans*), 37 ºC (*E. coli*, *B. subtilis* subsp. *spizizenii*, *S. aureus*, *K. pneumonia*, *P. aeruginosa*, *Y. pseudotuberculosis*, *E. faecalis*, *B. cereus*, *A. baumannii*) for 18-20 h. Bacterial growth was

detected by addition of 40 µL of an INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) alcoholic solution (0.2 mg/mL) (Sigma). The trays were again incubated at the temperatures given above for 30 min, and in those wells, where bacterial growth occurred, INT changed from yellow to purple. The MIC was defined as the concentration in the well containing lowest compound dose that observed no growth. Ampicillin, amikacin, and fluconazole were used as standard antibacterial and antifungal drugs, respectively.

3. Results and Discussion

Plant active compounds are gaining increasing popularity with their bioactive properties and they could be used as alternative medicinal sources. In our study herein, the extract of *O. nitida*, *H. cappadocicum*, *E. laguroides*, *E. macrophylla*, which had strong antioxidant and antimicrobial activity, was investigated with regard to 10 phenolic acid compounds (gallic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, rosmarinic, and benzoic acid) and 2 flavonoids (rutin and quercetin) Figure 1. When compared direct and indirect result from literature, the present study could be evaluated as a wide spectrum perspective with different bioactivity analysis. Also, the results of all plant samples were given as mg phenolic per g extract in Table 3.

Table 3. Phenolic constituents of the analyzed plants by RP-HPLC-DAD.

	Phenolic Compounds (mg phenolic/ g extract)	*O. nitida*	*H. cappadocicum*	*E. laguroides*	*E. macrophylla*
1	Gallic Acid	0.109	0.103	1.452	0.535
2	Protocatechuic Acid	0.953	1.668	1.935	3.745
3	*p*-hydroxybenzoic Acid	1.727	1.435	2.419	2.179
4	Chlorogenic Acid	n.d.	n.d.	n.d.	n.d.
5	Vanillic Acid	0.942	0.518	7.662	2.965
6	Caffeic Acid	0.053	0.043	0.248	0.092
7	Syringic Acid	0.077	0.271	1.231	0.668
8	*p*-coumaric Acid	0.713	0.507	5.329	2.525
9	Rutin	n.d.	n.d.	19.434	11.808
10	Rosmarinic Acid	n.d.	n.d.	n.d.	n.d.
11	Benzoic Acid	n.d.	n.d.	4.329	n.d.
12	Quercetin	n.d.	0.135	0.812	0.385

n.d.: not detected

Phenolic compounds known as secondary metabolites are separated as two groups named simple phenols (phenolic acid, coumarins) and polyphenols (flavonoids, stilbenes, lignans, tannins). Phenolic compounds are formed by some metabolic pathways which are the shikimic acid and acetic acid. These pathways can give chance for conversion and derivatization to each other. In the present study, phenolics could be seen some members of hydroxycinnamic acids, hydroxybenzoic acids, flavonols, and some ester derivatizations. Generally, ester derivatives could not be detected. Cholorogenic acid is a hydroxycinnamic acid ester and rosmarinic acid is a caffeic acid ester of 3,4-dihydroxyphenyllactic acid, they could be given as an evidence for this absence (Table 3).

With the exception of cholorogenic acid and rosmarinic acid, components were identified at various levels in sample extracts from all four types (Table 3). When the obtained results from each plant types were examined individually, different major compounds were seen as an answer. For example, rutin, the glycoside combining quercetin and rutinose, was dominate for *E. laguroides* (19.434 mg/g extract) and *E. macrophylla* (11.808 mg/g extract), protocatechuic acid for *H. cappadocicum*, and *p*- hydroxybenzoic acid for *O. nitida*.

Previous reports were generally concerning about *Onobrychis viciifolia*. It is well known as a source of proanthocyanidins which are member of tannin. There are common studies in this area. Malisch et al. [29] analyzed proanthocyanidin concentrations and simple phenolic compounds of individual plants of 27 *Onobrychis viciifolia* with Liquid chromatography-electrospray ionization triple quadrupole mass spectrometry (LC-ESI-QqQ-MS/MS). There was another study about the evaluation of polyphenolic compounds of *Onobrychis viciifolia* in order to support to its application for sustainable agriculture management in Mediterranean regions [30]. Both other actual reports which were not cited and these two studies showed us that *Onobrychis* and its subspecies had valuable bioactive sources. Although there was so many data about *Onobrychis*' studies, there was no study about the phenolic compounds of *O. nitida*. For this reason, comparisons were done with other subspecies of *Onobrychis*. Similar to our own findings, one previous study reported that *Onobrychis armena* had moderate level for *p*-hydroxybenzoic acid [13].

There is no data value about *H. cappadocicum* in scientific area. When the focus was on HPLC result, two beneficial components which were protocatechuic and *p*-hydroxybenzoic benzoic acids could be noticed. Protocatechuic acid is well known as the valuable human metabolite, combines with derivatizations of some cyanidin-glucosides. Semaming et al. [31] investigated the pharmacological properties of protocatechuic acid and their findings included antioxidant, anti-inflammatory, antihyperglycemia, antiapoptosis/proapoptosis, and antimicrobial activities. They emphasized that significant biological potential of protocatechuic acid through the modulation of cellular signals involved in the control of oxidative stress agents. Merkl et al. [32] determined the biological activities of some phenolic acids alkyl esters in the range of methyl and hexyl. It was reported that the increasing length of the alkyl chain was seen as parallel with the degree of antimicrobial effect of phenolic acid derivatives, and protocatechuic acid and *p*-hydroxybenzoic acid were possessed the bioactivity.

Besides *Onobrychis nitida* and *Hedysarum cappadocicum*, in terms of citation there wasn't any study concerning about *Ebenus laguroides* and *Ebenus macrophylla.* So, gathered results were compared with previous studies about other *Ebenus* species. Abreu et al. [22] reported some phenolic compounds from isolated of *Ebenus pinnata* aerial parts. Finally, their results showed a source of flavonoid as the present study.

There was another actual study from Turkey related to bio-properties of *Ebenus hirsute* such as some enzyme inhibitory effects, degrees of antioxidant, antimicrobial and antigenotoxic. The plant was evaluated as a remarkable biological activity source due to its rich phenolic compounds (*p*-coumaric acid, rutin, hyperoside, hesperidin, and tannic acid) which were applied by using liquid chromatography coupled with mass spectrometry (LC–MS/MS) technique [21]. Both of studies showed to us that rutin was a remarkable agent for the *Ebenus* species as in the current study.

As it is known, antioxidants mainly come from plants in the form of phenolic compounds such as flavonoid, phenolic acids. Also, phenolics have been received much attention for their effective antioxidant properties which have related with donating electrons, scavenging free radicals, and reducing power [33].

In an evaluation study written by Ince et al. [34], the antioxidant effects of the different extracts of *Onobrychis viciifolia* were designed. The antioxidant power was found 521.85±5.33 µmol/g of extract by using phosphomolybdenum method. Another study written by Karamian and Asadbegy [14] was concluded that *Onobrychis sosnovskyi* Grossh., *Onobrychis viciifolia* Scop. and *Onobrychis melanotricha* Boiss extracts represented strong antioxidant activity. In that study, Folin-Ciocalteu method, aluminum chloride colorimetric method, DPPH activity, metal-chelation activity, and ß-carotene/linoleic acid model were carried out, respectively. Although the results showed different value with the current study, because of different species

and other variable parameters (climate, region, elevation etc.), the general opinion emphasized in our study is that *Onobrychis* species suggested as antioxidant agents for special uses.

The total phenolic contents could be performed as a basis for rapid screening of antioxidant activity due to some bioactive groups such as phenolic acids, coumarins, flavonoids, stilbenes, tannins etc. known as plant secondary metabolites. Generally, total phenolic and other antioxidant assays show the strong correlation, but sometimes this situation could be change as a disadvantage. In our results, *Onobrychis nitida* had the highest value with 101.73 mg GAE/g sample. When the results were analyzed, its FRAP value showed moderate effect with 518.87 μM $FeSO_4.7H_2O$/ g extract. This uncorrelated data between TPC (Total phenolic content) and FRAP (Ferric reducing antioxidant power) could be explained with the interference of reducing ingredient such as ascorbic acid [35].

The reducing capacity of natural compounds is regarded as an impressive indicator of their potential antioxidant activity. FRAP has known as simple, quick, reproducible assay. FRAP results are calculated with linearly calibration graph related to the concentration of the concerned antioxidants. From this perspective, FRAP method was carried out to evaluate reducing capacities of the plant extracts. The results obtained showed that all samples contained high FRAP value. The highest results were in *Ebenus laguroides* with 719.09 μM $FeSO_4.7H_2O$/ g extract followed by *Ebenus macrophylla* (571.95 μM $FeSO_4.7H_2O$/ g extract), *Onobrychis nitida* (518.87 μM $FeSO_4.7H_2O$/ g extract), and *Hedysarum cappadocicum* (429.21 μM $FeSO_4.7H_2O$/ g extract). In the light of these current FRAP results, it was observed that all extracts showed nearly the same power at analyzed concentration. The main cause of small differences of these results was various bio-contents of plant species. But sometimes same species could have different bioactive values, since many factor are responsible for this changing such as storage time, geographic origin, harvesting date environment and technological factors [35].

Kedare and Singh [27] claimed that 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay is considered as precise, sensitive, simple, and commercial method to determine radical scavenging activity of antioxidants, because the radical compound is unchangeable and need not be produced. Recent studies displayed significant radical scavenging activity of natural component extracts [36, 37]. Each natural compounds show different degrees of DPPH scavenging activities depending on the capacity of antioxidant contents with losing hydrogen and converting the structural configuration [38]. *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides* and *Ebenus macrophylla* have various compounds, for this reason DPPH results could be changed as seen in the Table 4. According to our results, *E. macrophylla* extract confirmed the highest DPPH scavenging activity (IC_{50}: 69.45 μg/mL). Others were lined up by *O. nitida* (IC_{50}: 77.29 μg/mL), *E. laguroides* (IC_{50}: 88.00 μg/mL) and *H. cappadocicum* (IC_{50}: 108.32 μg/mL), respectively.

Results of antimicrobial activity of *Onobrychis nitida, Hedysarum cappadocicum, Ebenus laguroides and E. macrophylla* and standard antibiotics (Ampicillin, Amikacin and Fluconazole) were presented in Table 5. MIC values of the current extracts were among of 1.25-5 mg/mL, excluding *A. baumannii, E. cloacea* and *P. aeruginosa* bacteria and yeast *C. albicans*, while the analyzed extracts had no activity (MIC > 5 mg/mL). *O. nitida*'s antimicrobial activity was similar to that of other extracts against some microorganisms but was usually somewhat higher. The highest MIC values of *O. nitida* were 1.25 mg/mL against *B. subtilis* and *S. aureus*. All of the extracts showed no anticandidal activity. Some of these extracts showed antimicrobial activity (antibacterial activity), but their antimicrobial potentials were much lower than the standard antibiotics.

Table 4. Total phenolic contents, ferric reducing antioxidant power, and radical scavenging activities of plants.

	O. nitida	*H. cappadocicum*	*E. laguroides*	*E. macrophylla*
TPC (mg GAE/g extract)	101.73±0.52	50.96±1.01	64.88±1.13	56.97±1.01
FRAP (µM $FeSO_4.7H_2O$)	518.87±0.02	429.21±0.00	719.09±0.01	571.95±0.01
DPPH (IC_{50}:µg/mL)	77.29	108.32	88	69.45

When the TPC and HPLC analysis results presented in Table 3 and Table 4 were evaluated, some assumptions could be made about the better antibacterial activity of *O. nitida*. Higher total phenolic content might be responsible for higher antimicrobial activity. Moreover, low concentration of compounds (total concentration of standard phenolic compounds) identified by HPLC indicates that the phenolic or phenolics were responsible for antimicrobial activity, and it might be from unidentified phenolics. Our results showed that the Gram-positive bacteria were more susceptible for plant extracts, particularly *O. nitida*, than Gram-negative bacteria. This phenomenon might be associated with a thin peptidoglycan and extra lipopolysaccharide layer, which provides strong hydrophilic and less permeable properties [39].

Table 5. Minimal inhibitor concentrations (MIC) of plant extracts and reference antibiotics.

	O. nitida	*H. cappadocicum*	*E. laguroides*	*E. macrophylla*	Ampicillin	Amikacin	Fluconazole
E. coli	-	5000		5000	7.8	0.49	-
B. subtilis	1250	-	-	-	0.98	0.49	-
S. aureus	1250	2500	2500	2500	0.49	0.98	-
K. pneumonia	2500	2500	2500	2500	-	0.49	-
P. aeruginosa	-	-	-	-	-	0.49	-
Y. pseudo tuberculosis	2500	5000	5000	5000	125	31.2	-
E. faecalis	2500	5000	2500	-	1.9	62.5	-
B. cereus	5000	-	-	-	-	0.49	-
A. baumannii	-	-	-	-	7.8	0.98	-
E. cloacea	-	-	-	-	-	0.98	-
C. albicans	-	-	-	-	-	-	1.5

In the literature, there were no reports about the antimicrobial activity of *O. nitida, H. cappadocicum, E. laguroides and E. macrophylla*. So, results were compared with limited previous studies about other *Onobrychis, Hedysarum* and *Ebenus* species. In most of these reports, antimicrobial activities were determined by disc diffusion method, which did not provide a reliable and valid result for the determination of activity. In one of those studies, antimicrobial activity of water-insoluble and water-soluble leaves extracts of *Onobrychis armena* against *E. coli*, *S. aureus*, *B. subtilis*, and *C. albicans* were investigated and it was determined that there was no activity in water-insoluble extract. Similar to our results, water-soluble extract showed slightly antimicrobial activity against *S. aureus* (MIC 0.625 mg/mL) and *B. subtilis* (MIC >1000 mg/mL) [40]. Karakoca et al. [13] reported that the *O. armena* extract did not show antimicrobial activity against *E. coli*, *S. aureus*, *B. subtilis*, and *C. albicans*. They also determined that root extract had moderate antimicrobial activity (MBC: 22.50 mg/mL).

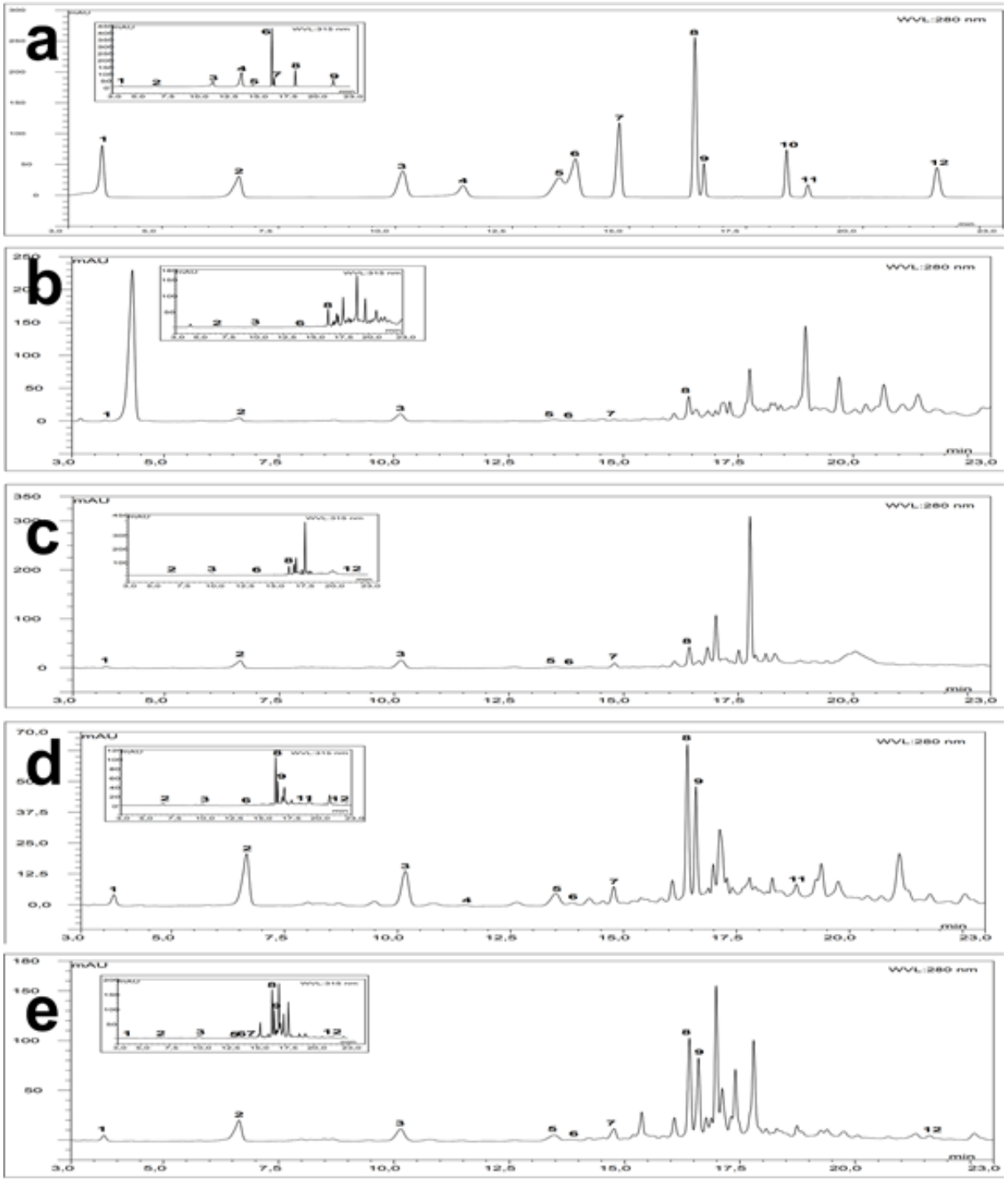

Figure 1. RP-HPLC-DAD chromatogram of plant extracts (a) standard phenolics (b) *O. nitida* (c) *H. cappadocicum* (d) *E. laguroides* (e) *E. macrophylla* (1) gallic acid (2) protocatechuic acid (3) *p*-hydroxybenzoic benzoic acid (4) chlorogenic acid (5) vanillic acid (6) caffeic acid (7) syringic acid (8) *p*-coumaric acid (9) rutin (10) rosmarinic acid (11) benzoic acid (12) quercetin

4. Conclusion

This research underlines the effort to analyze of phenolic compounds, antioxidant, and antimicrobial properties of the extracts from *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides* and *Ebenus macrophylla*. At this occasion, some different general parameters of bioactivity assays were combined and evaluated in four plant species for the first time. According to the results, it was noted that some species had strong characteristics in some branches of bioactive assays due to the changing of chemical components. Overall, there are broad agreements between bioactivity and chemical components that are based on types of plant characteristics. Also, there are some reasons for the decrease or increase of chemical properties of plants. The first factor is plant species and the others could be numbered as season, climate, and elevation. The efficient results of these endemic species may fill in the huge blanks of scientific reports and it may lead the way to enhance the further multidisciplinary experiments.

Conflict of Interest

The authors declare that there is no conflict of interests in this current study.

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Chemical Composition and Insecticidal Activity of *Origanum syriacum* L. Essential Oil Against *Sitophilus oryzae* and *Rhyzopertha dominica*

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Abstract: *Origanum* genus belonging to the Lamiaceae family is aromatic and medicinal plant. It has been used in many countries for medicinal and pharmaceutical purpose. Aerial part of the *Origanum syriacum* L. was dried at shade. The essential oil was generated by steam distillation and compounds were identified by GC-MS analysis. γ-terpinene (26.7%), thymol (26.6%) and carvacrol (22.9%) was detected as the mail constituents. The essential oil was tested for insecticidal activity against adult of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) using fumigation method. Essential oil revealed the excellent fumigant effect on *R. dominica* adults with a median lethal concentration (LC_{50}) value of 0.124 µL/L and 0.107 µL/L for 48 h and 72 h respectively. LC_{50} values of *S. oryzae* were found as 0.173 µL/L and 0.135 µL/L for 48 h and 72 h respectively. As a result, essential oil of *O. syriacum* has a potency to be a natural insecticide.

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Essential oil

1. Introduction

Plants include numerous fascinating compounds revealing plenty of biological activities [1-4]. Therefore, they play an important role in drug discovery and development process. *Origanum* genus belonging to Lamiaceae family, mainly distributed through the Mediterranean region and the Balkans has been represented almost 50 species through the world, fourteen of which are endemic for Turkey [5]. *Origanum* plant consumed as herbal tea and has been used for traditional medicine since ancient times as stimulating, antirheumatic, antispasmodic, and has antibacterial effects to treat various illness such as revulsion, dyspepsia, muscle contraction, diarrhea and infection diseases [6]. The secondary metabolites isolated from *O. rotundifolium* and *O. majorana* displayed the significant antiproliferative and antioxidant activities [7, 8]. *Origanum* species are well known for their essential oils which employ in flavoring agent mainly for meat, fish, soup and bottled food [9]. Carvacrol, caryophyllene, terpinene and thymol are the dominant essential oil compounds of *Origanum* revealing the significant biological activities [10]. *Origanum* demand has been increasing steadily in the world market, on accounts of the significance in food, pharmaceutical and cosmetic industries [11].

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Insect pests have threatened the crop production all over the world. Synthetic insecticides such as methyl bromide and phosphine fumigants have been used extensively to destroy stored pets and to protect stored food, feedstuffs, and agricultural materials [12]. However, widespread usage of fumigants has brought about the development of resistance in stored-product insects [13]. Moreover, owing to the depleting of ozone layer, residue forming, and carcinogenicity, some synthetic insecticides have been prohibited [14, 15]. As a result, it is essential to find novel fumigants which should be harmless to the environment, ecosystem, and non-target organisms to combat stored product insects. Natural products, especially essential oils have gained the great interest to replace synthetic products in fighting pets recently [16]. The rice weevil, *Sitophilus oryzae* and bean weevil, *Rhyzopertha dominica* are the most widespread and destructive insect pests of stored grains as well as stored products [17, 18].

Essential oils (EOs) exhibiting a broad spectrum of biological activities are generated from various aromatic plants. EOs have been extensively used in food and pharmaceutical industry [19]. Moreover, due to the their insecticidal effect, they have been employed in agricultural purpose [20].

Herein, the essential oil of *O. syriacum* was generated by steam distillation and identified by GC-MS. In addition, insecticidal activity test of essential oil was carried out against *S. oryzae* and *R. dominica.* The results revealed that the essential oil exhibited excellent insecticidal activity against these pets.

2. Material and Methods

2.1. Plant Material

The aerial parts of cultivated *Origanum syriacum* were harvested from the Medicinal and Aromatic Plant Field of Gaziosmanpasa University, Tokat, Turkey and identified by Prof. Dr. Ozgur Eminagaoglu. A voucher specimen was deposited in the Herbarium of the Faculty of Forest Engineering (ARTH: 5256) Artvin Coruh University.

2.2. Isolation of the Essential Oils

Aerial parts of plant material (50 g) were diluted with distillated water (250 ml) then subjected to hydrodistillation for 4 h, using a Clevenger apparatus. The oil was dried over sodium sulphate and stored in the freeze (4 °C) until to be analyzed.

2.3. GC and GC–MS Analysis

GC analyses were performed on a Perkin–Elmer Clarus 500 Series, in divided mode, 50:1, equipped with a flame ionization detector (FID) (Perkin-Elmer Clarus 500) and a mass spectrometer-equipped (Perkin-Elmer Clarus 500) BPX-5 capillary column (30 m × 0.25 mm, 0.25 um i.d., SGE Analytical science- Trajan scientific and medicinal). The temperature of injection was steady and FID was performed at 250 ºC. Helium was the carrier gas at a rate of 1.0 ml/min. The oven temperature was 50 ºC at the beginning then was raised to 220 ºC with a rate of 8 ºC/min. In the mass spectrometer, ionization energy was 70 eV and the temperature of transfer line was at 250 ºC. The standard components were used for the majority of the essential oil constituents and Kovats retention indices (RIs) were determined for all the sample components using the Van den Dool and Kratz equation according to homolog n-alkane series retention times.

2.4. Insect

The cultures of *S. oryzae* and *R. dominica* were supplied from Department of Plant Protection, Bozok University. One third of 5 L glasswares were filled with clean wheat, and then adult males and females were added to lay eggs to get a single aged population. After the adults were removed, cultures were incubated at 27 ± 2 ºC in a dark climate chamber with 60 ±

5% r.h. (relative humidity) for 48 h. The new generation of adults came into view by 45 day and 3-4 week-old adults were used in tests [21].

2.5. Dose-Response Bio-Assay

Insecticidal activity of essential oil was determined by fumigation method. Conducted dose effect on *S. oryzae* and *R. dominica* of *O. syriacum* was carried out. In the experiments, 5, 10, 15 and 20% (v/v) (essential oil/acetone) concentrations of the essential oil were used. Compressed rubber-capped glass tubes (20 ml) were used. Small paper discs were cut and then they are fixed to the cover and 10 adult specimens were situated in the each glass. 10 µl volume of the oil mixture was saturated to the filter papers by pipettes. After this process, it was waited for 5 minutes to volatilize the acetone. Then the caps were covered on the glasses and they were waited in the incubator at 25ºC in dark conditions for 24- 48 and 72 hours. The dead specimens were counted and recorded for each 24 hours interval. The randomized block design was used for the study. All experiments were repeated 3 times and each repetition consists of 3 replications [22].

2.6. Statistical Analysis

Dose-mortality response data from the bioassays were tested by POLO-PC probit analysis to determine the LC_{50}, LC_{90}, confidence level. The differences of LC_{50}, LC_{90} were executed by comparison of confidence levels [23].

3. Results and Discussion

Origanum species are well known with their rich essential oils contents which are clear red color, a pleasant and slightly spicy odor. In this study, EO analysis was executed by GC-MS and 14 components were identified with a rate of higher than 0.1%, representing 99.8% of the oil (Table 1). γ-Terpinene, thymol were the main compounds, at 26.7% and 26.6% respectively; carvacrol, p-cymene, monoterpenes which are the second most abundant compounds, were present at 22.9% and 13.3% respectively.

Table 1. Chemical composition of *O. syriacum* essential oil analyzed by GC-MS

No	Compounds	RI	Percent (%)
1	*α*-Thujene	935	0.71
2	*α*-Pinene	941	1.07
3	Camphene	955	0.13
4	Myrcene	991	2.69
5	*α*-Terpinen	1021	4.27
6	*p*-Cymene	1027	13.32
7	*γ*-Terpinene	1067	26.66
8	*α*-Terpinolen	1092	0.15
9	Borneol	1169	0.12
10	Terpinen-4-ol	1181	0.44
11	*α*-Terpineol	1192	0.12
12	Thymol	1291	26.62
13	Carvacrol	1305	22.91
14	*β*-Caryophyllene	1418	0.58
	Total		99.79

RI: Retention indices calculated against n-alkanes, % calculated from FID data

In this work, fumigation assay was executed to investigate the toxicity of *O. syriacum* essential oil against adults of *S. oryzae* and *R. dominica* at four different concentrations. EO revealed the most fumigant effect on *R. dominica* adults with a median lethal concentration

(LC_{50}) value of 0.124 µL/L at the confidence level of 0.100-0.140 for 48 h. Fumigation effect of EO increased with the time-dependence. LC_{50} value of *R. dominica* was 0.107 µL/L which was the best activity among the trials for 72 h. However, LC_{50} values of *S. oryzae* adults were calculated as 0.173 µL/L and 0.135 µL/L for 48 h and 72 h respectively. While comparing LC_{90} for *R. dominica* adults and *S. oryzae* adults, significant differences were detected. LC_{90} values of *R. dominica* adults and *S. oryzae* adults were calculated as 0.198 µL/L and 0.272 µL/L for 48 h respectively. Moreover, these values (LC_{90}) were found as 0.192 µL/L and 0.222 µL/L for *R. dominica and S. oryzae* for 72 h respectively (Table 2).

Table 2. Fumigant effect of *O. syriacum* essential oil on *R. dominica* and *S. oryzae*

Insects	hours	Slope±SD	LC_{50} (µL/L) (Confidence level)	LC_{90} (µL/L) (Confidence level)
R. dominica	48	17.166±2.790	0.124 (0.100-0.140)	0.198 (0.181-0.226)
	72	15.114±2.233	0.107 (0.082-0.125)	0.192 (0.174-0.218)
S. oryzae	48	13.014±1.520	0.173 (0.153-0.206)	0.272 (0.231-0.360)
	72	14.713±1.508	0.135 (0.123-0.147)	0.222 (0.202-0.251)

These results revealed a few differences regarding to the compounds and quantities with the literatures. For instance, *O. syriacum* collected from August at altitudes of 250-1900 m consisted of carvacrol and *p*- cymene at 60.5% and 7.4% respectively. In the same work, it was reported that *O. onites* essential oil constituted carvacrol (69.0%) as a chief product. Furthermore, carvacrol (%39.1), thymol (22.2%), and *p*-cymene (9.3%) were found in EO of *O. vulgare* [24]. These results emphasized the important variability of chemical composition of *Origanum* oils through their components. These differences could be on account of various criterions such as part of the plant, harvest period, location, altitude, climate, environmental conditions. Indeed, the synthesis and secretion of oils are influenced by these factors [25].

Insecticidal activity of *Origanum* species EOs was executed against various insects that EOs displayed the considerable activity. For instance, *O. majorana* EO displayed the good fumigant activity against *Spodoptera littoralis* which was the serious pest of some crops such as cotton, chili, and tobacco [26]. The major compounds of *O. majorana* EO were detected as thymol (38.8%), carvacrol (32.9%), *p*-cymene (7.9%), and *γ*-terpinene (5.1%). The chemical compounds of *O. majorana* have been similar with *O. syriacum*. Therefore, the insecticidal activity of *O. syriacum* may be due to the major constituents of essential oil components. Another work carried out in Algeria revealed that *O.glandulosum* has highly toxic potential power against *Rhyzopertha dominica* by both contact and fumigant assays [27].

Insecticidal components of most essential oils are usually monoterpene [28]. Because of the high volatility, essential oils have fumigant and gaseous action that may be significance for stored product insecticides. The carvracrol, a monoterpene has extensive insecticidal and acaricidal activity against agricultural stored product, and medicinal pests and act as a fumigant [29].

A good natural insecticide has been accepted that it influences a limited range of pest insects, has no harmful effects on non-target organisms and the environment, act in many ways on various types of pest complex [30]. Most plant extract and essential oils have been known to possess repellent and insecticidal activity against various stored product insects [31]. Some essential oils such as mustard oil, cinnamon oil and horseradish oil were reported to have strong

effect against *S. oryzae* insect. In addition, essential oils generated from *Pinus longifolia*, *Eucalyptus obliqua* and *Coriandrum sativum* were tested for contact and fumigant activities against rice weevil, *Sitophilus oryzae*, *Callosobruchus chinensis* and *Corcyra cephalonica* and the results varied with the test material, insect species and exposure time. In fumigation assay, *Corcyra cephalonica* and *Eucalyptus obliqua* oils at 130 µg/cm^2, led to 100% toxicity to all the species within the 24 h of the treatment [32].

4. Conclusion

O. syriacum essential oil has a potential for applications for stored-grain pests due to its high volatility and fumigant activity. In addition, EO of *O. syriacum* might be applicable for managing *S. oryzae* and *R. dominica* insects in enclosed spaces such as storage bins, glasshouses, or buildings due to their fumigant action. These results suggested that the insecticidal action of the oils might be due to the fumigant action. In other word, the oils could be toxic by penetrating the insect body via the respiratory system. The significant insecticidal activity of *O. syriacum* could be caused from γ-terpinene, thymol and carvacrol which were the major compounds of EO content. The major three compounds have methyl and isopropyl moiety. Thymol and carvacrol are constitutional isomers. In other words, both compounds have same molecular formula, $C_{10}H_{14}O$ but different structural formulas. Methyl, isopropyl and hydroxyl connected to the phenyl group in thymol and carvacrol. Hence, methyl, isopropyl and hydroxyl bonded to the phenyl may be effective for the activity.

Conflict of interest

The authors declare that they have no conflict of interest.

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Features of the Proline Synthesis of Pea Seedlings in Depend of Salt and Hyperthermia Treatment Coupled with Ionizing Radiation

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Abstract: The proline is an important amino acid that takes part on live cell protection as well as adaptation processes to adverse environment stress factors. The effects of ionizing radiation coupled with salinity or hyperthermia stress factors on pea seedlings were investigated. Different growth reactions and free proline content in root of the *Pisum sativum L.* seedlings for all treatments were evaluated. The received results of growth parameters show that some doses of ionizing radiation assists to plants in resistance to salt and temperature stressors, however this resistance is short-term. Deviation of plants reactions from additive effect to synergism or antagonism that can represent crosstalk of signal system was observed. This work proves that concentration of proline depends of stressors kind, their combinations and doses. The free proline level is a result of opposite processes of its synthesis and destruction, release and binding. The quantification of this amino acid is useful to assess the physiological status of signal systems crosstalk and more generally to understand stress tolerance of plants.

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1. INTRODUCTION

Investigation of plants response to different stress factors and their combination are going relevant in conditions of contrast changes of climatic situation and currently unstable ambient [1-2]. Special attention is paid to understanding the pathways and interconnections of signal systems and search for specific and nonspecific aspects of plant response during adaptation process. An abiotic stress is considered to be one of the main reasons for the loss of more than 50% harvest of different crops worldwide. For example, drought and salinity decrease crops yield by 20-40% and temperature increase above optimum as well as by 15% [3]. It is known that a pea is one of the most significant agricultural legumes. This dicotyledonous plant is nutritious and rich in protein, and is one of the very first an agricultural crop that was domesticated worldwide. Every year more than 7 millions hectares are planted with this crop, and up to 11 (dry) or 18 (green) millions tons of pea are produced [4]. It could be noted that influence of each stress factors on agrarian plants crop-producing is well studied separately. Nevertheless, in natural environment all living organisms are influenced by a combination of

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different stress factors that affect plant's general condition and can lead to decrease in yields and speed up degradation and/or aging ones [5-6].

The proline (pyrrolidine-α-carbon acid, $C_5H_9NO_2$) is heterocyclic amino acid where Nitrogen atom is included in secondary but not primary amine and it is one of the most universal and multifunctional secondary metabolite in plants. The proline effect of a plant is most pronouncedly manifested in response to the influence of abiotic stress factors. Increase in proline level is widespread response to different stresses, particularly osmotic stress. Therefore, quantities analysis of this amino acid is a very useful tool for evaluation of plant's physiological condition and in for understanding plant's tolerance to stress influence. Currently it is considered that in addition to well-established osmoprotective function, proline may have chaperone, antioxidant signal regulating and other functions [12-13].

Signal systems crosstalk. Particularly important is research of changes of plant's response to one stress factor under influence of another that is search of interaction points — crosstalk. Combination of the response strategies of the living organisms to different factors may lead to crosstalk of signal systems [7]. It provides the optimal and adequate response and form biochemical pathways of plant's active reaction [8-9]. For example, both heat shock and salinity stress lead to changes in SOD (superoxide dismutase) and catalysis activity [10-11]. It serves a number of functions such as signaling for transitioning to flowering/bloom [14] or supporting normal development of the pollen and seed [15]. Proline content have a direct relation to antioxidant, osmo- and membrano-protective influence, takes part in regulation of antioxidant enzymes genes expression and in binding of metals with variable valence as well as influences NAD(P)H/NAD(P) balance. To detect the signal systems crosstalk we suggest set of experiments that based on morphometrical [16] and proline level measurements of plants. It was intended to identify respective stressors combinations in time to determine the role of each signal system on an example of stress proteins' spectrum and their concentration [17]. The known specificity and non-specificity of plant's response to different stress factors indicates the certain level of correlation between parts of different metabolic subprograms and reduce or eliminate the damaging influence of the stressor to the plant organism.

The aim of our research is to study the influence of ionizing radiation in combination with other abiotic stress factors such as salinity and temperature on plant's response. These data could integrally characterize molecular, genetic, structural and metabolic changes in pea seedlings on their initial growth phase.

2. MATERIAL AND METHODS

2.1. Plant Material

The pea seeds (*Pisum sativum L.*) of the variety "Aronis" grew up in a thermostat in roll culture. The parameters of the pea seedlings growth after influence of stress factors were evaluated. The seedlings were grown in hydroculture at a temperature of 23±2°C and illumination of 2286±224 Lx on mode a dark-light cycle of 16 hours of light and 8 hours of darkness.

2.2. Scheme of Experiment

Pea seedlings at the stage of 3 days-old seedlings were exposed to stress factors. Acute X-ray irradiation was used as a stressor. The plants were irradiated by X-ray in apparatus RUM-17 (National Institute of Problem Oncology and Radiobiology of NASU, Kiev, Ukraine) in dose rate of 89 cGy/min (photon energy 180 keV), and the total doses were varied within 0 - 25 Gy (previous experiments show that irradiation in dose 25 Gy is critical for pea seedlings and can cause visible long-term defects). Additionally, some seedlings after irradiation were exposed for one hour to hyper thermal stress (at the temperature 44°C for 4-8 minutes in hydroculture)

or to osmotic stress (NaCl solution at a concentration of 0.22 Mol). After that all seedlings were returned to hydroculture and continued growth in standard environment. Each experimental group contained 25-35 seedlings. Experiments had been repeated for three times.

In the course of two weeks after stressors exposure, parameters of seedlings growth were determined. The relative growth rate (RGR) was calculated as the ratio of growth of the main root in the experimental treatment in compare with control variant. The magnitude and sign of the modifying effect antagonism, additive or synergism of the preliminary irradiated plants were evaluated by comparing the current value of the RGR. The resulting RGR ratio was expressed as a percentage. The levels of proline content in the roots of plants were measured during the month. Mode of treatments of experimental groups is shown in Table 1.

Table 1. Stress factors and the scheme of experiment .

Experimental Group	Radiation (Gy)	Stress Factors Salinity (NaCl, Mol)	Heat stress (°C)
1	0	0	RT
2	0	0.22	RT
3	0	0	44
4	5	0	RT
5	5	0.22	RT
6	5	0	44
7	10	0	RT
8	10	0.22	RT
9	10	0	44
10	15	0	RT
11	15	0.22	RT
12	15	0	44
13	20	0	RT
14	20	0.22	RT
15	20	0	44
16	25	0	RT
17	25	0.22	RT
18	25	0	44

RT - room temperature.

2.3. Measurement of Free Proline

Ninhydrin reacts with all amino acids having an α-amino group, giving a violet staining, with the exception of proline or hydroxyproline, in the reaction with which ninhydrin gives a yellow (orange) color. This colorimetric analysis is quantitative and provides reliable data on proline content, its sensitivity is about 1 nMol.

Proline is very soluble and can be easily extracted by heating the plant explants for 30 minutes in pure ethanol or in water. For measurement content of free proline the ninhydrin method was used [18]. Plant roots were chose to eliminate the influence of additional dyeing factors from vegetative materials on the color of the reaction. The plant material was frozen at -40°C or dried at room temperature. Proline was extracted from dry samples. Mixture an ethanol and water at a ratio of 70:30 was used for extraction of proline. The roots of the seedlings were homogenized and centrifuged. The supernatant was collected in a clean tube. A mixture of 1% ninhydrin in acetic acid and ethanol was added to the reaction mixture and incubated at 95°C

for 30 minutes. The tubes were cooled sharply on ice to stop the reaction. Mixture was centrifuged for 1 minute at 10000 rpm. The supernatant was taken out and transferred to the cuvettes. The optical density of the solution of ninhydrin-proline was determined at a wavelength of 520 nm on a spectrophotometer SF-26. The content of proline was determined from a calibration curve (Figure 1) constructed using standard solutions of L-proline at concentrations ranging from 0.04 to 1 mM. The level of free proline in control was taken at 100%. Statistical processing of data on plant growth parameters and proline content was carried out at a p <5% confidence level using the standard methods of the Microsoft Excel 2007 package.

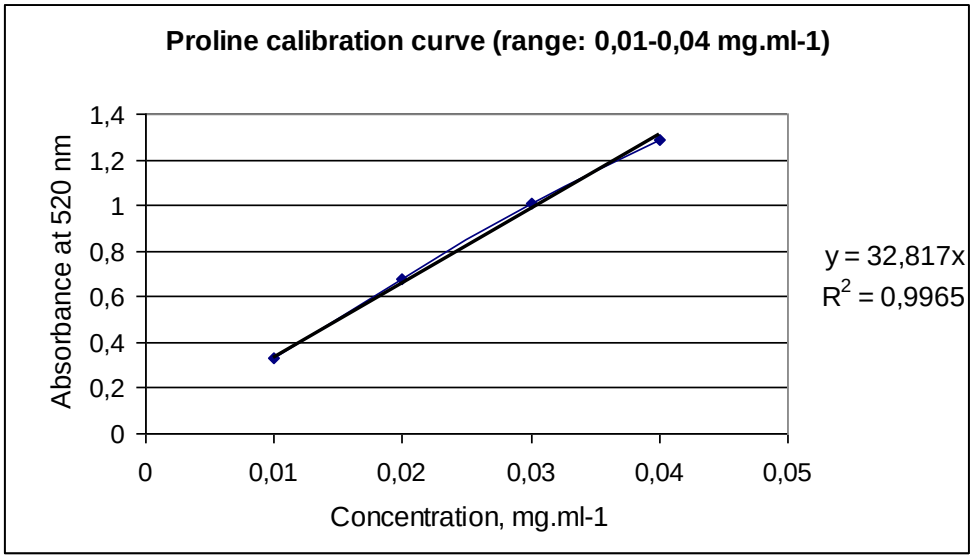

Figure 1. Calibration curve for evaluation of the free proline concentration

3. RESULTS AND DISCUSSION

3.1. The Relative Growth Rate (RGR) Analysis of Seedlings after Stress Factors Impact

According to measurements RGR of pea seedlings that were carried out 2 days after the influence of stress factors was built a chart. When the seeds are pre-irradiated at dose of 5 Gy and treated by heat shock the suffered effect significantly exceeded than was expected theoretically. It may indicate synergism between factors at appropriate doses (Figure 2, "5 Gy + t" group). In this case the experimental group which was under the affect of salt stress after irradiation at the same 5 Gy showed absolutely opposite result. It may indicate the role of ionizing radiation as a "mitigating" salinity stress factor in this particular case.

The similar behavior of pea seedlings RGR was observed with groups that undergo preliminary radiation with 10 Gy coupled with hyperthermia or salinity stress (Figure 2). At irradiation 15 Gy we could clearly observe growth suppression of the stem and root but with relatively high survivability of plants. On the fourth day after 10 Gy irradiated and heat shock influenced, the seedlings had high RGR, in comparison with the group that only was irradiated. This may indicate that the radiation modifies responses to hyperthermia and switches on some mechanisms of protection in plants.

It was found that plant growth reactions and adaptations to stressors can be influenced by content of polyamines. Obviously, increase of their concentration may have adaptive role only while they don't exceed the physiologically optimal limits. For example, for plants with protein type metabolism (e.g. pea, bean etc.) acute salinity can cause severe protein metabolism disorder and eventually accumulation of polyamides to the toxic levels that later lead to necrosis

[19]. While measuring growth reaction on term of RGR similar behavior of the relationship was observed especially when combining salinity influence with preliminary irradiation doses within 10-15 Gy.

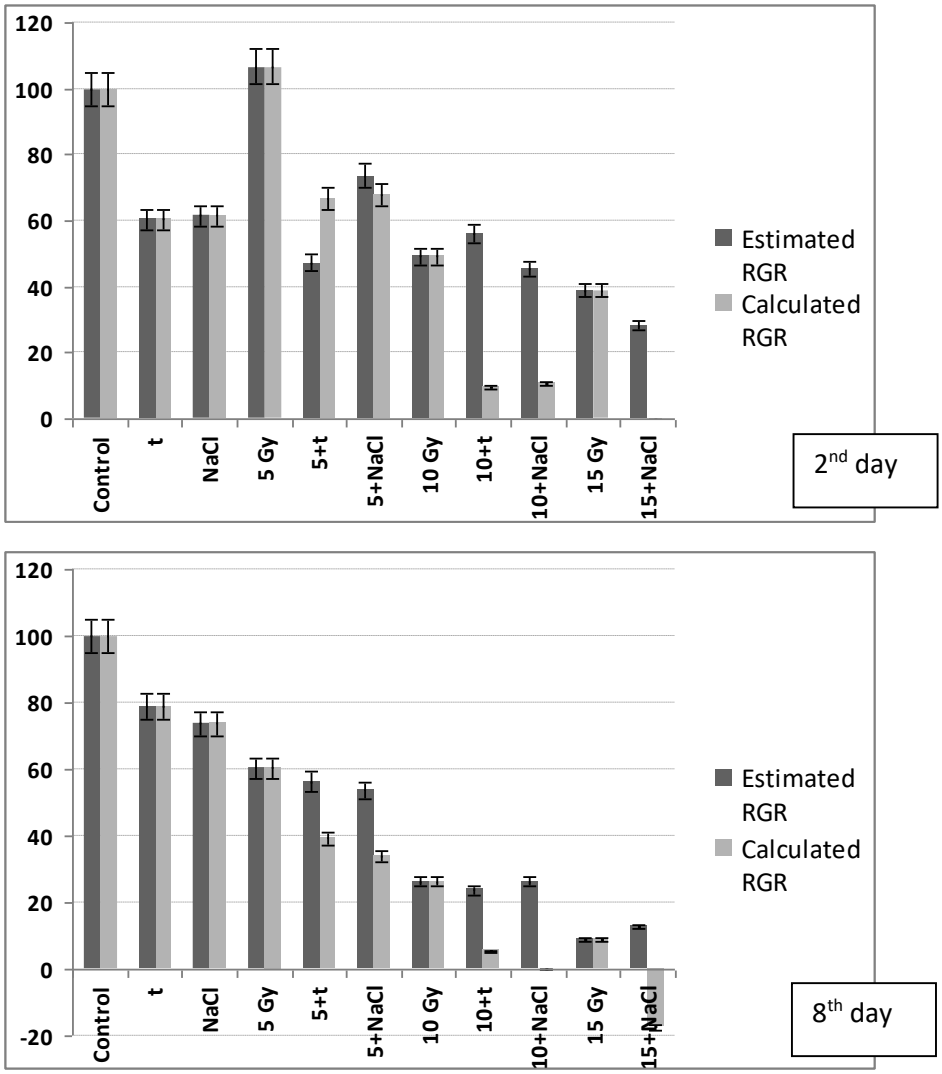

Figure 2. The impact of different doses of radiation coupled with hyperthermia or salinity stresses on the RGR of pea seedlings roots formation. 2nd and 8th days post-radiation term. (On Y axis – RGR, %. On X axis – type of impact).

Plants condition begins gradually recover in time after stress affect. The influence of different doses of acute radiation and other stress factors are presented on Figure 2. The data between 2nd and 8th day show similar response to stresses. At the same time, we could clearly observe antagonistic effects of stress factors of different origin (either salinity stress or hyperthermia after irradiation of 10 Gy and 15 Gy). It was found that plant's growth parameters exceed expectation.

On the 13th day of the experiment the length of the main root did not exceed theoretical expectations in any experimental groups. However, peripheral roots demonstrated compensatory growth after full or partial suppression of the main root's function. Similar to the stem growth it is difficult to identify a clear pattern of peripheral roots formation in response to the impact of different stress factors. Stimulation of peripheral roots growth was observed at the 5 Gy dose. But irradiation of 10 Gy and 15 Gy suppressed peripheral roots appearing. On the 13th day of pea seedlings development there was no clear relation between the stem growth

and stressor dose but there was a positive correlation between stem and main root length during the experiment (R^2=0.89). At the same time the root development was suppressed more, than that stem of seedlings.

3.2. Proline Content Measurement

3.2.1. Salt Stress in Combination with Preliminary Heat Shock

In experiment carried out on wheat seedlings exposed to salinity with hyperthermia stresses was shown [19] that the ratio of amino acids (as well as proline) in roots has a positive correlation with stems. The roots of pea seedlings were used for the proline content measuring in our studies. We suppose that results obtained on roots can be projected on the whole plant.

The content of proline was measured in roots of pea seedlings in one week after stress impact. The amino acid level was significantly higher after heating with or without salinity stress (Figure 3). It can be suggested that high temperature has a stronger or more prolonged effect on pea seedlings than salinity stress only.

Short-term heating in combination with following salinity affect decrease level of proline content in the plant's roots more drastically than chloride salinity stress only. However, proline concentration in pea roots reached to its maximum after longer period of heating. It was significantly higher in compare to control group or any groups of treated plants (salinization with/without hyperthermia). In this case heat impact has modifying influence on proline accumulation process. Previously it was reported [19] that the inhibition of the corn seedlings growth by salinization may be increased by following hyperthermia. However, short-term heating of plants up to 40°C can significantly decrease the content of the proline level.

In two days after the simultaneous impact of hyperthermia and salinization on wheat seedlings the highest lever of proline accumulation was observed. Concentration of amino acid in experimental plants was 4-5 times higher in compare to control group [19]. It can be noted that heating seedlings up to 44°C for 4 and 8 minutes provokes acute increase of proline content up to about 40% above control level. This amino acid concentration significantly varies in groups of plants after heating and following salinization. In case of 4 minutes hyperthermia proline level decreased almost twofold in compare to salinization-only group; in case of 8 minutes – increased by quarter.

As it is known increase of proline level after either ionizing radiation or osmotic shock can be explained by its origin. Proline is a product of the cell membrane proline-containing proteins disintegration. It is fixed fact that the proline rich proteins (PRPs) are involved in the formation of cell wall [20-21].

In our experiment the plants after salinization and relatively long term heating for 8 minutes can have following disintegration of all proteins that leads to plant growth suppression and significant increase of proline content in roots. It is known that impact of any stress factors is accompanied by producing of reactive forms of oxygen (ROS) superoxide anion, hydroxylate anion, hydrogen peroxide etc. They can participate in nuclear acids or proteins damage and cause peroxidation of lipids in the live cell membrane [22].

Previously it was shown that preheating of pea seedlings to 35^0C neutralized the decrease of proteins and DNA after salinization [19]. We can observe the same behavior in our experiments in groups of plants after combination of salinity and 4 minutes hyperthermia stresses. The following explanation of the different proline accumulation in different groups should not be excluded. The ability of spermine and proline to be long-distance transported through xylem and phloem from organs that undergo heat shock to other parts of plants is well known. Considering the transit nature of proline accumulation under the hyperthermia impact, we can assume that it participate in a stress signal transduction systems [23-24].

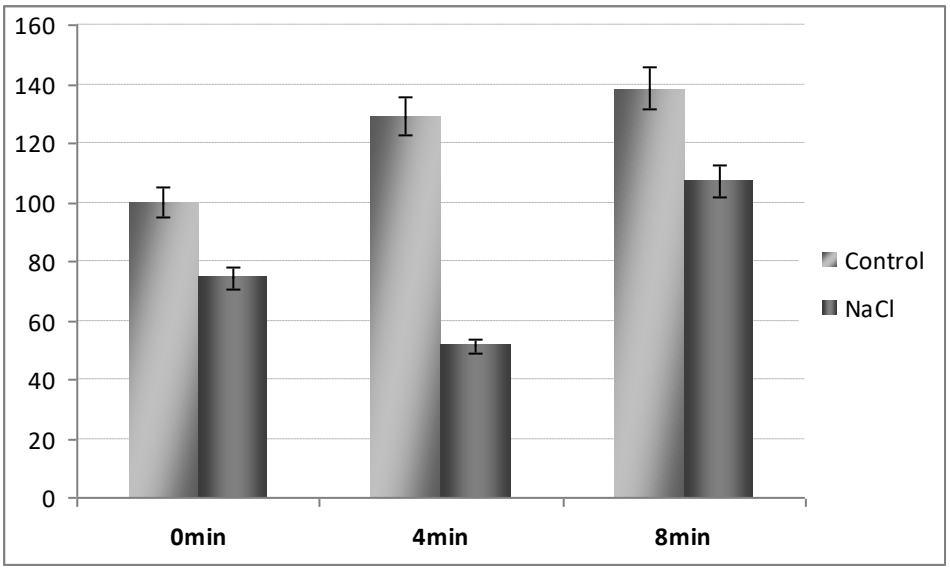

Figure 3. The proline content in pea seedlings roots in depend of salt or high temperature treatments. (On Y axis – % in compare to control; On X axis – high temperature impact duration). 7 days after stressors impact.

For example, similar proline and spermine accumulation in *Mesembryanthemum crystallinum* leaves under the influence of salinity has been shown. At the same time, increased temperature combined with salinity does not lead to significant changes in proline concentration. It can indicate that proline biosynthesis is regulated by osmotic factors in the similar manner to biosynthesis of spermine [25]. Therefore, we can assume that salinization after hyperthermia, which affected only roots of seedlings triggered the signal transmission to other organs of plants and triggered the synthesis of protective compounds there.

The proline content in plants after the combination of salinity and high temperature impact (during 4 minutes) was below the control values. It can be explained by changes in proline-rich proteins metabolism, the synthesis of proline and the transport of proline to pea stem and leaves (for example as a signal molecule). Tere is opinion that heat shock by itself can not induce proline synthesis. It just makes the plant to be competent to response to following salinization and farther accumulation of this osmolite. The nature of the receptor by which the plant cell can perceive temperature signal is still not clear. There are presumptions that changes in cell membrane fluidity can case opening of calcium channels [26] and activation of calcium-dependent pathways of signal transduction to the genome. Among other important intermediates may be active forms of oxygen or the products of lipids peroxidation [19] that can be formed by the influence of different stress factors on the living organisms.

3.2.2. Heat shock in Combination with Preliminary Irradiation

To study reaction of plant and accumulation of proline in response to radiation we suggest another set of experiments. It was also performed with heat shock in the form of 4 minutes heating to 44°C, but in this case modification factor was acute X-ray in doses up to 25 Gy.

Amino acids have different resistance to ionizing radiation. It depends on different factors, one of which is whether they are in a free state or included in the protein molecule. Thus, after irradiation the protein tyrosine breaks down faster than free tyrosine, but phenylalanine, arginine, proline and histidine in the proteins are more stable. Aromatic and heterocyclic amino acids can be put in the following order by sensitivity to ionizing radiation: histidine > phenylalanine > tyrosine > tryptophan. It is known that the most stable to irradiation

are proline and hydroxyproline. In two days after stress factors impact plant material was gathered and amino acid levels (Figure 4) were measured and analyzed.

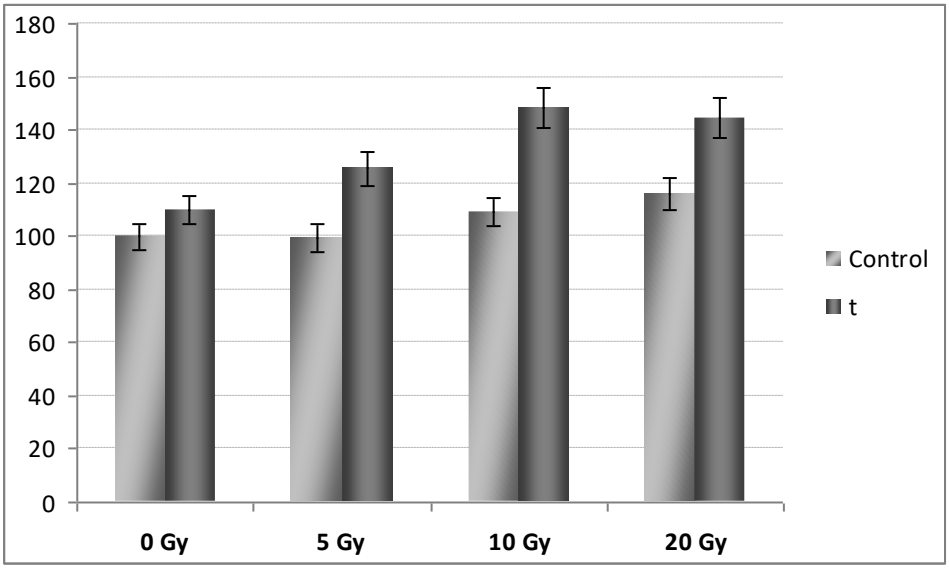

Figure 4. Proline accumulation in the pea seedlings in response to hyperthermia with and without preliminary irradiation (On Y axis – % in compare to control; On X axis – dose of radiation impact). 2 days after stressors impact.

The amino acid level was not changed significantly in response to radiation as in response to other treatments. However, it is remarkable that the proline levels increased in the case of preliminary irradiation and undergo hyperthermia impact. This type of interaction of stressors can be classified as potentiation. It means that the radiation itself does not affect the proline concentration in the plant roots but it can modify responses to hyperthermia. The evidence of this is the increase of amino acid 1.5 times in group of both stress treatments in compare to either control group or the group of hyperthermia impact only.

3.2.3. Free Proline Concentration in Pea Seedlings after Salinization with Preliminary Irradiation

As it was mentioned previously in a lot of plants the concentration of free proline in stem and in roots or other organs differs insignificantly and mainly correlates. Although some studies indicate that this amino acid accumulation in different parts of seedling supposes to be the tissue-specific.

Concentration of proline in different parts of the plant is equalized in later term suffer the stressors impact. The authors [27] note that amount of proline in both roots and leaves of corn plants has no difference in control on the 14^{th} day. In our study we evaluated amino acid content only in roots of pea seedlings to increase the reliability of measurements (by excluding chlorophyll-contained tissues from colorimetric estimations). The growth of proline concentration can be used as a quantitative measurement of stress reaction.

Proline was suggested as phyto-indicator for chemical contamination identification, because this amino acid is able to be accumulated in plant's vegetative organs in abiotic stresses environment [28]. It is known that the stronger a plant is affected by unfavorable environmental conditions, the higher the level of proline in its tissues. It has been repeatedly shown that the level of this amino acid rises more in plants that are more sensitive to stress than in resistant species [29].

Pea is sufficiently radio- and salinity-sensitive plant species so it was expected to see a fairly strong response of the metabolic apparatus and variability of the proline concentration. number of authors suggest that in annual plants the increase in proline concentration can be barely traced in 30 minutes after salinity stress, e.g. in case of millet [30], 4 hours in case of arabidopsis [31] and up to 7-10 days in case of tobacco [32].

A distinguishing feature of NaCl impact is its dual nature in compare to other abiotic stress factors: both toxic affect on ionic overflow excess and osmotic stress [33-35]. Salinity stress cause over reduction of photosynthetic chain, production of active forms of ROS and consequently oxidative stress [36]. In case of salinization Na^+ and Cl^- ions penetrate into the cells. These toxic ions lead to increase of proline accumulation, as expected, since the role of proline as a detoxicant is well known [12]. The same phenomenon was found in studies of another salts. It has been discovered that Cd^{2+} ion is one of the most severe ecotoxicants, which entails the activation of cellular mechanisms of plant stress tolerance that is caused by free proline accumulation [37].

In our studies on the 2nd day after the stress impact the highest increase of proline level was detected in plants after high doses of ionizing radiation. At 15-25 Gy the content of proline rise up to about 140% in compare to control. At the same time we observed minor increase of proline content for plants after 5 Gy with or without salinity (Figure 5).

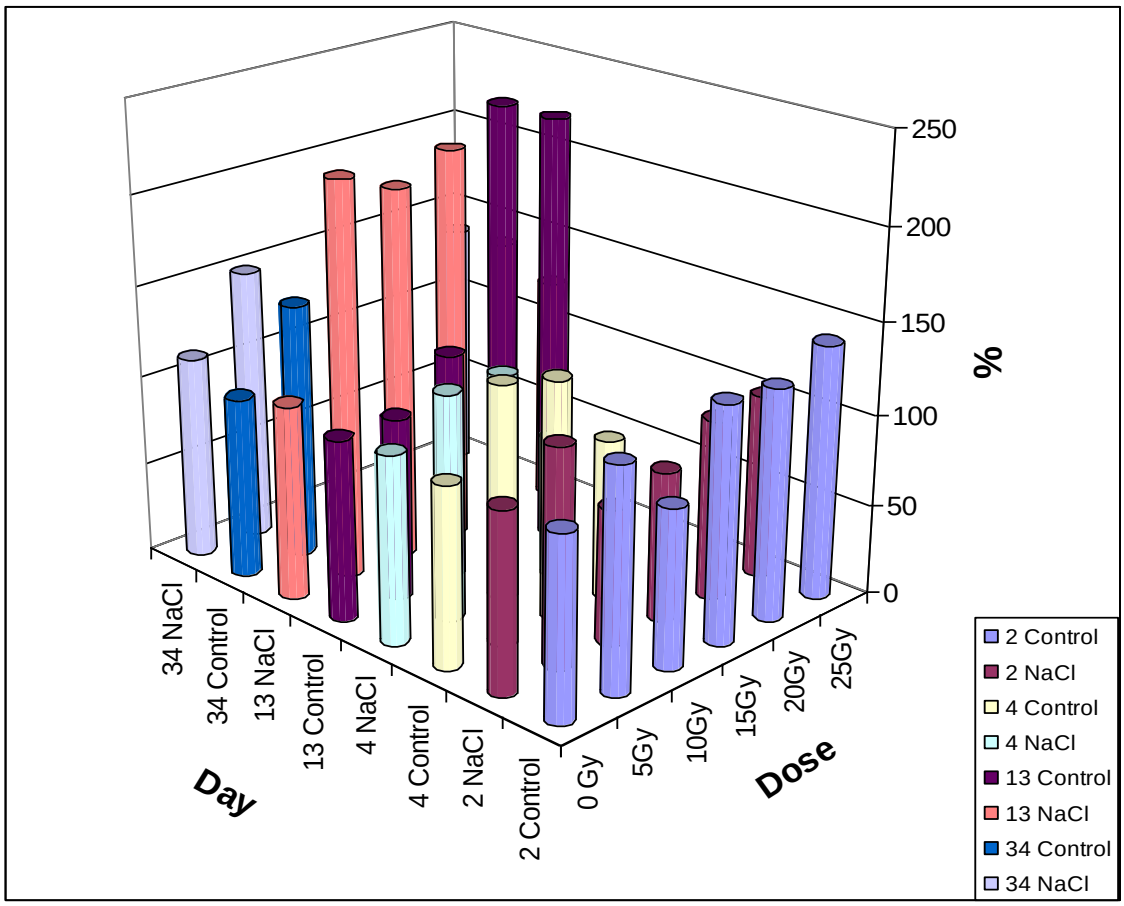

Figure 5. Free proline concentration in pea roots. 2-34 days after salinity, with or without irradiation. (% in compare to control).

It may be caused by the activation of genes expression that is involved in proline synthesis and disintegration. For instance, for Arabidopsis leaves it was shown that in 12 hours after the stress treatment in 100 and 150 mM sodium chloride, the mRNA level of the gene encoding the

key enzyme in proline synthesis - P5CS (pyroline-5-carboxylate reductase) increases by 2.5 and 3.5 folds, respectively [38].

In higher plants more than 20 genes are involved in processes of proline synthesis and disintegration [39]. Among them, two genes are especially worth mentioning - *AtP5CS2* and *AtP5CS1*. It is known that both genes are activated by low temperature, drought, high salt concentrations, and osmotic stress. They control the synthesis of P5CS (pyrroline-5-carboxylate synthetase) enzyme (key bi-functional enzyme of proline biosynthesis biochemical pathway). It is a "narrow bottle neck" in the biosynthesis of this amino acid and plays a major role in reactions to stress [40]. Another important enzyme of proline synthesis is ornithine aminotransferase (OAT). However, the role of this gene in controlling plant stress tolerance is still doubtful, since its type of expression differs from typical stress-specific variants [41].

The *P5CS* gene cloned from *Vigna aconitifolia* encodes a bifunctional enzyme with the catalytic activities of γ-glutamyl kinase and glutamyl-γ-dimial dehydrogenase. This enzyme catalyzes the conversion of glutamate to Δ'-pyrroline-ν 5-carboxylate, which is then restored to proline. Transgenic tobacco plants (with *P5CS* gene) produced up to 10-18 times more proline and higher total biomass than control plants. Increasing the concentration of osmotic active compound forward to an increase in osmotic potential (reduction of water potential) and to facilitate survival under conditions of water deficiency.

For our experiments under radiation or salinity impact it would be informative to search for similar genes and intermediate compounds that affect the level of their expression and signal transduction to explain the role of proline in the cross-adaptation of plants.

It is well known that among a large group of plant proteins there are special proline rich proteins (PRPs). They are included in the cell walls [42]. PRPs molecules containing several repeats of a short proline-rich sequences to which hydroxyproline can be added. Intra- and intermolecular cross-links that creates the structure of the cell wall are formed.

On the 13th day we noted significant growth of proline concentration. There are concerns to all groups where stress factors affect individually or in combinations. Exception was the group of 5Gy irradiated plants, in which the concentration of proline did not differ much from the control group. This may be due to the fact that previous studies of the seedlings growth reactions showed not suppression, but stimulation of root length growth for such a non-critical dose.

On the second day after stressors impact content of amino acid was 20% higher than in control group. Perhaps the proline that appeared on the first days of plant's response to minor damage was subsequently involved in the synthesis of other substances necessary for the cell in that moment. At the same time high doses of radiation still inhibited the synthesis of proline after salinization, in contrast to doses up to 10 Gy. In this dose range, apparently, as in the experiment with tobacco, we can agree with the authors' statement. They believe that as the pathological effect of salt is associated with the penetration of toxic ions into the cell, on the 7th-10th day (in our case - on 13th day) their negative impact on the internal structures of the cell may have began. This led to activation of plant protective reactions, both to the active response of cells and to the enhancement of its synthesis, and to the destruction of cell walls structures with the release of free proline [32].

On the 34th day of experiment the amount of proline in plants after salinization with or without preliminary irradiation was slightly higher than the control values on about 20%. Significant difference (up to 50%) was observed only in case of salinization combined with high radiation dose of 25 Gy. Apparently as time passes a process of restoration of plants and therefore alignment of amino acid concentrations takes place.

Another interesting fact was that according to our data the accumulation of proline in all groups turned out to be lower than the in control ones on 90th day after the stressors impact on pea seedlings. The authors [30] arrived at a conclusion that hydrogen peroxide produced by NADPH-oxidase plays role in activating the plant reactions that necessary to salinization adaptation, in particularly proline accumulation. Preliminary treating the seedlings with hydrogen peroxide or NADPH-oxidase inhibitor before the salinization reduced the usual response to salinity stress by decrease concentration as of hydrogen peroxide as proline.

It should be noted that under the impact of ionizing radiation in addition to direct energy transfer to molecules, various radiolysis products are formed in the presence of oxygen. These products have oxidative properties and include hydrogen peroxide H_2O_2. So H_2O_2 could be the reason for proline increase in irradiated seedlings (15-25 Gy). When radiation (10 and 15 Gy) was combined with salinity stress, proline concentration was 20-30% below control level. It is true for early stages of organism's response to radiation impact. In this time changes in physical, chemical and biological features of molecules defines damages of cells and cells active response is formed. The duration of this phase can be prolonged for a long period up to the lifespan of the organism. Thus on the following steps the indirect impact of radicals and hydrogen peroxide on proline level is eliminated and its concentration begins to depend on other factors. For example, the accumulation of proline can be regulated by abscises acid (ABA) and Ca^{2+} ions [43-44]. ABA activates the synthesis of proline and whole arrays of stress proteins: aquaporins, dehydrins, osmotin, proline synthesis enzymes. It assists in the accumulation of hydrated water in osmotic stress, inhibits the synthesis of RNA and normal proteins. It is shown that Ca^{2+} and a number of other inorganic ions change the activity of proline dehydrogenase, an enzyme that controls the catabolism of this amino acid [44-45].

On 13-34th day after irradiation and salinization seedlings accumulated more proline than after salinization only that indicates the loss of radiation's protection effect on late phases of pea development. At the same time the growth of the main root passes into the deceleration phase while growth of lateral roots increases. It is associated with destruction of the cell walls and activation of the intercalary meristem of the main root, which leads to the increase in proline quantity especially on 13-34th days after stress factors impact [46].

Proline is more characterized by not osmoregulatory but protective function. It affects the intracellular regulation between the cytoplasm and vacuoles, regulates the pH of cytosol, protects enzymes and intracellular structures and inactivates free radicals. It is the source of carbon and nitrogen for recovery after stress [47] takes part in signal transduction under the stress in plant. Ambiguity of proline accumulation under the impact of various stressors and their combinations may indicate the appearance of other compounds that affect the amino acid synthesis system and its degradation, signal transmission during certain stages of plant's response [48]. The experiments show that the level of proline raises first and later polyphenolic compounds are added to it [31].

A high level of free proline in both salt-tolerant and salt-sensitive plant genotypes is a display of differently directed reactions in tobacco plants. Therefore its absolute values can not be an unambiguous indicator of tolerance to salinity [32]. In addition, it is also necessary to consider the probability of other protective osmotically active substances appearance.

Further experiments on the adaptation of pea with different stress factors or their combinations, similar to experiments on pea with cross-cultivation in polyethylene glycol (PEG) and NaCl solutions [49] or our experiments, can be useful not only to achieve a certain level of proline in the tissue of seedlings to increase their integrated resistance to stress. But they also are promising for understanding the general mechanisms of crosstalk signal transduction response of the plants to stress.

4. CONCLUSION

Plants are supposed to undergo two different strategy stages to survive in harmful environment non-specific stress reaction and specialized adaptation that differs in their biological functions and duration. We observed deviation of plants reactions under combination of stress factors from additive perceive effects to synergism or antagonism that can represent crosstalk of signal system.

Radiation can modify salinity and heat stress tolerance of seedlings. Irradiation of seedlings over 10 Gy stimulated accumulation of proline on the first stages of plant's response. Following after irradiation high temperature stress increased proline level in roots in all range of radiation. On the contrary, following after irradiation salinity stress decreased proline concentration for high doses (15-25 Gy). High temperature has more strong effect on the seedlings than salinity stress ones. Heating causes modulating effect on the process of proline accumulation after salt treatment in depend of the duration of the stress influence on the plants. Unlike the growth parameters of the seedlings proline accumulation was not changed as significantly in response to ionizing radiation.

The highest level of proline was observed at 2-3rd week after stressors impact (up to three fold higher than control value) for plants irradiated 5-25 Gy coupled with salinity and after 15-20 Gy irradiated only. It may be consequence of proline synthesis enhancement and the destruction of cell walls PRPs. The non-linear relationship between growth reactions and proline concentration may indicate the presence of other (specific) adaptive plant responses to stress. It is possible that cross talk between signal systems takes place. This may lead to synthesis interact other substances that influence on proline synthesis and its degradation, signal transduction and plant's adaptive potential in general.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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Antiradical and Antibacterial Activity of Essential Oils from the *Lamiaceae* Family Plants in Connection with their Composition and Optical Activity of Components

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Abstract: Antiradical activity of essential oils of korean mint (*Agastache rugosa* (Fisch. et Mey)), blue giant hyssop (*Agastache foeniculum* (Pursh) Kuntze), hyssop (*Hyssopus officinalis* L.), lavander (*Lavandula angustifolia* L.), peppermint (*Mentha piperita* L.), lemon mint (*Mentha piperita* var. *citrata* (Ehrh.) Briq), monarda (*Monarda fistulosa* L.), oregano (*Origanum vulgare* L.), common sage (*Salvia officinalis* L.), clary (*Salvia sclarea* L.), and winter savory (*Satureja montana* L.) cultivated in the Central Botanical Garden of NAS of Belarus was investigated in the reaction with the cation-radicals of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{+\bullet}$). The most pronounced antiradical activity was observed for the essential oils with a high content of phenolic compounds: winter savory and monarda. Antiradical properties of the essential oils and individual phenolic and terpene compounds (eugenol, carvacrol, thymol, citral, (+)-pulegone) in the reaction with $ABTS^{+\bullet}$ significantly differ in aqueous solutions and ethanol-water mixtures.

High antibacterial activity of selected components of essential oils from the *Lamiaceae* plants, carvacrol, citral, and linalool, towards test organisms *Sarcina lutea, Escherichia coli, Staphylococcus saprophyticus, Pseudomonas fluorescens, Bacillus megaterium, Pseudomonas putida* was shown. The antibacterial activity of enantiomers of pinene and limonene was determined. The dextrorotary isomer of α-pinene possesses a significantly higher level of activity as compared with the levorotary one. S-(-)-limonene proves itself as a more active antimicrobial component towards *Sarcina lutea* and *Staphylococcus saprophyticus* than R-(+)-limonene. Both enantiomers show comparable activity towards *Escherichia coli*. Due to the high antibacterial activity the essential oils from *Satureja montana* and *Monarda fistulosa* can be considered as effective antibacterial agents.

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1. INTRODUCTION

The consumption of aromatic plants in the pharmaceutical, cosmetic, and food industries has been increasing steadily. The *Lamiaceae* (Lindl.) family consists of about 3500 species

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widely distributed all over the world and many plants are of interests as a source of essential oils, because they diverge sufficiently in the level of accumulation and variety of fragrant substances [1].

Essential oils distilled from plants show a wide spectrum of biological activities that depends on their composition and content of individual constituents [1-2]. Some aspects of the activity, such as antimicrobial or anticonvulsant properties, are affected by chirality of compounds biosynthesized in selected plants and their distribution in a given oil [3,4]. In its turn, the composition of essential oils originated from plants depends on many factors, such as difference in chemotypes, plant growing conditions, technologies of production and storage of vegetative raw materials [1,2]. The evaluation of antioxidant activity of essential oils frequently produces sufficiently different results that do not correlate with each other [5,6]. One of the reasons is the specificity of interaction of essential oil compounds with different media (solubility, hydrogen bonding with solvent molecules and other constituents, etc.). Antimicrobial activity of essential oils is mainly due to the content of specific components and their interaction with selected parts of bacterial cell [7,8].

We report on high antioxidant and antibacterial activity of selected essential oils distilled from raw plant material of the *Lamiaceae* family species cultivated in the central region of Belarus. The essential oil composition and properties of individual compounds are the main factors responsible for a certain type of oil biological activity. The comparison of antiradical activity of essential oil in two different solvents, water and 80 % ethanol, allows for qualitative evaluation of its major antioxidant constituents. For antibacterial activity, both essential oil composition and the content of enantiomers in it are important.

2. METHOD

2.1. Plant material

The aerial parts of korean mint (*Agastache rugosa* (Fisch. et Mey)), blue giant hyssop (*Agastache foeniculum* (Pursh) Kuntze), hyssop (*Hyssopus officinalis* L.), lavander (*Lavandula angustifolia* L.), peppermint (*Mentha piperita* L.), lemon mint (*Mentha piperita var. citrata* (Ehrh.) Briq), monarda (*Monarda fistulosa* L.), oregano (*Origanum vulgare* L.), common sage (*Salvia officinalis* L.), clary (*Salvia sclarea* L.), winter savory (*Satureja montana* L.), basil (*Ocimum basilicum* L.) plants were collected from the Herbs and Spice Collection of the Central Botanical Garden, National Academy of Sciences of Belarus. A half of the collected sample was immediately investigated and the other half was dried in shade at room temperature for two days and then analyzed.

2.2. Extraction and isolation of essential oils

The plant material was subjected to hydrodistillation in a Ginsburg type apparatus (0.2 kg per each loading) for 1 h. The collected samples of essential oils were dried over anhydrous Na_2SO_4 for 24 h and stored at 4-8 ºC until analyzed.

2.3. Chromatography

A Tsvet-800 gas chromatograph (TsvetChrom LTd., Russia) equipped with a HP-5 column (Agilent Technologies Inc., 5% phenyl 95% methylpolysiloxane: equivalent to USP Phase G27, 30 m x 0.25 mm, film thickness 0.25 μm) and a flame ionization detector (FID) with N_2 as a carrier gas was used. The column temperature was ranged from 50 to 200 ºC with an increase programmed at 3 ºC/min. The characterization of essential oil composition was achieved on the basis of retention indices (RI) using a homologous series of *n*-alkanes (Supelco) and standard substances.

The enantioselective capillary GC analysis was conducted on a Cyclosil B (Agilent Technologies Inc.) capillary column (30 m x 0.32 mm, film thickness 0.25 μm, 30% hepatkis

(2,3-di-O-methyl-6-O-t-butyl dimethylsilyl)-ß-cyclodextrin in DB-1701). The column temperature was ranged from 70 to 200 ºC in five consecutive steps: a 5 min isotherm at 70 ºC, an 3 ºC/min increase to 115 ºC, an isotherm for 20 min, a 4 ºC/min increase to 200 ºC and an isotherm for 10 min. Nitrogen was used as a carrier gas at the linear speed of 30 cm/s, the split ratio was 1:50. The identification of terpene enantiomers was performed on the basis of retention indices of the substances ((−)-α-pinene (P7408, Sigma), (+)-α-pinene (W290238, Kosher, Aldrich), (−)-β-pinene (402753, Aldrich), (+)-β-pinene (80607, Fluka), (−)-limonene (62128, Fluka), (+)-limonene (62118, Fluka)) in a standard mixture. Triplicate analysis of each oil sample was performed and quantitative results were presented as a mean of the data derived from the GC-FID analyses. The relative amounts of individual components (in %) were calculated on the basis of GC peak areas without using correction factors.

2.4. Antiradical activity

The antiradical activity of individual compound and essential oil was evaluated using its reaction with the cation-radicals of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{+\bullet}$) [9, 10]. In a typical experiment, a solution of individual compound or essential oil under investigation (5–200 μL) was added to 3.0 mL of $ABTS^{+\bullet}$ solution at 25 °C. The change of absorbance at 734 нм ($A_0 - A$) was measured 6 min after mixing.

The comparative evaluation of antiradical activity of different compounds and essential oils was carried out using values of antiradical parameter (AP) and antiradical activity (ARA). The value of AP was considered as a tangent of linear dependences of $A_0 - A$ on the concentration of individual compound or essential oil. ARA was calculated as a quotient of AP of the substance under investigation and AP of the standard antioxidant compound Trolox. ARA is equivalent to the amount of Trolox that has the same effect in the reaction with $ABTS^{+\bullet}$ as 1 mole of individual compound or 1 mL of essential oil. To avoid and quantify solvent effect we evaluated AP and ARA values of selected essential oils and individual terpene and phenolic compounds found in the oils in two different media, pure water and aqueous ethanol with 80 % content of alcohol.

2.5. Antibacterial activity

Antibacterial activity of essential oils was evaluated using the agar disc-diffusion method [11]. The method consists of determining the diameter of growth inhibition zone of test-cultures on meat-peptone agar medium (MPA) which is formed under the action of the substance diffusing in the medium from a disc impregnated with a substance that presumably has antimicrobial activity.

MPA (20 mL) was placed in a Petri dish under aseptic conditions. The bacterial culture was added to the top of agar plates as a continuous lawn with a standardized inoculum of the test microorganism using a Drigalski spatula. Then, sterile filter paper discs of 8 mm in diameter were placed on the inoculated with a test microorganism agar surface. An aliquot of essential oil (10 μL) was placed on every disc. The Petri dish was incubated under suitable condition for 48 h. The diameters of inhibition growth zones for test-cultures were measured to the nearest millimeter. For liquid individual compounds, the experiments were carried out in the similar way.

3. RESULTS AND DISCUSSION

3.1 Essential oil composition

The identified active substances and their content in essential oils obtained from different plants are summarized in Table 1. Many terpene and phenolic compounds are found in all species but some are specific for certain plants.

Table 1. Major components (%) in essential oils

Compound	RI	Agastache rugosa	Agastache foeniculum	Hyssopus officinalis	Lavandula angustifolia	Mentha piperita	Mentha piperita var. citrata	Monarda fistulosa	Ocimum basilicum	Origanum vulgare	Salvia officinalis	Salvia sclarea	Satureja montana
α-thujene	927					0.05/0.05		1.24		0.23	0.26/0.05		
camphene	950					0.08/0.05		0.11			3.14/1.21		
sabinene	971					0.15/0.18		0.18		2.25	0.37/0.05		0.12
α-pinene	976	0.47/0.02		0.50	0.12	0.35/0.43	0.31/0.40	0.49	0.43	0.51	2.38/1.03		
β-myrcene	992					0.22/0.31	0.99/1.31	1.87		1.38			1.64
1,8-cineole	1017	0.13/0.26		0.53	1.43	0.42/0.46		23.36	7.65	2.66	12.69/10.09		
β-pinene	982.1	0.06		8.52	0.10	0.27/0.47	0.31/0.44	1.14	0.49	0.94	3.69/1.41		
3-octanol	977.6									0.21			
α-phellandrene	996					0.03/0.06		0.68					0.49
β-phellandrene	1028									2.35			
p-cymene	1029					0.42/0.36				6.38			3.66
limonene	1033.2	8.63/10.02	13.5		1.61	1.79/2.86	8.25/9.73	1.44	0.17		2.06/1.96	1.4	
cis-ocimene	1035									10.24			
trans-ocimene	1050							0.46		11.24			
γ-terpinene	1063							3.49		18.02			3.88
trans-sabinenehydrate	1074					0.18/0.11					0.14/0.18		
γ-terpinolene	1078							0.45		0.41	0.52/0.31		
cis-sabinenehydrate	1089							0.16		0.1			
α-terpinene	1106							0.27		1.47	0.52/0.41		0.80
linalool	1108	0.93/0.61		0.84	43.65		0.17/0.20	1.8	44.11	1.17		24.7	
α-thujone	1114										38.82/35.11		
β-thujone	1124				1.02						3.77/4.51		
camphor	1155		0.09		0.37						13.44/13.72		0.01
menthone	1160	7.50/4.53	4.28	0.44	0.71	23.24/23.56	0.63/0.46						
pinocamphone isomer (T)	1168												0.57
isomenthone	1156	38.8/27.60				3.56/3.95							
borneol	1176							0.42			0.12/0.09		
menthol	1168					51.49/51.77							
isomenthole	1184					2.45/2.34							
terpinen-4-ol	1173							0.93		5.73	2.83/4.04		2.54
α-terpineol	1148							0.53		1.30	0.37/0.31		

Table 1. Continuation of Table-1

Compound	RI	Agastache rugosa	Agastache foeniculum	Hyssopus officinalis	Lavandula angustifolia	Mentha piperita	Mentha piperita var. citrata	Monarda fistulosa	Ocimum basilicum	Origanum vulgare	Salvia officinalis	Salvia sclarea	Satureja montana
methyl chavicol (estragol)	1207.5	33.37/34.10								0.23			
carvone	1221					0.17/0.16				0.21			
geraniol	1229							9.12					
thymol	1273		0.11		0.18			28.46		0.77			0.02
carvacrol	1288							14.01		0.31			73.4
citral (neral B)	1248		0.16	0.16			82.74/80.38					3.0	
pulegone	1250	3.59/20.8				1.21/0.81					2.85		
piperitone	1251					0.99/1.46	0.32/0.28						
linalyl acetate	1253				17.90			0.42		0.04		32.3	
bornyl acetate	1275							0.42		0.05			0.04
menthyl acetate	1289					0.96/1.81							
eugenol	1356		0.19	0.22					3.45		0.08		
ceranyl acetate	1363							0.02					
α-copaene	1391									0.04			
carvacryl acetate	1370							0.04		0.06			
β-bourbonene	1391	3.53/2.02				0.26/0.23	0.16/0.14	0.11		0.27			
β--elemene	1399					0.27/0.24	1.28/0.87	0.2		0.17			
β–caryophyllene	1436	0.84/1.46	0.88	0.38	0.75		1.55/1.33	0.8		7.17	3.59/6.73		1.56
aromadendrene	1443					1.78/1.70				0.04	0.06/0.11		
α–caryophyllene	1455										4.31/8.76		0.08
α-humulene	1472									0.92			
γ–muurolene	1483							0.03		0.19			
germacrene D	1490							1.87		6.10			0.22
γ-cadinene	1505							0.11					0.20
α-muurolene	1518									0.13			
α-cadinene	1524							0.18		0.14			
α–farnesene	1537									1.67			
viridiflorol	1595										0.1/0.07		
caryophyllene oxide	1605									0.45			0.28
T–muurolol	1636										0.1/0.11		
Other components													

RI- for column 1, * - fresh/dried

β-Myrcene is the main representative of the group of acyclic triene monoterpenes in most investigated essential oils. The essential oil of *O. vulgare* is distinguished by a high content of another compound of this group – *cis*-ocimene. At the same time, only the essential oil of *M. fistulosa* contains *trans*-ocimene. Among the functional derivatives of acyclic monoterpenes, linalool found in eight essential oils is the most common component. Linalool and linalyl acetate dominate in oils distilled from *L. angustifolia* and *S. sclarea* (61.55 and 57% of total composition). Citral is the major component in *M. piperita var. citrata* but it is also found in the essential oils from *S. sclarea*, *H. officinalis*, and *A. rugosa*. Geraniol, another derivative of acyclic monoterpenes, was observed exclusively in the *M. fistulosa* oil.

Monocyclic monoterpenes of the essential oils differ in variety. Among the investigated plants, 1,8-cineole is found in a high quantity in the essential oils from *M. fistulosa* (the second most abundant component) and *S. officinalis* (the third one). The central metabolite of the biosynthesis of monocyclic monoterpenes of the menthane group, limonene, is found in practically all essential oils with the exception of *H. officinalis*, *O. vulgare*, and *S. montana*. Limonene is contained in high amounts in *A. rugosa, A. foeniculum,* and *M. var. citrata* oils. Menthone is the second most abundant constituent after menthol in peppermint essential oil. The sum content of mentone and isomenthone exceeds 46% in *A. rugosa* oil. Monoterpene ketone pulegone in high amounts presents in essential oil from the *A. rugosa* dried materials, while no traces of the compound is found in the oil from *A. foeniculum*. Pulegone is also observed in minor amounts in *M. piperita* and *S. officinalis*. Another monoterpene ketone piperitone was found in both *Mentha* sp.

Bicyclic monoterpenes present in practically all of the studied essential oils. The sum of pinenes is high in essential oils from hyssop and common sage, but still their combined content is lower than 10%. Thujones and camphor prevail in the essential oil from *S. officinalis*, their sum reaches 56.03% and 53.34 % in the oils from fresh and dried plant materials accordingly.

The amount of sesquiterpenic hydrocarbons is the largest in the *O. vulgare* essential oil, but did not exceed 18% of all compounds. The presence of bicyclic sesquiterpene β–caryophyllene is confirmed in all analyzed oils with rare exception, its high content is found in the oils of *M. piperita var. citrata, O. vulgare,* and *S. officinalis*.

Among the investigated essential oils the highest content of phenolic compounds was observed in ones from monarda and winter savory, thymol and carvacrol combined give 42.61% of the oil in the first plant, and carvacrol alone constitutes up to 73.4% in the second with a light admixing of thymol. One more phenolic compound eugenol is found only in minor amounts in the oils from *A. rugosa, H. officinalis*, and *S. officinalis*.

The composition of essential oils from spice and medicinal plants of the *Lamiaceae* family varies significantly indicating the complexity of synthetic pathways of secondary plant metabolites in every species.

3.2. Distribution of enantiomers in essential oils

(-)-α-Pinene is found as the dominating form in the essential oils from all investigated *Mentha* and *Agastache sp*., while the dextrorotary form of the compound prevails in the oils of *M. fistulosa, L. angustifolia*, and all *Salvia sp*. (Table 2).

Table 2. Enantiomer components (%) in essential oils from dried plants

Compound	RI	*Agastache rugosa*	*Agastache foeniculum*	*Hyssopus officinalis*	*Lavandula angustifolia*	*Mentha piperita*	*Mentha piperita var. citrata*	*Monarda fistulosa*	*Salvia officinalis*	*Salvia sclarea*
1*S*-(-)-α-pinene	985	0.02		0.35	0.02	0.26	0.26	0.03	0.60	0.05
1*R*-(+)-α-pinene	989	-		0.15	0.10	0.14	0.16	0.15	1.70	0.12
1*R*-(+)-β-pinene	1031	0.01		1.72	-	0.29	0.44	0.3	0.80	-
1*S*-(-)-β-pinene	1036	0.05		6.80	0.10	0.21	0.32	0.1	0.20	-
4*S*-(-)-limonene	1068	0.20	0.16	-	0.94	2.09	5.66	-		-
4*R*-(+)-limonene	1077	9.82	13.34	-	0.67	0.19	2.36	6.70		-
(-)-linalool	1225	0.61	-	-	43.65	-	-	-	-	20.49
(+)-linalool	1229			0.84	-			0.25		12.06
S-(-)-camphor	1267		0.09	-	0.17				2.48	-
R-(+)-camphor	1269				0.20				15.78	-
(–)–menthone	1240	4.53		0.44	0.12	21.10	0.83		-	-
(+)–menthone	1246		4.28		0.59		-		0.52	-

RI – for column 2 (chiralic).

For β-pinene, the (-)-enantiomer is found in the dominant amount in the *Hyssopus* oil. A higher content of this form as compared with the (+)-isomer is observed in essential oils from *Lavandula* and *A. rugosa*, where the terpene is detected in trace amounts. At the same time, the (+)-enantiomer is prevalent in both *Mentha* species, *Monarda*, and *S. officinalis*. Limonene exists exclusively as the (+)-form in *Agastashe*, while in the essential oils from the *Mentha* kind the (-)-form is abundant.

In the *Lavandula* essential oil, the sole presence of (-)-linalool is detected while in the essential oil from *S. sclarea* it is accompanied by (+)-linalool, the amount of the later was twice as low. In the oil from *S. officinalis*, the (+)-isomer of camphor dominates while in the oil of *L. angustifolia* the excess of (+)-camphor over (-)-isomer was minor.

3.3 Antiradical activity of individual compounds

The choice of phenolic and terpene compounds for testing antiradical activity in the $ABTS^{+\bullet}$ system is based on the chromatographic analysis of essential oils from the *Lamiaceae* family species, as indicated in Table 1, and availability of pure individual components. AP and ARA of the individual compounds are summarized in Table 3.

Table 3. Antiradical activity of selected compounds

Compound	AP, L/mol		ARA, mol Trolox/mol	
	water	80% ethanol	water	80% ethanol
eugenol	$2.5 \cdot 10^5$	$2.0 \cdot 10^5$	65.8	52.6
carvacrol	$3.5 \cdot 10^4$	$5.5 \cdot 10^3$	9.2	1.5
thymol	$1.5 \cdot 10^4$	$7.0 \cdot 10^2$	4.0	$18 \cdot 10^{-2}$
(+)–pulegone	$3.7 \cdot 10^3$	0	1.0	0
(±)–citral	0	$0.2 \cdot 10^2$	0	$5.3 \cdot 10^{-3}$

Individual phenolic constituents of essential oils, such as eugenol, thymol, and carvacrol, show extraordinary antiradical activity exceeding that of Trolox (Table 3). It is evident that the specificity of ARA of the phenolic compounds is determined by their structure. The activity of phenolic compounds in radical reactions depends on two factors: the strength of O–H bond and the presence of bulk substituents in the ortho-position of phenol ring that create steric hindrance [12]. Thus, shifting bulk isopropyl substituent from meta-position relatively to –OH group as in carvacrol molecules into ortho-position as in thymol results in a decrease of ARA by a factor of 2.3 in aqueous and by a factor of 8.1 in ethanol medium. Eugenol shows ARA that exceeds that of carvacrol 7.1 times in water and 36.3 times in ethanol.

Among the investigated monoterpenoids, pulegon solely demonstrates the ability to react with $ABTS^{+\bullet}$ in water, while citral does in ethanol. The later discolors the cation-radical solution in the concentrations of the order of 1 mmol/L. Both substances have α, β – unsaturated carbonyl moiety, from which they derive the potential properties to act as radical scavengers, covalently bind to target proteins, or act as antioxidants, for example, by thiol trapping [13].

The significant difference of ARA of the compounds in different media presumably can be explained by the interaction of individual components of essential oils with solvent molecules, by specific and non-specific solvation in particular. Phenols can form hydrogen bonds with molecules containing hetero-atoms or π-bonds [12]. In such solvents, phenolic-type inhibitor (InH) exists in two forms: as free molecule and associated with solvent through hydrogen bond (InH и $InH...OH_2$ accordingly). Free radicals attack phenolic O–H bond which is not involved into such complexation, and lower value of rate constants in such solvents is due to decreasing concentration of free, and thus more active, molecules InH. Depending on

the phenol structure the association can be different. Moreover, the cation-radicals $ABTS^{+\bullet}$ themselves are highly polar particles and presumably form complexes with alcohols.

By ascending ARA, the investigated compounds can be arranged in the following orders: in water: linalool = 1,8–cineole = linalyl acetate = menthone = limonene = β–caryophyllene = citral (zero activity) < pulegone < thymol < carvacrol < eugenol; in 80% ethanol: linalool = 1,8–cineole = linalyl acetate = menthone = limonene = β-caryophyllene = pulegone (zero activity) < citral < thymol < carvacrol < eugenol. The values of ARA of the compounds in aqueous solutions are higher than those in the medium with a high ethanol content, but their ranking is similar in both media.

3.4 Antiradical activity of essential oils

As one can see from Table 4, the investigated essential oils vary in antiradical activity. Taking into account the presence of radical scavenging compounds among the constituents of different essential oils and the character of ARA changes in water and ethanol aqueous media the investigated essential oils can be arranged in three nominal groups with carvacrol/thymol, eugenol/pulegone, and citral dominating types of antiradical activity.

Table 4. Antiradical activity of essential oils

Essential oil	AP, L/mL		ARA, mol Trolox/mL	
	water	80% ethanol	water	80% ethanol
Agastache foeniculum	0.04	0.02	0.01	$5 \cdot 10^{-3}$
Agastache rugosa	3.5	1.5	0.9	0.4
Hyssopus officinalis	4.3	0.4	1.1	0.1
Lavandula angustifolia	-	0.02	-	$5 \cdot 10^{-3}$
Mentha piperita	4.6	0.6	1.2	0.2
Mentha piperita var. citrata	1.8	1.4	0.5	0.4
Monarda fistulosa	42.9	17.6	11.3	4.6
Ocimum basilicum	21.1	2.5	5.6	0.7
Origanum vulgare	7.9	2.6	2.1	0.7
Salvia sclarea	2.2	1.9	0.6	0.5
Salvia officinalis	1.8	0.8	0.5	0.2
Satureja montana	182.4	108.3	48.0	28.5

The essential oil of *S. montana* shows the highest ARA both in water and 80% ethanol due to the domination of phenols, carvacrol and thymol, in this oil. Two other oils with a high ARA value, *M. fistulosa* and *O. vulgare*, also contain carvacrol and thymol in sufficient amounts. The comparative analysis of ARA values shows that in aqueous ethanol, as compared with pure water, the essential oils of *S. montana*, *M. fistulosa*, and *O. vulgare* decrease their activity towards $ABTS^{+\bullet}$ similar to pure carvacrol and thymol, but to a lesser degree than these individual compounds.

A tendency of 2-10 times decreasing ARA in ethanol solutions was also observed for the oils with intermediate ARA values, such as *M. piperita*, *H. officinalis*, *A. rugosa*, *S. officinalis*, and *A. foeniculum* containing eugenol and pulegone/piperitone in reasonable amounts. The essential oil of *O. basilicum* with the highest eugenol content among investigated oils [14] shows relativly high activity in the discoloration of $ABTS^{+\bullet}$ in water and 8 times lower ARA value in ethanol. In the oils of *Lavandula* and *A. foeniculum*, a low content of eugenol and

thymol is combined with a high concentration of easily oxidazable terpenes, thus their ARA is extremely low in both solvents.

Despite the fact that citral alone has no antiradical activity in pure water, the oils of *S. sclarea* and *M. citrata* containing high concentrations of citral retain comparable ARA level both in water and aqueous ethanol solutions. Piperitone found in moderate amounts in the *M. citrata* oil can also host some antiradical activity.

The results above suggest a complex character of the interaction of $ABTS^{+\bullet}$ with essential oils which are multicomponent systems of interacting with each other components. The properties of essential oils as a whole are sufficiently different from simple additive mixtures of individual constituents.

3.5. Antimicrobial activity of essential oil components

Many constituents of essential oils are reported to have high antibacterial activity. Besides being related to physicochemical characteristics of the compounds (such as lipophilicity, water solubility, molecule polarity), antibacterial effect appears to be dependent on the lipid composition and net surface charge of the bacterial membranes. Furthermore, the active compounds might cross the cell membranes, penetrating into the interior of the cell and interacting with intracellular sites critical for antibacterial activity [11,15].

According to the obtained experimental results carvacrol completely inhibits proliferation of *E. coli, S. lutea*, and *S. saprophyticus* bacteria (Figure 1). High antimicrobial activity of carvacrol and compounds with similar structure (thymol, cymene, carvacrol methyl ester) is well known and associated with the presence of system of delocalized electrons and enhanced by phenol hydroxyl group. Carvacrol presumably adsorbs in phospholipid bilayer, thus fluidizes the cytoplasmic membrane that weakens and expands [11,15]. As a week acid, carvacrol disturbs the pH gradient and ionic transport across the cytoplasmic membrane. This results in collapse of the proton motive force and depletion of the ATP pool and eventually lead to cell death [8]. Eugenol showed lower activity against microorganisms than carvacrol and thymol, due to its capacity to make intramolecular hydrogen bonding with the neighboring ether groups; therefore, it has diminished ability to make intermolecular hydrogen bonding [16].

Oxygenated monoterpenes exhibit strong antimicrobial activity while hydrocarbon derivatives possess lower antimicrobial properties, as their low water solubility and limited hydrogen bonding capacity decrease their diffusion through the medium. Ketones, aldehydes, and alcohols are considered as more active antimicrobial compounds, but with varying specificity and levels of activity. Their activity is related to the presence of functional groups and influenced by hydrogen bonding parameters in all cases [16, 17]. As compared to antibiotic streptomycin, thymol, carvacrol, and menthol reportedly showed higher antibacterial activity, while linalool, linalyl acetate, camphor, and 1,8-cineole showed the same or slightly higher level of activity than the antibiotic [7]. The sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone that have traditionally been used as flavorants and aroma compounds in the food and perfume industries enhance bacterial permeability and susceptibility of *Lactobacillus fermentum*, *Staphylococcus aureus*, and *Escherichia coli* to a number of exogenous antimicrobial compounds [18].

Our experimental results prove citral being an effective antimicrobial agent toward all test-organisms while linalool was highly active toward *E. coli* and *S. lutea*, and less active against *S. saprophyticus.* However, other terpenes show a sufficiently lower level of activity. (+)-Pulegone is half as active as citral. (-)- Carvone and (+)-menthofuran inhibit slightly the proliferation of two types of bacteria, while (-)-thujon was active exclusively against *E. coli*. 1,8-cineole shows no antibacterial properties under experimental conditions (Fig. 1).

S-(-)-limonene is more active towards *S. lutea* and *S. saprophyticus*, while both enantiomers of the compound were equally effective against *E. coli*. (R)-(+)-limonene is known to be especially effective in inhibiting the proliferation of a variety of microorganisms that cause crop damage or food spoilage, including *Aspergillus niger*, *Colletotrichum falcatum*, *Bacillus subtilis*, and *Staphylococcus aureus* even though some studies declare that it exerts almost no antimicrobial activity [19].

The agar diffusion test shows that levorotary isomers of pinenes do not have any bacteriostatic properties while both (+) - α- and β-pinenes are active toward *S. lutea*, and (+)-α-pinene - towards *E. coli*. It is in good agreement with other observations that only the dextrorotary enantiomers of the α- and β-isomers of pinene are microbiologically active [20].

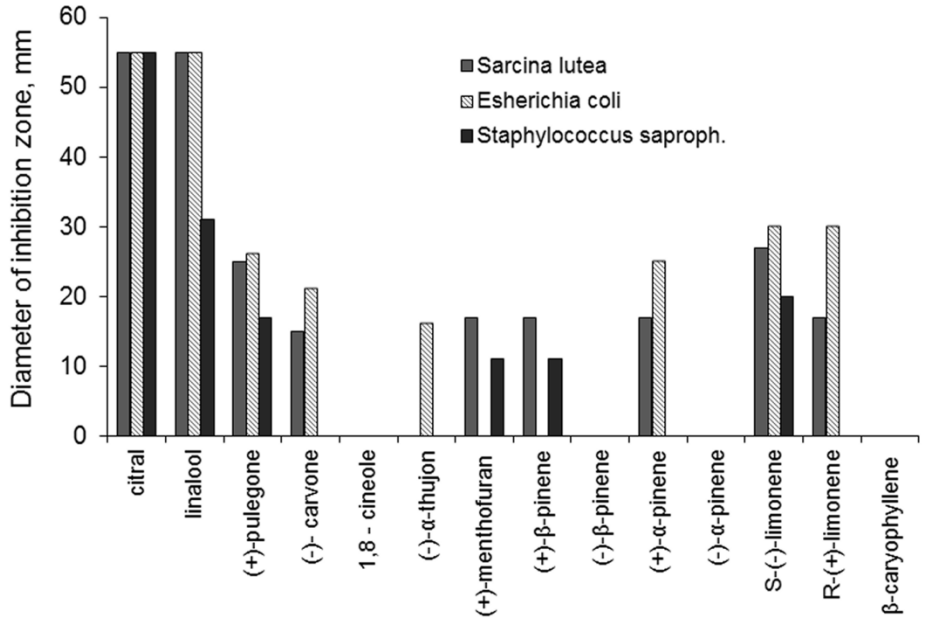

Figure 1. Antimicrobial activity of selected components of essential oils.

3.6. Antimicrobial activity of essential oils

In the used assay the oils of *S. montana, L. angustifolia, M. fistulosa, H. officinalis*, and *S. officinalis* are highly active toward *Sarcina lutea*. The oil of *S. montana* appears to be the most effective among them ensuring a proliferation inhibiting zone on a *Sarcina lutea* continuous lawn as high as 69 mm (Figure.2). The oils from *M. fistulosa* and *S. montana* were the most effective among investigated plants towards *Pseudomonas putida*. A higher efficacy of the essential oils towards gram-positive *S. lutea* as compared with gram-negative *Pseudomonas putida* can be explained by specific structure of cell walls of gram-positive and gram-negative cultures. The presence of additional outer membrane supports to higher resistivity of gram-negative bacteria to bactericidal agents of different nature.

The essential oil of *S. montana* also shows the highest activity towards *P. fluorescens* supporting the zone of proliferation inhibition of about 26 mm. Other oils were much less active or show no activity at all against specific bacteria. With the exception to *M. citrata* and *S. officialis*, all oils were effective towards *Bacillus megaterium*.

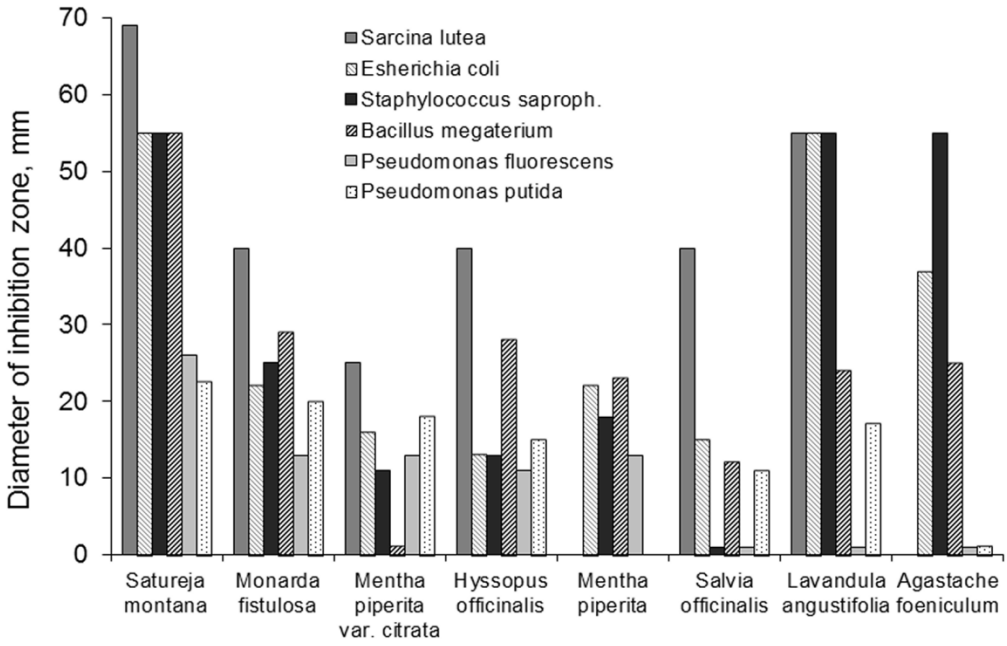

Figure 2. Antimicrobial activity of essential oils.

4. CONCLUSIONS

The antiradical and antibacterial activity of essential oils distilled from raw plant material of the *Lamiaceae* family species in model systems varies significantly and depends on their composition and properties of individual compounds.

Antiradical properties of essential oils and individual phenolic and terpene compounds that are components of the oils significantly differ in aqueous solutions and ethanol-water mixtures. The comparative analysis of antiradical activity of essential oils in the two different media, water and 80 % ethanol, allows us to distinguish three nominal types of essential oils related with their major antioxidant constituents. The highest antiradical activity in the reaction with the ABTS cation-radicals was observed for essential oils with high content of carvacrol and thymol: winter savory and monarda. The intermediate ARA values that are 2-10 times lower in ethanol than in water are associated with an eugenol/pulegon type of activity in peppermint, hyssops, korean mint, and common sage. The citral-related type of activity with comparable ARA levels in water and 80% ethanol is observed for clary and lemon mint oils. The comparison of antiradical activity in water and ethanol-water mixtures can be used as a tool for preliminary analysis of essential oils of unknown composition in order to evaluate the presence of certain classes of constituents in the mixture.

For antibacterial activity, both the essential oil composition and the content of enantiomers in it are important. A significantly higher antibacterial activity of the dextrorotary isomer of α-pinene as compared with the levorotary one was found. S-(-)-limonene proves itself as more active antimicrobial component towards *Sarcina lutea* and *Staphylococcus saprophyticus* than R-(+)-limonene, while both enantiomers show comparable activity towards *Escherichia coli*. Essential oils from the *Lamiaceae* family plants with a high content of carvacrol, citral, and linalool are traditionally chosen for high antibacterial activity towards a wide spectrum of bacteria. Due to high antibacterial activity essential oils from winter savory and monarda can be considered as the most effective antibacterial agents among the investigated essential oils.

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Fructan Determination in Transgenic Nicotiana Tabacum L. Plants Harbouring Human Inf α2b Gene Infected by Tobacco Mosaic Virus

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Abstract: Fructan is one of the main storage compounds in higher plants, their accumulation and synthesis is associated with the plant's adaptation. They take part in cryoprotection, osmoregulation and water balance and included in plant protection from pathogens (biotic stress). Fructan, anthocyanins, proline are involved in reduction of negative effects of biotic factors on plants.

Utilizing of genetic engineering methods makes it possible to create stress resistant plants. However, the impact of the transformation still remains unclear, because transformation can be regarded as biotic stress factor for plants. Investigation of the biotic stress effects on metabolic pathways in transformed plants is still actual.

The aim of our work was to identify changes in fructan accumulation caused by the presence of tobacco mosaic virus (TMV) in transgenic Nicotiana tabacum plants with human ifn - α2b gene.

Control wild type plants contained 6.8 + 0.08 mg/g of fructan. In the transgenic line №1, the level of fructan accumulation was 4.68 ± 1.02 mg/g. In the transformed line №3, its content increased to 30+1.8 mg/g. The fructan content in control plants increased from 6.8 + 0.08 to 8.2 + 1 mg/g, after viral infection. For transgenic line №1, the content of fructan increased to 25 + 1 mg/g. The growth of fructan concentration was not observed in the line №3.

The increase in fructan content in infected transgenic plants can be regarded as adaptive response to viral infection as biotic stress.

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Определение Содержания Полифруктанов В Трансгенных Растениях *Nicotiana Tabacum* L. С Геном Inf-*A2B* Человека, Инфицированных Вирусом Табачной Мозаики

Резюме: Были определены изменения в накоплении фруктанов в трансгенных растениях *Nicotiana tabacum* с геном *ifn - α2b* человека, вызванные наличием вируса табачной мозаики (ВТМ). Для получения экстрактов растительный материал взвешивали, гомогенизировали с добавлением фосфатного буфера (pH 7.4) и центрифугировали 15 минут при 15 тыс. g. После отбирали необходимую аликвоту для проведения анализов. Колориметрический метод (реакция Селиванова) применяли с использованием 0,1% спиртового раствора резорцина для определения количества полифруктанов. В результате было показано, что содержание фруктанов в контрольных растениях составило 6,8±0,08 мг/г. В разных линиях трансгенных неинфицированных растений было установлено как отсутствие достоверных различий накопления фруктанов по сравнению с их содержанием в контрольных нетрансформированных неинфицированных растениях, так и увеличение их содержания до 25±1 мг/г массы. В инфицированных трансгенных растениях содержание фруктанов было выше, чем их содержание в инфицированных нетрансформированных контрольных растениях и составляло до 30±1,8 мг/г и 10±2,2 мг/г соответственно.

Генетическая трансформация в ряде случаев приводила к повышению содержания фруктанов в трансгенных растениях. Наличие фитовирусной инфекции приводило к увеличению содержания фруктанов в инфицированных трансгенных растениях по сравнению с их содержанием в нетрансгенных инфицированных растениях. Вероятно, такое повышение является одной из реакций растений табака на фитовирусную инфекцию как на биотический стрессовый фактор. Следует отметить, что как генетическая трансформация, так и инфекция приводили к развитию растительного ответа, вызванного биотическим стрессовым фактором. Таким образом, нами было показано, что растения реагируют на влияние двух стрессовых факторов, что выражается в накоплении фруктанов, как одного из возможных защитных механизмов.

ИСТОРИЯ СТАТЬИ

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КЛЮЧЕВЫЕ СЛОВА

Nicotiana tabacum L,
ВТМ,
фруктаны,
биотический стресс,
интерферон.

1. ВВЕДЕНИЕ

Фруктаны являются одними из главных запасных соединений высших растений [9]. Выделяют несколько основных типов фруктанов: инулин (в основном у двудольных), леван, нео-инулин и нео-леван (у однодольных). У двудольных растений фруктаны накапливаются как долгосрочные резервные углеводы в основном в подземных органах [17]. Их накопления и синтез относят к адаптациинной реакции растения на стресс [8]. Известно, в частности, что фруктаны участвуют в процессах криопротекции и осморегуляции клеток и обеспечивают поддержание водного баланса, обеспечивая длительную защиту мембран растений от обезвоживания, препятствуют высушиванию при абиотических стрессах [13, 14], снижают негативное воздействие замораживания [6, 7]. Было показано, что они могут стабилизировать мембранные структуры и уменьшать отток воды из клеток [13]. Благодаря фруктанам поврежденные растения могут быстро восстанавливаться [6]. При биотических стрессах фруктаны включаются в синтетические процессы, направленные на защиту растительного организма от негативного воздействия патогена, стабилизируя мембранные структуры [15]. Явление, которое связано с повышенным синтезом фруктанов при биотических стрессах, получило название «сладкий иммунитет» [10]

Методами генетической инженерии возможно создание устойчивых к стресам растений, однако, известно, что сама генетическая трансформация является стрессом для растений и может приводить к накоплению фруктанов в растениях. Фитовирусная инфекция также является биотическим стрессовым фактором, поэтому актуально комплексное исследование действия биотических стрессов (трансформация и наличие вируса) на изменения синтеза фруктанов в трансформированных растениях [5].

Исходя из этого, целью нашей работы было исследовать изменения в синтезе фруктанов в трансформированных растениях *Nicotiana tabacum* с геном *ifn-α2b* человека до и после инфицирования вирусом табачной мозаики.

2. МАТЕРИАЛЫ И МЕТОДЫ

В качестве объектов исследования использовали полученные нами ранее растения *Nicotiana tabacum* L сорта *Petit Havana* с генами *ifn-α2b* человека и неомицинфосфотрансферазы II. Растения выращивали в стерильных условиях на без гормональной среде МС [11]. Опытные растения переносили в стерильную почву для дальнейшего роста в условиях теплицы при температуре +24°С с 16-часовом режимом освещения.

Вирусосодержаший материал получали из пораженных листьев растений табака с симптомами вирусной инфекции путем гомогенизации в фосфатном буфере (pH 7,4) с последующим центрифугированием в режиме 5 тыс. g 20 мин. Полученный материал был инокулирован в растения механическим втиранием в молодые листья верхнего яруса растения.

Для получения экстрактов растительный материал взвешивали, гомогенизировали с добавлением фосфатного буфера (pH 7.4) и центрифугировали 15 минут при 15 тыс. g. После отбирали необходимую аликвоту для проведения анализов.

Реакцию Селеванова проводили следующим образом: к 100 мкл экстракта добавляли по 100 мкл 0,1% спиртового раствора резорцина и HCl (5:1). Полученный раствор нагревали на водяной бане 5 мин при температуре 80^0С. После нагревания в течение 5 мин при температуре 80^0С и появления характерного вишневого окраса проводили измерение оптической плотности на автоматическом анализаторе Eppendorf biofotometr plus при длине волны 550 нм.

Исследование проводилось в трех проворностях. Для обработки статистических данных использовали пакет программ MS Exel 2003.

3. РЕЗУЛЬТАТЫ И ОБСУЖДЕНИЕ

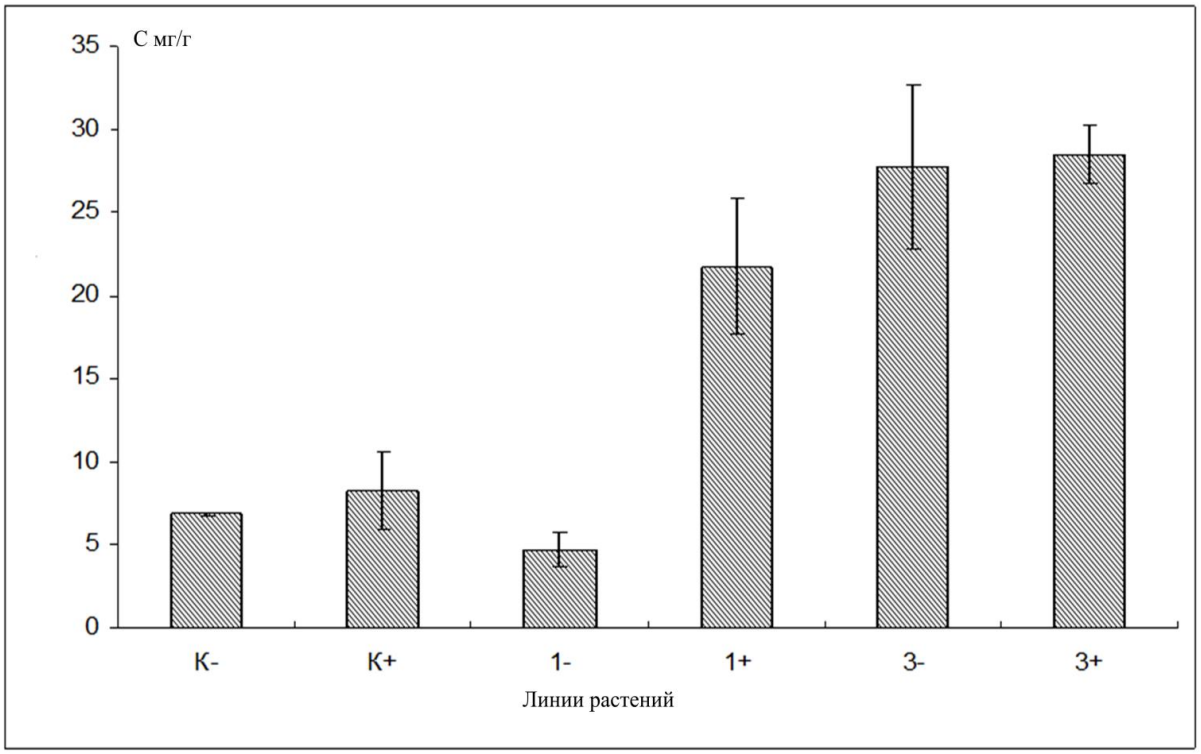
Ранее нами были получены трансгенные растение *Nicotiana tabacum* с геном *ifn-α2b* человека, их трансгенная природа была подтверждена молекулярно- биологическими методами. Для данного исследования были выбраны две разные линии трансгенного табака, которые были получены из разных исходных эксплантов и являющиеся разными трансформационными событиями.

В результате исследования было показано, что в растениях дикого типа содержание фруктанов составило 6,8±0,08 мг/г. В трансгенной линии №1 уровень накопления фруктанов достоверно не отличался от уровня этих соединений в контрольных растениях и составлял 4,68±1,02 мг/г (рис. 1). Трансгенной линии № 3 наблюдалось повышение содержания фруктанов до 30±1,8 мг/г (рис. 1).

В наших предыдущих исследованиях, проведенных на растениях цикория [2-4] и салата [1], которые были трансформованы *Agrobacterium rhizogenes* или *Agrobacterium tumefaciens* с векторной конструкцией с геном интерферона *ifn-α2b* человека, также было

отмечено, что содержание фруктанов в трансгенных растениях может как оставаться на уровне контроля, так и превышать контрольные показатели. Вероятно, это вызвано фактом переноса чужеродного гена в разные места в геноме растений. Следовательно, можно предположить, что растения разных видов могут подобным образом реагировать на генетическую трансформацию, которая в ряде случаев может приводить к повышению уровня накопления запасных соединений.

После инфицирования ВТМ происходили изменения в накоплении фруктанов. При инфицировании их содержание росло как в контрольных инфицированных растениях дикого типа, так и в трансгенных растениях с симптомами вирусной инфекции (линии №1 и №3). В контрольных растениях содержание фруктанов повышалось незначительно и составило 8,2±1мг/г. Для трансгенных растений линии №1 произошло увеличение содержания фруктанов до 25±1мг/г. Однако, в линии №3 после инфицирования не произошло существенного повышения содержания фруктанов. Этот факт можно объяснить тем, что синтез фруктанов при культивировании *in vitro* частично зависит от наличия субстрата (сахарозы) в среде. Поскольку содержание сахарозы в среде фиксировано, возможно, что именно это лимитирование привело к отсутствию дальнейшего роста содержания фруктанов в инфицированных трансгенных растениях линии №3. Вероятно, содержание этих веществ в клетках растений было лимитировано количеством субстрата.



РРис. 1. Содержание полифруктанов в растениях табака до и после инфицирования ВТМ [Примечание: - неинфицированные ВТМ растения, + инфицированные ВТМ растения; К: контрольные нетрансформированные растения; 1 и 3: линий трансгенных растений]

В результате нами было показано, что после инфицирования растений происходит повышение содержания фруктанов, что, вероятно, является результатом биотического стресса, вызванного персистенцией в их организме фитовирусной инфекции. Отсутствие значительного повышения содержания фруктанов в контрольных растениях с вирусом

по сравнению с содержанием этих соединений в контрольных растениях без вируса и увеличение содержания фруктанов в трансгенных инфицированных растениях можно расценивать как стрессовую и адаптационную реакцию трансгенных растений к вирусной инфекции. Вероятно, это вызвано физиологическими особенностями растений., поскольку каждая из полученных линий является отдельным трансформационным событием и реагирует на стресс по-разному. Однако в растениях, подвергшихся воздействию фитовирусной инфекции, происходило увеличение содержания фруктанов, что, вероятно, обусловлено развитием стресс-реакции и ответом растения на биотический стресс, вызванный циркуляцией фитовируса.

4. ОБСУЖДЕНИЕ

Генетическая трансформация в ряде случаев приводила к повышению содержания фруктанов в трансгенных растениях *Nicotiana tabacum* с геном *ifn-α2b* человека. Наличие фитовирусной инфекции приводило к увеличению содержания фруктанов в инфицированных трансгенных растениях по сравнению с их концентрацией в нетрансгенных инфицированных растениях. Вероятно, такое повышение является одной из реакций растений табака на фитовирусную инфекцию.

После трансформации, как и после инфицирования реакция растений была схожа и проявлялась в повышении содержания фруктанов. Следует отметить, что как генетическая трансформация, так и инфекция приводили к развитию растительного ответа, вызванного биотическим стрессовым фактором. Таким образом, нами было показано, что растения реагируют на влияние двух стрессовых факторов, что выражается в накоплении фруктанов, как одного из возможных защитных механизмов.

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EXTENDED SUMMARY

Introduction

Fructan is one of the main storage compounds in higher plants, their accumulation and synthesis is associated with the plant's adaptation. They take part in cryoprotection, osmoregulation and water balance and included in plant protection from pathogens (biotic stress). The increase of fructan synthesis under the biotic stresses has been associated with plant "sweet immunity". Fructan, anthocyanins, proline are involved in reduction of negative effects of biotic factors on plants.

Utilizing of genetic engineering methods makes it possible to create stress resistant plants. However, the impact of the transformation still remains unclear, because transformation can be regarded as biotic stress factor for plants. Genetic transformation can lead to the fructan accumulation in plants and to provide an effect on the plants metabolism. Investigation of the biotic stress effects on metabolic pathways in transformed plants is still actual.

The aim of our work was to identify changes in fructan accumulation caused by the presence of tobacco mosaic virus (TMV) in transgenic *Nicotiana tabacum* plants with human *ifn - α2b* gene. Selivanov reaction was used to determine fructan content.

Material and Method

Control wild type plants contained 6.8 $\pm$ 0.08 mg/g of fructan. In the transgenic line №1, the level of fructan accumulation was 4.68 ± 1.02 mg/g. In the transformed line №3, its content increased to 30$\pm$1.8 mg/g. In our previous study of transgenic chicory plants and salad were investigated. It was shown that *Agrobacterium* – mediated transformation can either increase the level of fructans or remain at control level. The phenomenon can be explained by position effect of T-DNA integration. We presume that each transformed line is a separate transformation event and has unique stress reaction.

Result

The fructan content in control plants increased from 6.8 $\pm$ 0.08 to 8.2 $\pm$ 1 mg/g, after viral infection. For transgenic line №1, the content of fructan increased to 25 $\pm$ 1 mg/g. In contrast, the growth of fructan concentration was not observed in the line №3. The fructan synthesis depends on the substrate (sucrose) in the medium during *in vitro* cultivation. The fixed sucrose content in the medium can limit further growth fructan accumulation. Probably, in line №3 the fructan content is limited by substrate in medium.

Discussion

The increase in fructan content in infected transgenic plants can be regarded as adaptive response to viral infection as biotic stress.

The increase of fructan content was observed both after transformation and viral infection. Genetic transformation or viral infection was the biotic stress factors leading to the development of plant response. Fructan accumulation is one of the possible adaptive mechanisms to the stress influence.

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Development and validation of modified QuEChERS method coupled with GC-MS/MS for 123 pesticide residues in food

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Abstract: In this study, a gas chromatography-tandem mass spectrometry (GC-MS/MS) instrument, which has been widely used in recent years and has high separation power, selectivity and ability to identify pesticides has been used. It is aimed that the main criterion of this analytical method, in which the QuEChERS methodology is used, is applicable to fast, easy, cheap, environmentally friendly and different matrices. At the same time with this method, 123 pesticide residues and their degradation products were quantitatively assayed by GC-MS/MS as well as method validations in tomatoe, lemon, lettuce, almonds, raisins, honey, green pepper, milk and flour. Tomatoe was selected as potential reference matrixes for the target. The steps of concentration and solvent exchange were performed in the resultant extracts for the purpose of improving analytical performance in terms of recovery, precision, linearity, of reducing the amount of co-extracts. Multiple reaction monitoring (MRM) was used to identify and quantify the pesticides. The samples were extracted with 1% acetic acid in acetonitrile, anhydrous magnesium acetate, anhydrous magnesium sulfate and clearing agent. For all pesticides, good linear calibrations with coefficients (R2) ≥0.99 for nearly all of the analytes were obtained. Limit of quantitation of most of the pesticides were in the range of 5-10 ng/g, and recovery of the method validation accuracy parameter was done at two different concentrations 10 ng/g and 50 ng/g were 88.6 - 99.7% and CV 1.60 – 14.0%.

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1. INTRODUCTION

Every kind of chemical compound used to protect agricultural products from disease, harmful and foreign weeds is called pesticide. A pesticide is known to be any compound or mixture of compounds that prevents, removes or protects from the spread of any unwanted organism (pest). The usage of pesticides in agriculture after World War II, the world has multiplied to increase food production. Since then, the development of different types of pesticides belonging to various groups has become important [1].

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As a result of producers' unconscious and excessive use of pesticides, resistant populations are formed, natural enemies are adversely affected and harmful effects occur in terms of environment and human health. Regular use of pesticides is detrimental to the ecosystem. Many international organizations and countries are extremely worried about pesticide residues. The pesticide maximum residue limit (MRL) determination [2,3] is being undertaken to protect public health and ensure food safety. Numerous studies have been carried out naturally on the simultaneous determination of analytical techniques, especially multiple residues, in the detection of pesticide residues [4,5]. This study is quite up to date on these aspects.

Various extraction methods have been stated in the literature including hydrolysis with chloric or sulfuric acid, soxhlet extraction, liquid-liquid extraction (LLE), extraction with organic solvent directly from the solid matrix, and more recently solid-phase microextraction (SPME) [6-10]. Purification might be needed to remove co-eluted matrix material and reduce analytical background noise. To date, purification techniques usually included elution of sample extracts with hexane and dichloromethane on chromatographic columns packed with acidified silica gel, deactivated alumina or florisil [11,12]. Recently, an attempt was made to replace these laborintensive clean-up steps by LLE or purification on solid-phase extraction (SPE) cartridges [10]. Different extraction and measurement methods have been used by various scientists for the detection of multiple classes of pesticides in many food, vegetables and fruit varieties [13,14].

In 2003, Anastassiades et al. [15] developed a fast, easy, inexpensive, effective, robust and secure (QuEChERS) method to overcome the critical deficiencies and practical limitations of existing methods. Then, Lehotay et al. 2010 developed a sample preparation method with the citrate-buffered QuEChERS procedure [16]. The main criterion for choosing any methodology is that analytical method is applicable to fast, easy, inexpensive and different matrices.

Developing a new methodology requires the resolution of a large number of problems. Like extraction solvent selection. Solvents used to identify pesticide residues in food matrices [17-19], and these solvents provide high analytical recovery. Acetone can be mixed with water, but it is not possible to separate water from the solvent without using apolar solvents. On the other hand, it is the part where ethyl acetate is mixed with water. It causes unnecessary addition of apolar solvents to separate water, but most of the highly polarized pesticides are not separated. The acetonitrile extracts of foods contain less interfering substances than ethyl acetate and acetone extracts and acetonitrile can be separated from the water quite easily (salt precipitation), which is the preferred extraction solvent of acetonitrile methodologies. The aim of the study is to develop and validate the modified QuEChERS method for 123 pesticide residue in food using GC-MS/MS.

2. MATERIAL AND METHODS

2.1. Reagent and Materials

Pesticide standards were purchased from Dr. Ehrenstorfer. Acetonitrile, methanol, acetic acid, anhydrous sodium acetate, ammonium formate, anhydrous magnesium sulphate (98% purity), Silica gel 60, PSA (Primary Secondary Amine) sorbent (40 µm particle size) were purchased from local supplier. HPLC-grade water (18.2 mΩ) was purified using a Millipore Elix Advantage 10 and Milli-Q Advantage A10 system that comprise reverse osmosis, ion exchange, and filtration steps.

The samples used in the study (tomatoes, lemons, lettuce, almonds, raisins, honey, green pepper, milk and flour) were supplied without pesticides from Fethiye, Datça, Marmaris. Fruits

and vegetables were purchased from producers of organic farming. This study has been selected as representative matrices by considering the directive of the European Union.

2.2. Pesticide Standards Main Stock Solution

All pesticide standard substances Dr. Ehrenstorfer are certified reference materials are taken from local supplier. The pesticide standards were weighed in a 50 mL volumetric flask as approximately 10 mg with a precision of 0.01 mg. The volume was completed with methanol. The main stock solutions are stored at -18 °C.

2.3. Pesticide Mix Solutions

The pesticide was withdrawn with an automatic pipette such that the concentration of each of the standard main stock solutions was 1 mg L^{-1} (1 ppm). The pesticide mix solutions for GC-MS/MS were prepared separately in methanol. The pesticide mixture solutions were stored at 4 °C.

2.4. Pesticide Working Solutions

To determine the GC-MS/MS conditions of the pesticides, the concentrations from the parent stock solutions were set to be about 500 ng mL-1 (ppb). The target ion, qualifier ions, collision energies and retention time (Rt) were determined separately in the device. For the method-device optimization, the acquisition method was created by dividing it into eight time segments.

2.5. Method

GC-MS/MS instrument coupled with Agilent Technologies 7890A GC gas chromatography 7000B Triple Quadrupole mass spectrometer was used in the study. Agilent Technologies 7693 autosampler and multi-mode inlet system were used in the system. This method involves extraction of the extract using acetonitrile pre-extraction and separating solid phase extraction. For liquid-liquid separation, salt precipitation is carried out using anhydrous magnesium sulfate, so that the water is separated from the sample.

Most of the methods applied worldwide at the clean-up level use Primary Secondary Amine (PSA) in most cases. PSA is an expensive chemical that significantly increases the cost of analysis. In this study, the sample extracts have been studied using silica-gel, which are cheaper, simple and effective chemicals, with GC-MS/MS to detect multiple pesticide residues.

The most common approach to derive from the matrix effect is to use matrix-match calibration standards [20,21]. However, a large number of blank matrices are required to work with matrix-match, which requires extra extraction. In this study, a separate matrix-match calibration curve is used for each matrix. Thus, it was already known how each pesticide will behave when interacting with the matrix.

Approximately 1 kg of the sample is disintegrated and homogenized with the aid of the sample shredder. Fifteen grams of the homogenized sample is transferred to a 50 mL falcon tupe. Add 15 mL 1% acetic acid in acetonitrile. It is vigorously shaken for 1.5 min. After addition of 1.5 g of anhydrous magnesium acetate and 6 g of anhydrous magnesium sulfate, the mixture is shaken vigorously for 1.5 min and 150 µL (5 ng mL^{-1}) of Internal Standard Solution is added. Centrifuge at 4000 rpm for 5 min. Then transfer to 8 mL of extraction tubes then add 400 mg of clearing agent (Silica-gel, PSA, C18 or GCB) and 1200 mg of anhydrous magnesium sulfate, shake, centrifuge. The extract is transferred to 2 mL vials and 50 µL 3-phenyl phosphate (TPP) is added, for the analysis using GC-MS/MS.

2.6. Instrument and Pesticide Optimization

In this study, the parent ion, daughter ions, cone voltage and collision energies and retention times (Rt) of each pesticide were determined separately in the device. Multiple

reaction monitoring (MRMs) were generated for the analysis method. The process method was established in which the ions of each pesticide were written at the time of retention. Retention time, parent and precursor ions, collision voltages for each of the pesticide were identified in order to obtain maximum signal. Standard main stock solutions were prepared in the concentration of 50-500 ng mL^{-1} and precursor and product ions of each the pesticide were determined in GC-MS/MS using SCAN mode. Ions were examined in the detected peak, and compared with molecular weight of each pesticide. It is examined by looking at the structure of the molecule, whether it is equal to the molecular weight or fragmented from the molecule. In order to detect target ion and breakdown ions in the MRM mode, different impact energy is tried in increments of 5 V between 5 and 30 for each transition of the analytes to detect the collision energy and the collision energy producing the highest area was detected. An example of this work is illustrated below for propham in Figure 1.

The pesticide representative, propham, three transitions were examined. Three different collision energies were tried, 10V, 20V, 30V. First is 179.1 > 92.2 *m/z*. The highest peak area was detected as 3.209 abundance at 20 V. Second is 93.0 > 66.0 *m/z*. The highest peak area was detected as 198.532 abundance at 20 V. With this study, for propham; 93 > 66 *m/z* and 93> 65 *m/z* transitions and the result of the collision energy optimization study have been determined.

2.7. Method Optimization

Tomato was used as the first matrix in the method optimization study. Four parallel runs according to the sample preparation procedure. The specimens were run separately for GC-MS/MS and the spikes at 50 ng g^{-1} were made in parallel from GC mixtures. Approximately 1 kg sample was crushed and homogenized with a chopper. A 15-g portion of the homogenized sample was weighed in an analytical balance with a 50 mL falcon tube and 150 µL 5 ppm di ethatyl ethyl (DEE) (internal standard) and 750 µL 1 ppm pesticide solution were added. 15 mL of acetonitrile containing 1% acetic acid was added. It was vigorously shaken by hand for 1.5 min. The previously weighed 1.5 g anhydrous sodium acetate and 6 g anhydrous. Magnesium sulfate was added, then vigorously shaken again manually for 1.5 min and centrifuged for 5 min at 4000 rpm. Previously, a 15 mL centrifuge tube was prepared by weighing 400 mg of cleaning agent (PSA/Silica gel) and 1200 mg of anhydrous magnesium sulfate, and after centrifugation, 8 mL of the supernatant was transferred, then shaken by hand and centrifuged at 4000 rpm for 5 min. 1 mL of the supernatant was transferred to 2 mL vials and 50 µL TPP was added. The tube was agitated in the mixer. The vial was analyzed in GC-MS/MS.

3. RESULTS AND DISCUSSION

In this study, all of the analyzed pesticide analytes could be successfully chromatographically separated using an Agilent Technologies 7890A GC gas chromatography and HP-5MS UI (5% phenyl methylsiloxane) 15 m x 250 µm x 0.25 µm capillary column and oven temperature program in MRM mode. The oven program was established in GC for chromatographic separation of analytes. Multiple reaction monitoring (MRM) was used to identify and quantify the pesticides with a precursor ion and at least two product ions in Agilent Technologies 7000B Triple Quadrupole MS/MS. Firstly, the instrument method optimization was accomplished. The pesticide standard working solutions were prepared separately for each of the analte, and then tested to determine the precursor and product ions, collision energies retention time in GC-MS/MS with scan mode. Ions were evaluated in detected peak by comparing its molecular weight. The collision energies were detected for the each of transision in the range of 5-30 V by increments of 5V. The parameters of GC-MS/MS and data related to method validation were displayed in Table 1.

Table 1. GC-MS/MS instrument and the pesticide method validation parameters

No	Pesticide	Rt min	Target ion *m/z*	Product ion *m/z*	Dwell time sec	Collision energy V	Average recovery %	S	v	CV%	n
1	Acrinathrin	15.39	181.1	152.1, 127.1	20	25, 30	95.5	4.44	19.69	4.65	120
2	Alachlor	8.50	188.1	160.1, 130.1	25	10, 42	94.5	2.40	5.75	2.54	119
3	Aldrin	9.25	263.0	193.0, 191.0	25	30, 30	94.6	4.07	16.54	4.30	120
4	Allethrin	10.89	123.1	81.1, 79.1	20	10, 20	98.5	3.73	13.95	3.79	120
5	Azinphos-ethyl	15.32	132.0	77.0, 132.0	20	12, 1	88.6	6.83	46.68	7.71	120
6	Azinphos-methyl	14.83	160.1	132.1, 77.1	20	5, 20	89.1	7.29	53.21	8.19	106
7	Benfluralin	5.86	292.1	264.0, 160.1	10	20, 15	91.6	3.85	14.81	4.20	120
8	Bifenthrin	14.44	181.1	166.1, 165.1	20	15, 30	96.4	3.29	10.83	3.42	120
9	Bromocyclen	7.59	358.7	278.0, 243.0	20	5, 20	94.3	2.63	6.94	2.79	120
10	Bromophos-ethyl	11.26	358.7	331.0, 303.0	20	5, 15	96.9	2.71	7.35	2.80	120
11	Bromopropylate	14.34	183.0	155.0. 76.0	20	15, 35	96.8	3.66	13.40	3.78	120
12	Captafol	4.15	79.0	77.0, 78.9	10	20, 20	96.6	3.14	9.86	3.25	119
13	Carbophenothion	13.33	342.0	96.9, 157.0	20	10, 10	93.7	3.10	9.63	3.31	120
14	Chinomethionate	10.94	234.0	148.0, 206.0	20	17, 9	89.1	12.48	155.68	14.01	120
15	Chlorbenside	10.91	125.0	99.0, 89.0	20	20, 20	93.7	1.84	3.40	1.97	120
16	Chlordane, cis-	11.01	372.7	266.1, 264.1	20	25, 25	96.1	2.40	5.77	2.50	120
17	Chlordane, trans-	11.41	372.7	266.1, 264.1	20	25, 25	96.1	2.40	5.77	2.50	120
18	Chlorfenapyr	12.64	247.0	227.0, 59.0	15	15, 10	90.9	4.86	23.60	5.34	119
19	Chlorfenprop-methyl	4.98	195.6	164.6, 101.8	10	15, 35	93.8	2.64	6.98	2.82	120
20	Chlorfenson	11.65	301.8	174.8, 111.1	15	5, 22	96.6	2.58	6.65	2.67	120
21	Chlorfenvinphos	10.80	267.0	159.0, 81.0	20	20, 40	94.5	4.22	17.78	4.46	120
22	Chlorobenzilate	12.72	139.0	111.0, 75.0	15	15, 30	95.3	2.98	8.87	3.13	120
23	Chloroneb	4.31	191.0	141.0, 113.0	10	10, 15	99.6	2.21	4.89	2.22	120
24	Chlorothalonil	7.40	265.9	230.9, 133.0	20	20, 40	92.5	5.57	31.08	6.03	120
25	Chlorpropham	5.54	213.0	171.0, 127.0	10	5, 10	97.0	4.77	22.73	4.91	120
26	Chlorpyrifos	9.62	196.9	168.9, 107.0	25	16, 44	93.7	2.51	6.32	2.68	120
27	Chlorpyrifos methyl	8.29	286.0	270.9, 93.0	25	20, 25	93.5	2.86	8.16	3.06	120
28	Chlorthal-dimethyl	9.72	299.0	221.0, 223.0	25	25, 25	98.8	2.49	6.20	2.52	120
29	Chlorthiamid	8.03	170.6	135.6, 99.7	25	15, 35	92.5	4.69	22.02	5.07	120
30	Chlozolinate	10.18	259.0	147.1, 188.0	25	15, 10	89.5	2.92	8.51	3.26	120
31	Cyanophos	6.88	242.5	124.8, 108.8	20	15, 15	91.2	2.40	5.76	2.63	120
32	Cyfluthrin	16.24	163.0	127.1, 91.1	20	5, 15	96.8	4.19	17.54	4.33	120
33	Cyhalothrin (I, II, III, IV)	15.22	197.0	171.0, 161.0	20	15, 10	90.5	4.75	22.61	5.25	118
34	Cyhalothrin, λ-	15.22	181.1	152.1, 127.1	20	29, 33	94.2	4.25	18.04	4.51	120
35	Cypermethrin	16.60	181.1	152.1, 127.1	20	27, 33	92.6	3.93	15.48	4.25	119
36	Dazomet	6.24	161.8	88.9, 72.9	10	5, 40	95.0	5.31	28.16	5.59	120
37	3,4- Dichloraniline	3.83	160.5	125.8, 89.9	10	15, 25	99.5	3.17	10.02	3.18	118
38	3,5- Dichloraniline	3.67	160.7	98.8, 89.9	10	25, 25	99.6	2.52	6.34	2.53	120
39	DDD, o,p'-	12.18	235.0	199.1, 165.1	15	15, 30	98.4	2.01	4.03	2.04	120
40	DDD, p,p'-	12.84	235.0	199.1, 165.1	15	20, 25	96.1	2.19	4.81	2.28	120
41	DDE, o,p'-	11.24	246.0	211.0, 176.1	20	20, 40	96.5	2.27	5.17	2.36	120
42	DDE, p,p'-	12.01	246.0	176.1, 175.1	15	40, 40	95.7	2.46	6.08	2.57	120
43	DDT, o,p'-	12.84	235.0	199.1, 165.1	15	20, 20	93.8	2.23	4.99	2.38	120
44	DDT, p,p'-	13.49	235.0	199.1, 165.1	20	20, 30	89.5	4.79	22.98	5.36	118
45	Deltamethrin	17.79	253.0	174.0, 93.0	20	6, 22	91.5	5.00	24.99	5.46	117
46	Demeton-S-methyl	5.25	88.1	60.0, 59.0	10	5, 20	92.2	4.15	17.25	4.50	120
47	Dibromobenzophenone, 4,4	12.29	339.9	185.0, 182.9	15	22, 21	98.0	2.24	5.01	2.28	120
48	Dichlobenil	3.40	171.0	136.0, 100.0	10	30, 15	97.2	2.36	5.57	2.43	120
49	Dichlofenthion	8.08	279.0	223.0, 205.0	25	10, 25	96.0	2.53	6.39	2.63	120
50	Dichlorobenzophenone, 4,4	9.60	249.9	214.9, 139.0	25	11, 10	97.2	2.23	4.98	2.30	120
51	Diclofop-methyl	13.84	339.3	252.4, 183.5	20	15, 35	91.2	4.85	23.55	5.32	120
52	Dicloran	6.28	206.0	176.0, 123.9	10	10, 25	93.7	3.24	10.47	3.45	120
53	Dieldrin	11.93	263.0	193.0, 191.0	15	30, 30	96.1	3.22	10.34	3.35	120
54	Dinitramin	7.39	304.6	260.6, 243.6	20	5, 5	97.7	5.86	34.35	6.00	108

No	Pesticide	Rt min	Target ion *m/z*	Product ion *m/z*	Dwell time sec	Collision energy V	Average recovery %	S	v	CV%	n
55	Diphenylamine	5.26	169.0	167.0, 168.0	10	20, 15	97.2	2.36	5.58	2.43	120
56	Disulfoton	7.27	88.1	60.0, 59.0	20	5, 25	91.1	2.59	6.70	2.84	120
57	Endosulfan sulfate	14.93	271.9	236.9, 116.9	20	16, 44	99.7	1.94	3.78	1.95	120
58	Endosulfan, α-	11.31	240.8	205.9, 136.0	20	16, 40	97.1	3.07	9.45	3.17	118
59	Endosulfan, β-	12.58	195.0	159.0, 125.0	15	9, 28	95.6	3.67	13.43	3.84	120
60	Endosulfan-sulphate	13.38	271.9	236.9, 116.9	20	16, 44	97.5	2.03	4.11	2.08	120
61	Endrin	12.38	263.0	193.0, 191.0	15	30, 30	95.3	3.36	11.27	3.52	120
62	Esfenvalerate	17.23	125.0	99.2, 89.1	20	25, 25	91.7	3.52	12.40	3.84	120
63	Ethion	13.01	231.0	175.0, 129.0	15	24, 10	92.7	3.51	12.29	3.78	120
64	Etridiazole	3.98	183.0	139.9, 108.0	10	20, 40	93.7	5.76	33.14	6.15	118
65	Etrimfos	7.58	292.0	181.0, 153.0	20	5, 20	95.1	3.53	12.48	3.72	120
66	Fenchlorphos	8.65	284.9	269.9, 93.0	25	15, 25	94.7	2.80	7.81	2.95	120
67	Fenitrothion	9.04	277.0	260.0, 109.0	25	4, 20	89.3	3.60	12.98	4.03	120
68	Fenson	9.83	141.0	77.1, 77.0	25	15, 20	94.1	2.10	4.39	2.23	120
69	Fenvalerate (I-II)	17.23	167.0	125.0, 89.1	20	10, 40	90.8	4.09	16.72	4.50	120
70	Fipronil	10.91	367.0	228.0, 213.0	20	30, 30	91.6	3.29	10.82	3.59	120
71	Fluchloralin	7.32	306.0	264.0, 206.0	20	15, 15	94.2	4.11	16.92	4.37	120
72	Flucythrinate (I-II)	16.61	199.0	157.0, 107.0	20	15, 5	92.4	4.06	16.52	4.40	120
73	Flumethrin	5.86	215.5	158.7, 76.9	10	25, 25	96.6	5.15	26.48	5.33	120
74	Fluvalinate-τ (I-II)	17.45	250.0	199.9, 54.9	20	23, 20	94.2	5.80	33.66	6.16	118
75	Formothion	7.62	170.0	93.0, 63.0	20	10, 25	94.1	7.30	53.34	7.76	115
76	Furalaxyl	10.97	241.6	94.9, 151.7	20	15, 12	93.1	2.77	7.69	2.98	113
77	Halfenprox	16.45	477.3	237.0, 171.0	20	10, 20	89.6	3.68	13.58	4.11	120
78	HCH, α-	6.04	181.0	145.0, 109.0	10	15, 30	95.4	2.30	5.29	2.41	120
79	HCH, β-	6.60	181.0	145.0, 109.0	20	15, 30	96.3	2.04	4.18	2.12	120
80	HCH, γ- (Lindane)	6.71	181.0	145.0, 109.0	20	15, 30	95.2	2.25	5.08	2.37	120
81	HCH, δ-	7.26	181.0	145.0, 109.0	20	15, 30	94.1	2.36	5.56	2.51	120
82	Heptachlor	8.39	271.9	236.8, 142.9	25	25, 40	92.0	2.73	7.48	2.97	117
83	Heptachlor endo-epoxide	10.47	183.0	154.9, 118.9	25	15, 30	95.4	3.59	12.89	3.76	120
84	Heptachlor exo-epoxide	10.35	352.9	281.9, 262.8	25	20, 25	96.8	2.45	6.03	2.54	118
85	Mirex	14.93	272.0	235.0, 216.9	20	25, 20	98.5	1.76	3.11	1.79	120
86	Nitralin	14.10	303.0	302.0, 145.0	20	10, 26	88.8	7.43	55.19	8.37	116
87	Nitrapyrin	3.97	193.8	167.0, 158.0	10	20, 20	95.6	4.92	24.25	5.15	120
88	Nitrofen	12.43	282.9	253.0, 202.1	15	10, 25	89.5	4.05	16.41	4.52	120
89	Nitrothal-isopropyl	9.93	236.1	194.1, 148.1	25	5, 20	92.8	3.17	10.04	3.42	120
90	2-phenylphenol	4.39	170.0	141.0, 115.0	10	15, 35	96.5	1.55	2.41	1.61	120
91	Parathion (-ethyl)	9.63	291.0	109.0, 81.0	25	10, 35	91.0	3.26	10.66	3.59	120
92	Parathion methyl	8.29	263.0	109.0, 79.0	25	12, 33	91.1	3.10	9.61	3.40	120
93	Pentachloroaniline	7.76	265.0	230.0, 194.0	25	10, 20	96.5	2.67	7.14	2.77	120
94	Pentachloroanisole	6.29	264.8	143.0, 117.1	10	35, 35	95.3	3.11	9.65	3.26	120
95	Permethrin	15.82	183.1	168.1, 153.1	20	14, 16	95.0	3.66	13.38	3.85	120
96	Perthane	12.57	222.8	179.2, 165.3	15	20, 25	94.5	3.28	10.78	3.48	120
97	Phorate	5.96	231.0	174.9, 128.9	10	10, 25	95.2	4.18	17.50	4.39	120
98	Phosmet	14.85	160.0	133.0, 77.1	20	15, 30	95.6	7.20	51.91	7.54	116
99	Phthalimide (Folpet)	4.00	147.0	76.0, 103.0	10	30, 6	95.2	3.35	11.21	3.52	120
100	Procymidone	10.98	283.0	96.1, 67.1	20	10, 39	98.4	2.85	8.15	2.90	120
101	Profluralin	7.01	318.1	284.1, 199.1	20	10, 15	89.9	2.78	7.74	3.10	120
102	Propham	3.96	93.0	66.0, 65.0	10	15, 25	102.7	2.45	6.02	2.39	120
103	Prothiophos	11.87	266.5	238.5, 240.6	15	5, 5	94.9	2.73	7.43	2.87	120
104	Pyraflufen-ethyl	13.72	349.0	307.0, 349.0	20	10, 10	96.3	4.11	16.91	4.27	120
105	Pyrimidifen	17.05	160.6	134.8, 90.9	20	15, 35	94.4	3.18	10.08	3.36	116
106	Quinalphos	10.84	146.1	118.1, 91.1	20	10, 30	95.0	2.76	7.60	2.90	120
107	Quintozene	6.83	236.9	142.9, 118.9	20	30, 25	91.8	2.93	8.61	3.20	120
108	Resmethrin	14.01	123.1	95.1, 81.1	20	5, 5	97.5	4.95	24.54	5.08	120
109	S421	8.66	130.0	130.0, 95.0	25	5, 20	93.7	3.54	12.54	3.78	120

No	Pesticide	Rt min	Target ion *m/z*	Product ion *m/z*	Dwell time sec	Collision energy V	Average recovery %	S	v	CV%	n
110	Spiromesifen	14.18	272.0	254.0, 209.0	20	5, 20	93.5	5.86	34.39	6.27	120
111	Sulprofos	13.19	322.1	155.9, 97.0	20	5, 25	93.0	3.49	12.18	3.75	120
112	Tecnazene	5.12	202.9	142.9, 83.0	10	20, 25	92.0	3.21	10.33	3.49	120
113	Tefluthrin	7.54	177.0	137.0, 127.0	20	15, 15	95.7	2.00	3.99	2.09	120
114	Terbacil	7.33	160.7	143.8, 116.9	20	15, 5	94.5	4.79	22.95	5.07	120
115	Terbufos	6.88	231.0	174.9, 128.9	20	10, 25	92.0	3.79	14.36	4.12	120
116	Tetrachlorvinphos	11.49	329.0	108.9, 93.0	15	20, 10	96.3	5.08	25.77	5.27	120
117	Tetradifon	14.71	355.8	159.0, 353.9	20	12, 5	98.0	3.32	10.99	3.38	120
118	Tetrahydrophthalimide	4.14	151.0	122.0, 79.0	10	11, 19	98.0	2.93	8.56	2.99	120
119	Tetrasul	13.03	251.8	216.9, 182.2	20	25, 25	95.9	2.64	6.95	2.75	120
120	Thiometon	6.17	125.0	79.0, 47.0	10	20, 10	93.4	2.98	8.90	3.19	120
121	Tolclofos-methyl	8.40	265.0	250.0, 93.0	25	15, 25	97.6	2.32	5.38	2.38	120
122	Trifluralin	5.81	306.1	264.0, 160.0	10	5, 26	92.2	3.36	11.26	3.64	120
123	Vinclozolin	8.31	212.0	145.0, 109.0	25	25, 40	96.1	2.54	6.45	2.64	120

Rt: retention time; S: standard deviation; v: variance; CV: coefficient of variance; n: number of samples

Tomatoes, lemons, lettuce, almonds, raisins and honey were selected as representing food matrix for the validation of the method performance. The pesticide matrix solutions (tomato, lemon, lettuce, almond, raisins and honey) were made ten repetitions for three different days for each level at two different concentrations, with concentrations of 10 ng mL^{-1} and 50 ng mL^{-1} for each analyte.

The specificity parameter of an assay is a measure of the extent to which the method can determine a particular compound in the analyzed matrices without interference from matrix components. The validation procedure should confirm the ability of the method to unequivocally assess the analyte in the presence of other components that may be present (for example, impurities, degradation products and matrix components). The chromatographic separation of all analytes from each other was accomplished successfully by an Agilent Technologies 7890A GC gas chromatography and HP-5MS 5% phenyl methylsiloxane (15 m x 250 µm x 0.25 µm) the capillary column, oven program in MRM mode.

The selectivity is that a method analyzes a given compound without interfering with the matrix components in the matrix. It is accomplished by Agilent Technologies 7890A GC gas chromatography 7000B MS/MS Triple Quadrupole GC-MS/MS system. The identification, validation, calculation of an analyte takes place with at least one precursor ion and at least two product ions in the MRM mode.

The limit of detection (LOD) is the limit lowest residue concentration that result could not submit. The limit of quantitation (LOQ) is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met [22]. In the European Commission Regulation (EC/299) [23], maximum residue limits (MRLs) are specified using LOQ values.

With the method validation studies, the LOD - LOQ parameter was used in all studied matrices. In the repeatability study of the analysis, the recovery of the method validation accuracy parameter was done at two different concentrations, 10 ng g^{-1} and 50 ng g^{-1}, with percent recovery and % CV values. The recovery results obtained in GC-MS/MS for contaminants for authenticity and precision subparameters were 88.6 - 99.7% and CV 1.60 – 14.0%.

In the reproducibility study data is evaluated, the recovery obtained for each residue at 10 ng g^{-1} of the matrix containing GC-MS/MS pesticides is 70.1% -120.0% and CV% 1.80 - 31.92%. The recovery achieved for 50 ng g^{-1} level is 70.7% - 120.7% and CV is 2.48 – 27.59%. It was found that the recovery obtained for each residue in the concentration range of 10-50 ng

g^{-1} of the matrix containing GC-MS / MS pesticides varied between 91.7-98.9% and CV 2.21-5.67%. Combining the recovery results from all the matrices and pesticides in GC-MS/MS resulted in 95.0% average recovery and 4.91% reproducibility % CVR of laboratory data.

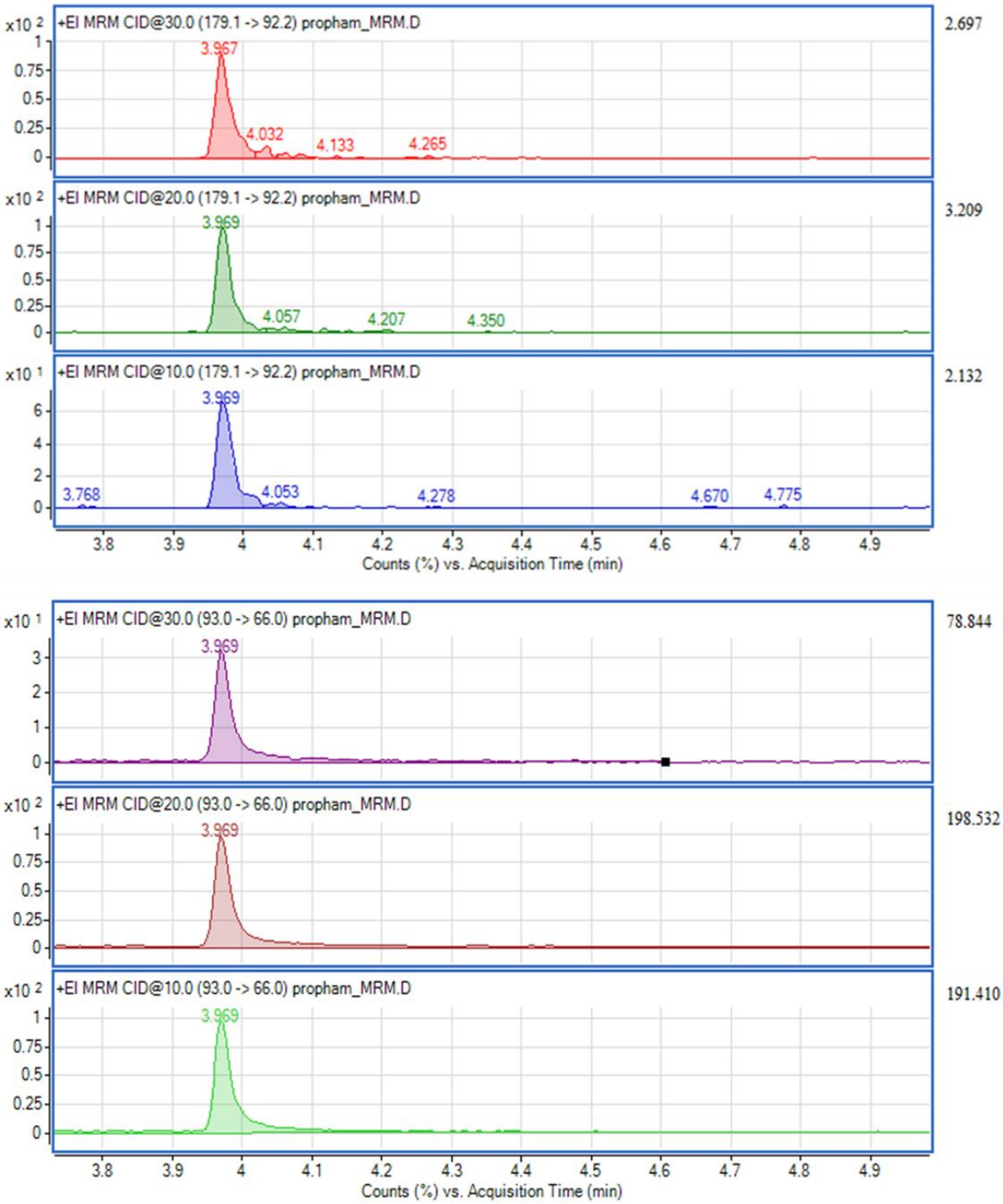

Figure 1. GC-MS/MS pesticide optimization evaluation for propham pesticide residue

4. CONCLUSION

This method is fast, both as an analysis method and as a method of reading on devices. The device can be analyzed in about 16 minutes in the European Union and in Russia with about 123 pesticides (with their metabolites) that are required for analysis.

Thus, approximately 123 pesticides were validated and quantified in GC-MS/MS. It covers a wide range of products from selected indications to vegetable origin food and food of animal origin. In addition, the method reduces time for analysis by providing time gain. On the other hand, the analysis cost has been reduced, and significant gains have been achieved on the basis of day-month-year as well as instrument consumables, analytical column, vial, working life of all working parts etc.

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Conflict of Interest

The authors declare that there is no conflict of interests in this current study.

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Extraction, purification, antioxidant properties and stability conditions of phytomelanin pigment on the sunflower seeds

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Abstract: Phytomelanin pigment, a rare secondary matabolite in plants, has potential for use in the cosmetic and food sectors. This pigment has antioxidant, antimicrobial and ultraviolet (UV) light absorbing properties, so it can be used as natural food coloring and in the field of cosmetics. In this study, extraction, purification, antioxidant properties and stability conditions of phytomelanin pigment found in sunflower (*Helianthus annuus* L.) seed coat were investigated. NaOH, KOH and NH_4OH solutions were tested at different concentrations for extraction. It has been determined that the most suitable solvent for extraction is 0.3 M NaOH. The purification process involves precipitation with HCl followed by washing with ethyl alcohol, ethyl acetate and acetone. The findings show that the proportion of phytomelanin in sunflower seeds is 1.95% and that the antioxidant capacity is 9.8% ascorbic acid equivalent. The purity degree of the purified phytomelanin pigment and that of the synthetic phytomelanin pigment were compared by thin layer chromatography (TLC). Chromatography findings have shown that the purification performance is quite high. It was determined that the pigment was slowly deteriorated in temperature and light, and was not affected by air. Consequently, sunflower seed coat can be a convenient and economical source of producing pure phytomelanin for industrial use.

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1. INTRODUCTION

The use of colorants in the food and cosmetics production has begun in ancient times. There are many examples of the use of natural colorants in recorded history [1]. Today, there is an increasing interest in food colorants derived from natural sources as an alternative to synthetic dyes due to legal regulations and consumer demand [2]. Pigments have an important place in food additives and the demand for pigments has increased in recent years. Natural pigments are regarded as safe compared to synthetic pigments in terms of nutritional value and therapeutic effects. At present, most natural pigments are obtained from plant material by appropriate extraction methods [3].

Melanin is a black, insoluble pigment that is not degraded by concentrated acids but can be whitened with oxidizing agents. This pigment can be found in plants, animals and microorganisms. The form found in plants is called phytomelanin or phytomelan [4].

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Phytomelanin is especially found in the seed coats of Asteraceae species. There are only a few published literature on the structure, chemical composition and formation of the composite outer zone, as known for over 50 years. Pigment; polymeric, highly insoluble, resistant to alkali and acid, and has a black color at high concentration. Although the natural function is not fully known, it has been suggested that black coated seeds can survive for years under the soil, and may be resistant to weather conditions, soil acidity and microbial attack [5, 6].

Melanins are high-molecular weight polymers formed by the oxidation of phenols. These nitrogenous compounds which are derived from tyrosine, are called eumelanin [7]. Complex polymeric structured melanin compounds are classified as: eumelanin, pheomelanin and allomelanin. Allomelanines are black pigments produced by plants and fungi. It is found in black spots of leaves, in flowers, in fruits and seeds (eg *Osmanthus fragrans*), in fungal spores (eg *Tuber melanosporum*). Fungal melanins are produced by *Aspergillus nidulans* and *A. niger*, *Alternaria alternata*, *Cryptococcus neoformans*, and *Wangiella dermatitis* [8]. Phytomelanins are isolated from leaves of *Tea sinensis* and their radical scavenger properties are shown [9]. Phytomelanin is found in fruit pericarp of species belonging to *Eupatorium* and *Helianthus* genus from Asteraceae family. In these tissues, it forms a mechanically hard, durable layer of brown-black color [6]. Phytomelanin acts as a protective layer that protects the pericarp, providing external protection against attacks by insects, insects and macro-micro organisms. In addition to the destructive effects of insect pests, it is also resistant to bacterial deterioration [10].

Phytomelanin is used especially in cosmetics sector and has usage potential in many different sectors. Various sources indicate that both antioxidant, antimicrobial and ultraviolet light absorptive properties are present, as well as their potential for use as natural food colorants [8, 9].

In this study, optimization of extraction and purification methods of phytomelanin pigment in sunflower seeds were carried out. In addition, antioxidant properties of phytomelanin pigment were determined and evaluated for stability in various conditions such as air, light and temperature.

2. MATERIAL AND METHODS

2.1. Material

In these experiments, black colored seeds produced by Cypriot variety of sunflower (*Helianthus annuus* L.) were used. Seeds were obtained from seed markets in Mersin/Turkey.

2.2. Optimization of extraction conditions

Melanin in animal and fungus species is highly stable in acidic solutions, but soluble in dimethyl sulfoxide (DMSO) at low levels. Phytomelanin can be successfully extracted from various plant tissues by standing in alkaline solutions for 24 hours [11]. Three different alkaline chemicals (NH_4OH, KOH and NaOH for analysis from MERCK) were used to determine the optimum solvent and concentration for extraction. The prepared 0.5 M stock solutions were then diluted to 0.3 M, 0.1 M, and 0.05 M, respectively.

1.0 g of sunflower seeds were placed in 100 ml of solution in the absence of oxygen and at room temperature for 24 hours. The extract was filtered through filter paper (Whatman No 1) and then centrifuged at 3500 xg for 5 minutes. The extract was diluted 1/3 with distilled water (Comecta destilation 3.1) and the absorbance at 280 nm was measured [11, 12]. The amount of melanin in the samples was calculated using standard curve prepared with pure melanin pigment (Sigma Aldrich M8631).

The most suitable solute / solvent ratio was determined by making experiments with seed quantities ranging from 1.0 to 5.0 g. The most successful extractions were with 4.0 g seeds in 100 mL of solvent. The subsequent extractions were carried out at these ratios. In order to determine the effect of temperature and time on extraction, 4.0 g of seed was incubated for 24 hours at 4, 24, 35 and 50 ˚C in 100 ml of 0.3 M NaOH solution. At specific time intervals, 50 µl samples were taken and final volume was adjusted to 3.0 ml with distilled water and absorbance at 280 nm was measured by spectrophotometer.

2.3. Purification process

The phytomelanin extract was filtered and adjusted to pH between 1.0 and 3.0 with a 2.0 M HCl solution. After being allowed to stand at room temperature for 2 hours, it was centrifuged at 4000 xg for 5 minutes. The supernatant was removed and the precipitated material was taken up with a spatula. It was boiled in 6.0 M HCl for 2 hours to remove carbohydrates and proteins, cooled and then centrifuged at 4000 xg for 5 minutes. The supernatant was removed and then suspended with distilled water, vortexed and centrifuged at 4000 xg for 5 min. The supernatant was removed and the precipitated melanin pigment collected using a spatula. The obtained melanin pigment was shaken for 30 minutes by adding ethyl alcohol and centrifuged at 5000 xg for 15 min. The supernatant was removed and washed with distilled water. The same process was repeated with ethyl acetate and acetone [13]. The purified phytomelanin was dried in a glass petri dish at 100 °C and stripped from the glass surface.

2.4. Antioxidant capacity

The prufied fitomelanin pigment was dissolved in methanol and quantified. 20, 40, 60, 80, 100 µg ml^{-1} ascorbic acid solutions were prepared to form a standard curve. 0.3 ml of melanin and ascorbic acid solutions were mixed with 2.7 ml of the reagent solution (containing 0.6 M H_2SO_4, 28.0 mM Na_2HPO_4 and 4.0 mM ammonium molybdate in water). The reaction mixture was kept at 90 °C for 90 min and then cooled. Finally, absorbance measurements were performed at 695 nm spectrophotometer (Chebios Optimum One UV-VIS) [14].

2.5. Stability tests

10 mg of pure melanin was dissolved in 250 ml of 0.1 M NaOH to determine the stability of the melanin pigment in high temperature conditions. The melanin solution was exposed to a temperature of 100 ° C for 2 hours. The volume was adjusted to 250 ml before sampling for measurement. The samples were taken at the beginning and 30 min intervals. 0.1 ml of the sample was diluted with 2.9 ml of 0.1 M NaOH and the absorbance was determined at 280 nm on spectrophotometer.

Stability tests of melanin pigment in presence of light and oxygen were performed at room temperature. 0.1 ml of the melanin pigment solution was taken and diluted with 2.9 ml of 0.1 M NaOH solution and the absorbance at 280 nm was measured by spectrophotometer. The solution transferred into the four different tubes was kept in the light, dark, air + light and air + dark conditions. The tubes were fully filled and closed to create an airless environment. Measurements of absorbance were performed at varying intervals for 35 days.

2.6. Thin layer chromatography (TLC)

Thin layer chromatography was performed to compare the pigment of the purified phytomelan with the pigment purity of the synthetic melanin [15]. Purified and dried phytomelanin pigment and synthetic melanin pigment dissolved in 0.1 M NaOH solution. solvent system of n-butanol: acetic acid: pure water (70:20:10) was used [16]. It was carried out on silica gel layers (MERCK TLC Silica Gel 60 F254) [17]. Purity levels were compared with the spots on the chromatogram.

2.7. Determination of purity level

A synthetic melanin pigment from Sigma-Aldrich was used to determine the amount of pigment of the purified phytomelan. 500 mg L^{-1} melanin stock solution was prepared for analysis. This solution was diluted to give serial solutions such as 0, 10, 20, 40, 80 and 100 mg L^{-1}. The absorbance at 280 nm was measured on spectrophotometer. Quantitative analysis was performed on the standard curve generated by the measured absorbance values.

2.8. Statistics

All analysis were repeated at least three times. The significance levels of the difference between groups were determined with ANOVA test. Results of statistics analyses were shown with P values and significance levels in Table and Figure legends. Data were indicates as arithmetic means and standard deviations (±SD) in Tables and Figures.

3. RESULTS

Different concentrations of three alkaline chemicals were tested in order to determine the most suitable solvent for pigment extraction of phytomelanin pigment and the findings are shown in Table 1. The measurement results showed that the most suitable solvent was 0.5 M NaOH solution. However, in order to avoid problems resulting from high concentrations of NaOH, 0.3 M NaOH solution was used in subsequent processing.

Table 1. Mean absorbance values of phytomelanin pigment extracted at different concentrations of three alkaline solvents at 280 nm wavelength on a UV-VIS Spectrophotometer. Values are given as averages and standard deviations of three repetitions. (Statistics: Solvent P = 0.031 (*), concentration P = 0.00 (**), interaction P = 0.00 (**)).

Concentrations (M)	NaOH	KOH	NH_4OH
0	0.32±0.04	0.32±0.04	0.32±0.04
0.05	1.05±0.15	0.58±0.06	0.74±0.05
0.1	1.07±0.17	0.97±0.08	0.92±0.12
0.3	1.50±0.25	1.30±0.16	1.28±0.18
0.5	2.05±0.22	1.34±0.12	1.58±0.21

1.0-5.0 g of sunflower seeds were incubated in 0.3 M 100 mL NaOH solution, 24 h, at room temperature to determine the optimum amount of phytomelanin pigment that could be dissolved in solvent. A slight difference between 4.0 and 5.0 g was observed with an increase from 1.0 g to 4.0 g (Figure 1). The ideal material / solvent ratio was determined as 4.0 g seed / 100 ml.

Experimental results to determine the appropriate temperature and duration for extraction are given in Figure 2. Measurement was carried out at four different temperatures (4, 24, 35 and 50˚C) during one hour of the experiment. Findings show that the most successful extraction takes place in 7 hours at a temperature of 50 ˚C. The increase in phytomelanin after 6 hours is considerably reduced.

Total antioxidant capacity analysis was performed by phosphomolybdenum complex formation method in order to determine the antioxidant properties of phytomelanin pigment. The antioxidant capacity was determined to be the ascorbic acid equivalent. The antioxidant capacity of the purified 1000 mg L^{-1} phytomelanin pigment was determined to be 98 mg L^{-1} ascorbic acid equivalent, i.e., 9.8%.

In order to determine the stability of the purified phytomelanin pigment at high temperature, the phytomelanin solution was boiled for 2 hours at 100 °C. Absorbance measurements were made every 30 minutes from the start and the amount of phytomelanin was

calculated. The concentration of phytomelanin pigment was not significantly changed for 90 minutes at 100 ˚C but decreased after 120 minutes (Figure 3).

The levels of stability of the purified phytomelanin pigment in daylight and in the air have been determined. The amount of phytomelanin in the solutions was measured with a spectrophotometer at certain time intervals and the results are given in Table 2. At the end of the 35th day, loss of phytomelanin was determined as 61.6% in light and 25.3% in darkness. The loss of phytomelanin in air exposed samples was found to be less than those in air-free controls. These findings show that the destructive effect of light is stronger than the effect of air.

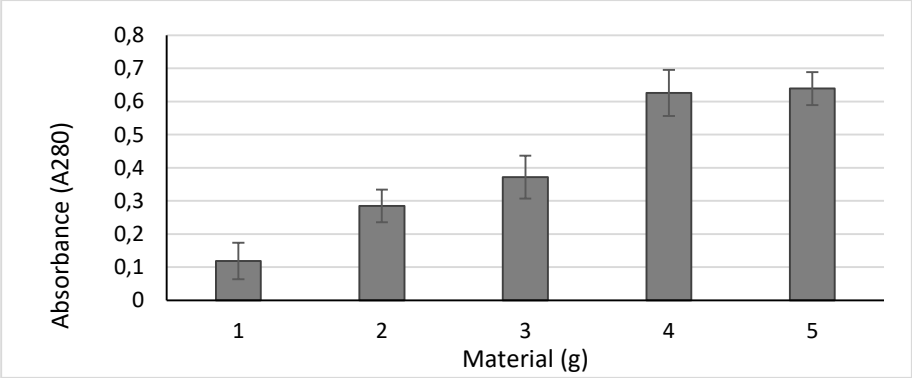

Figure 1. Absorbance values measured at 280 nm wavelength on extracts of phytomelanin from sunflower seeds in different amounts (1.0-5.0 g). Data represent the mean and standard deviation of the triplicate measurements.

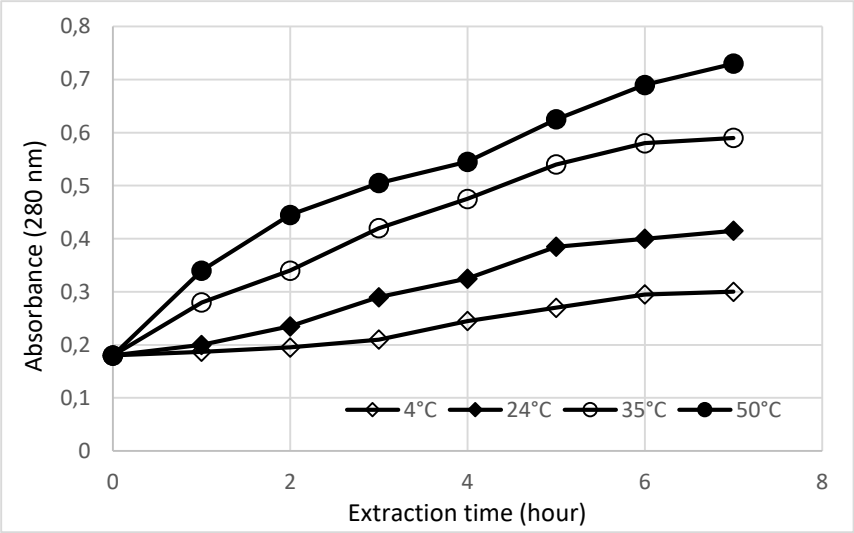

Figure 2. Temperature and time-dependent change in extraction performance of phytomelanin pigment. Extraction was carried out with 0.3 M NaOH solution. (Statistics: Temperature P = 0.00 (**), time P = 0.00 (**), interaction P = 0.00 (**)).

Thin layer chromatography was applied to compare the purity level of phytomelanin pigment purified from sunflower seeds with synthetic melanin pigment. Since the melanin pigment is insoluble in the acidic mobile phase, the applied chromatographic process distinguishes the impurities. The comparison results obtained are given in Figure 4. It has been determined that the procedure applied in this study provides 90% purification.

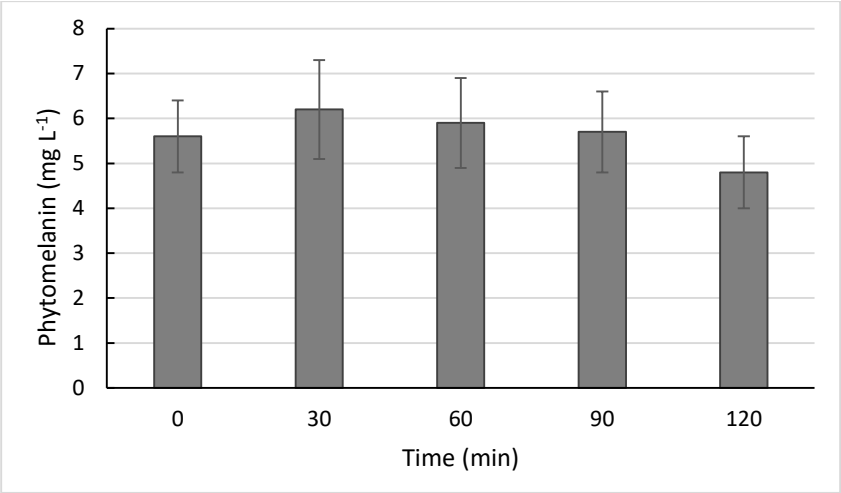

Figure 3. Time-dependent changes of phytomelanin pigment concentration at 100 ˚C. Data represent the mean and standard deviation of the triplicate measurements.

Table 2. Time-dependent changes in amount of phytomelanin pigment (mg Kg^{-1}) exposed to daylight and oxygen. (Statistics: light P = 0.034 (*), air P = 0.563, time P = 0.29, interaction P = 0.151).

Time (day)	Light	Light+Air	Dark	Dark+Air
0	11.1±0.0	11.1±0.0	11.1±0.0	11.1±0.0
1	11.1±0.2	11.2±0.2	11.1±0.1	11.1±0.1
2	11.2±0.2	10.9±0.2	10.9±0.2	11.5±0.3
3	11.3±0.3	11.1±0.2	10.8±0.2	11.1±0.1
7	6.6±0.5	10.3±0.3	9.2±0.3	11.4±0.3
14	6.5±0.5	9.5±0.4	9.2±0.3	11.3±0.3
21	6.1±0.5	9.1±0.5	8.5±0.4	11.1±0.3
35	4.3±0.8	8.3±0.6	8.3±0.5	10.9±0.4

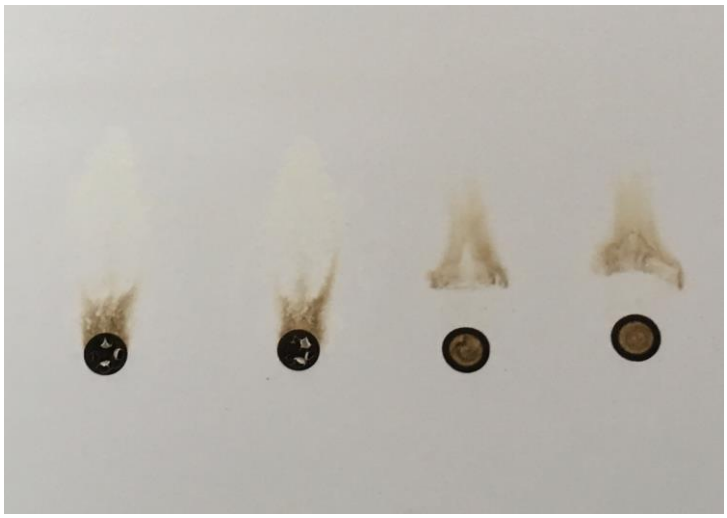

Figure 4. Time-dependent changes of phytomelanin pigment concentration at 100 ˚C. Data represent the mean and standard deviation of the triplicate measurements.

4. DISCUSSION

In plants, phytomelanin can be solubilized at a low level with dimethyl sulfoxide (DMSO), although it is highly stabilized in pigmented acidic solutions. Phytomelanin dissolved in water

and organic solvents is completely soluble in 0.5 M NaOH or KOH. The dark brown material which can be extracted with alkali solutions is precipitated when hydrochloric acid is adjusted to pH 2 or when the medium $FeCl_3$ is added. For this reason, precipitation is an important step in the purification process of phytomelanin. [11]. It has been observed that sedimentation at high temperatures takes place in a shorter time. Coloring is achieved with oxidation agents such as $KMnO_4$, H_2O_2, and color can be reproduced with $AgNO_3$ and ammonia solution [13].

Because of the difficulty of extraction and purification processes, the economic value of pure phytomelanin pigment is high. For this reason, it is important to obtain abundant and good quality pigment in a short time by optimizing pigment purification processes. The success rate has been increased by making some changes in the extraction and purification processes in the literature. Thin layer chromatography was used to determine the degree of purity of the obtained pigment [16]. Chromatograms taken with the acid-solvent mixture on the silica gel layer indicate that the pigment is above 90% of the purity level. This purity grade is sufficient for industrial use. For analytical use further purification by thin layer or column chromatography is necessary.

The pigment of phytomelanin is very resistant to deterioration due to its inert nature. However, when exposed to heat and light, it was observed to be damaged in a certain rate. The most common problems in the use of pigments in the food sector are stability and toxicity. In order to obtain the desired color in the formulations, the formulation experts must know the interactions with the other compounds and other factors that determine the color qualities. Although the pigment of phytomelanin is a natural product, toxicity tests are required [5].

Antioxidants may delay or prevent the staling of foodstuffs and the deterioration of their taste. Antioxidants inhibit degradation in two ways: primary antioxidants are effective by scavenging free radicals (such as phenolic compounds), while secondary antioxidants are effective by binding metal ions, converting hydroperoxides to non-radical products, UV absorbing, or singlet oxygen deactivation [18]. The antioxidant activity of phytomelanins may well be due to a combination of chelating and scavenging charecteristics [9, 19].

Park et al. [20] show that phytomelanins accumulate mainly in the outer epidermis and palisade layers of the wild-type *Ipomoea purpurea* seed coats, The outermost epidermal layer, in which phytomelanins accumulated extensively, appeared to overlap with the proanthocyanidin accumulating layers. Phytomelanin deposition in the outer epidermis and palisade layers, probably because the precursors of phytomelanins can migrate from synthesized cells into the space where phytomelanins are polymerized [21]. The biosynthesis and/or accumulation of seed phytomelanins are controlled by *bHLH2* gene, even though both the chemical properties and biosynthetic pathway of phytomelanins remain unknown [20].

5. CONCLUSION

Natural melanin found in plants and animals affects various biological activities with its antioxidant properties. Due to the antioxidant properties of melanin, it is of interest in the protection of health and the production of useful foods. Consequently, sunflower seed coat can be a convenient and economical source of producing pure phytomelanin for industrial use. Phytomelanin posses a potential to the development of new products in cosmetics and food industries.

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Transgenerational Transmission of Radiation-Induced Expression Patterns of *Arabidopsis Thaliana (L.) Heynh. Rad51* and *Rad1* Genes

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Abstract: Transcription rates of the genes *AtKu70*, *AtRAD51*, *AtRad1*, involved in maintaining *Arabidopsis thaliana* genome stability, in relation to the modification of phenotypic characteristics in irradiated plants and their progeny after the action of acute and fractionated X-ray radiation were studied. Differences in the transcription rate were measured by densitometric analysis of cDNA, synthesized by reverse transcription at the template of mRNAs, extracted from fresh leaves after 2 hours irradiation treatment. The doses 3 Gy, 12 Gy, 15 Gy and 21 Gy with 1.48 Gy/s specific dose rate were applied. Significant correlation between phenotype modifications in F_0 and F_1 generations, between phenotype traits and caretaker genes activity in irradiated F_0 plants were shown. Also preservation of changes in the pattern of *AtRad1* and *AtRAD51* but not *AtKu70* expression in F_1 plant leaves had been revealed. Changes in F_1 compared with F_0 generation do not correspond to the extrapolation of dependence between the phenotypic modifications and DNA repair genes transcription rate in the leaves of irradiated plants. Based on the obtained data it could be suggested that the altered transcriptional activity of *AtRAD51* and *AtRad1* reflects the transfer of DNA lesions from parent to offspring.

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DNA repair,
Transgenerational inheritance

1. INTRODUCTION

Nowadays there is greatly increased interest in the study of the mechanisms of long-term changes in the genome stability of living organisms under the influence of stress factors such as exposure to chemical mutagens, heavy metals, UV, ionizing radiation, adverse extreme climate changes [1-3]. One of the manifestations of such arrangements is to transfer the "memory" of radiation exposure through mitotic and meiotic barrier, what appears on the organism level. Previously, we have identified non-linear wave-like dose-effect dependence for some morphometric characteristics of *Arabidopsis thaliana* irradiated at the late vegetative stage of development, which characterized by the presence of the "critical points"– sublethal extremums at 3 Gy, 12 Gy, 15 Gy, 21 Gy. Given dependence was more statistically significant in the case of dose fractionation into three equal portions with the time interval between fractions of 24 h. Comparing the dose-effect curves with early changes in transcriptional activity of *AtKu70* (NHEJ DNA DSBs repair pathway, telomeric length maintenance and transcriptional silencing [4-7]), *AtRAD51* (HR DNA DSBs repair pathway, replication-coupled

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DNA repair, DNA interstrand crosslinks repair, cluster DNA damage repair [5, 7-9], *AtRad1* (DNA excision repair, SSA and MMEJ error-prone DNA repair, telomeric length maintenance, DNA interstrand crosslinks repair, cluster DNA damage repair [2, 9-15] genes, that participate in the maintaining of the genomic integrity, a close correlation between the level of activity of these loci and phenotypic features of irradiated plants on the 30th day after irradiation was observed [16]. This means that at least part of long-term effects could be associated with radiation-induced modification of the caretaker genes activity.

2. MATERIAL AND METHODS

Experimental methods and research design have been described in [16]. The relative concentration of the *AtKu70*, *AtRAD51*, *AtRad1* mRNAs in rosette leaves of 35-days-old *A. thaliana* plants after acute and fractionated whole-plant irradiation with 180 keV X-rays was studied. The exposure specific dose rate was 1.48 Gy/s and doses, previously described as sublethal "critical points" [16], were 3 Gy, 12 Gy, 15 Gy and 21 Gy. Experiments had been repeated for three times.

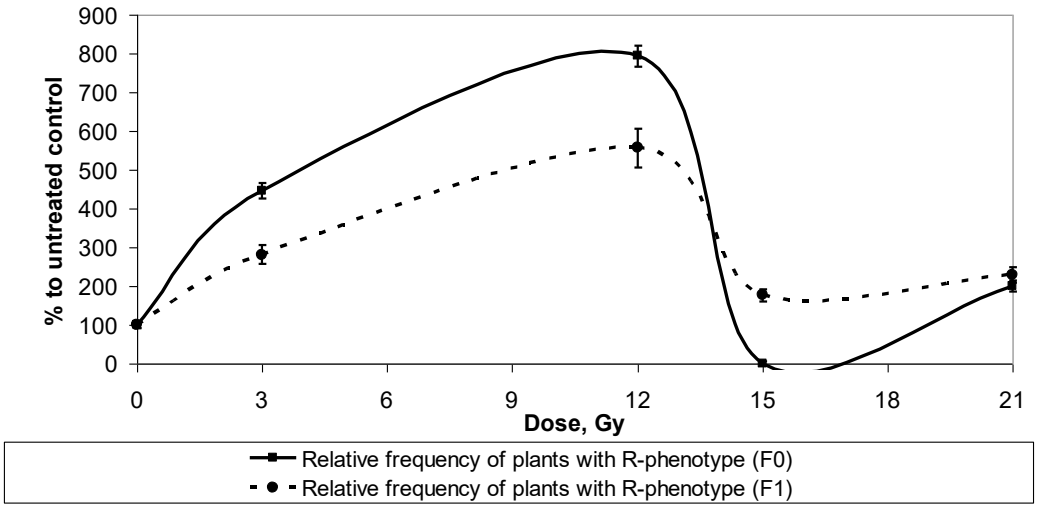
Two generations of plants were used: F_0 - irradiated on X-ray generator RUM-17 (National Cancer Institute, Kiev, Ukraine) and their non-irradiated progeny F_1. cDNAs was synthesized by reverse transcription of mRNAs, extracted from fresh leaves 2 h after irradiation. Then a PCR amplification of the cDNAs complementary to the mRNAs of the studied genes was performed. Differences in the relative level of expression were measured by densitometric DNA analysis using the ImageJ software package (National Institutes of Health, USA) after agarose gel electrophoresis [17]. The results were normalized to the cDNA concentration of the reference housekeeping gene *AtEf1a*. Statistical processing of data was carried out in MS Excel 2003 (Microsoft Corporation, USA) and SPSS 13.0 (IBM, USA).

3. RESULTS AND DISCUSSION

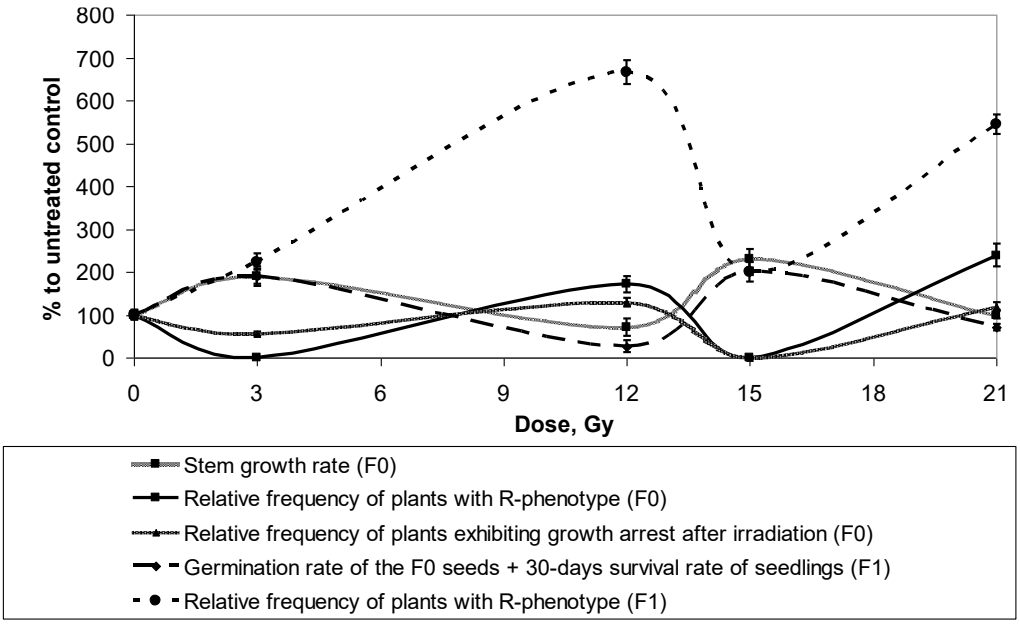
In connection to the previously obtained data, the question has been arisen about the effects that occur in the generation F_1, grown from the seeds of irradiated F_0 plants. Such effects, correlated with in F_0, revealed for the seeds germination rate, 30-days-old seedlings survival and for the proportion of plants with abnormal habitus, which we have referred as R-phenotype. R-phenotype is “reduced” phenotype, which characterizes by dwarfism, stopping on the vegetative phase of development or poor flowering, sterility, small or twisted rosette leaves of dirty-green/"anthocyanin" color with hypertrophic trichomes [16, 18]. Frequency of plants with the described phenotype also increase in response to other abiotic (drought and cold) and biotic stresses (pathogen invasion or insect attack). We have find that percentage of R-plants in F_1 closely related to the percentage of R-plants in F_0 after acute and fractionated radiation exposure, to the relative frequency of plants exhibiting growth arrest and to the stem growth rate after fractionated irradiation (Figure 1, Table 1). Interestingly, that after fractional X-ray irradiation correlations between a larger number of phenotypic features of irradiated plants and their progeny than after acute irradiation are observed.

Phenotype “inheritance” can realized through the transgenerational transition of DNA lesions, which cause modified phenotype and in parallel activates the transcription of DNA repair genes, or through the transmitting of expression pattern of such genes to the generation of non-irradiated plants. In this case pattern in turn leads to a deviation of the plant phenotype from normal. Both variants assume a stable relationship between the phenotype and the genes activity, as well as the cross-generational correspondence of expression pattern of the studied loci. In full accordance with the assumptions made, our results showed, first, significant correlations of “inherited” radiation-modified group phenotypic traits with *AtRad1*, *AtRAD51* and *AtKu70* transcription rates in F_0 generation (Table 2, Table 3), second, preservation of

changes in the pattern of expression of *AtRad1* and *AtRAD51* in leaves of the F_1 generation (Figure 2).



A. Acute X-irradiation.



B. Fractionated X-irradiation.

Figure 1. Dose-response curves for some related characteristics of acute (A) and fractional (B) X-ray irradiated F_0 and non-irradiated F_1 *Arabidopsis* plants. Every point on the graph represents the average of three experimental repeats with standard error bars.

Table 1. Pearson's correlation coefficients between related characteristics of irradiated F_0 and non-irradiated F_1 *Arabidopsis* plants.

	Relative frequency of plants with R-phenotype, acute irradiation (F_1)	Relative frequency of plants with R-phenotype, fractionated irradiation (F_1)
Relative frequency of plants with R-phenotype, acute irradiation (F_0)	0.94*	-
Relative frequency of plants with R-phenotype, fractionated irradiation (F_0)	-	0.75*
Relative frequency of plants exhibiting growth arrest after irradiation, fractionated irradiation (F_0)	-	0.64*
Stem growth rate, fractionated irradiation (F_0)	-	-0.59*

* $P \leq 0.05$

Table 2. Pearson's correlation coefficients between radiation-modified group phenotypic characteristics (F_0) and AtRad1, AtRAD51, AtKu70 transcription rates (F_0).

	AtRad1	*AtRAD51*	*AtKu70*
Relative frequency of plants with R-phenotype, acute irradiation (F_0)	0,86*		
Relative frequency of plants with R-phenotype, fractionated irradiation (F_0)	0,56*	-0,79*	-0,62*
Relative frequency of plants exhibiting growth arrest after irradiation, fractionated irradiation (F_0)	0,74*	-0,55*	-0,86*
Stem growth rate, fractionated irradiation (F_0)	-0,77*	0,60*	0,88**

* $P \leq 0.05$, ** $P \leq 0.01$

Table 3. Pearson's correlation coefficients between radiation-modified group phenotypic characteristics (F_1) and *AtRad1*, *AtRAD51*, *AtKu70* transcription rates (F_0 and F_1).

	AtRad1 (F_0)	*AtRAD51* (F_0)	*AtKu70* (F_0)	*AtRad1* (F_1)	*AtRAD51* (F_1)	*AtKu70* (F_1)
Relative frequency of plants with R-phenotype, acute irradiation (F_1)	0,72*			0,97**		
Relative frequency of plants with R-phenotype, fractionated irradiation (F_1)					0,93*	
Germination rate of the F_0 seeds + 30-days survival rate of seedlings, acute irradiation (F_1)		0,90*				
Germination rate of the F_0 seeds + 30-days survival rate of seedlings, fractionated irradiation (F_1)	-0,75*	0,92*		-0,64*	0,93*	

* $P \leq 0.05$, ** $P \leq 0.01$

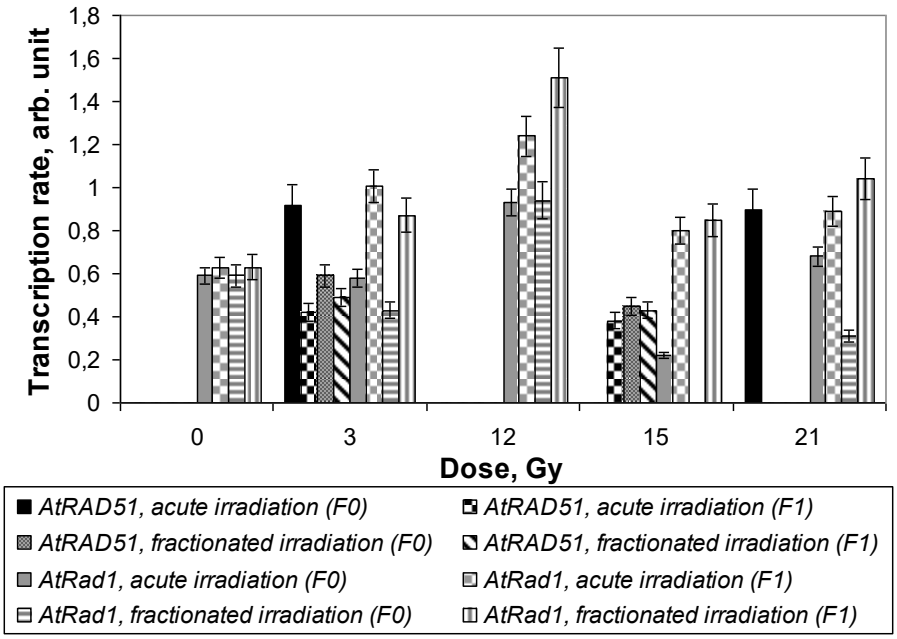


Figure 2. Relative transcription rate of *AtRAD51* and AtRad1 genes in rosette leaves of *A. thaliana* plants of irradiated F_0 and non-irradiated F_1 generations. Each bar in a diagram represents the average of three repeated experimental measurements, normalized to the *AtEf1a* reference transcription activity in leaves of non-irradiated plants with standard error bars.

There were no changes in transcription rate of AtKu70 in progeny of irradiated plants in comparison with progeny of control plants. At all tested doses and irradiation modes, the level of expression of AtRad1 was higher in rosette leaves of non-irradiated plants of the F_1 generation versus irradiated plants of the F_0 generation (Figure 2). As for the marker gene of double-strand DNA breaks of AtRAD51, the main factor of homologous recombination, its expression in the progeny increases only after acute irradiation of the parental plants at a dose 15 Gy. This is the solely dose of acute irradiation, at which the relative frequency of R-plants increases in F_1 (Figure 1(A)). With fractionated irradiation, given index in F_1 is higher than in F0 at all doses used (Figure 1(B)). Thereby, changes in F_1 in comparison with F_0 generation do not correspond to the extrapolation of the relationship between the phenotype and the level of caretaker genes expression in the leaves of irradiated plants. This indirectly confirms the second of the above-mentioned inheritance mechanisms for the radiation-modified phenotype, transgenerational transition of unrepaired DNA lesions (probably single-stranded lesions, which is indicated by an increase in the transcriptional activity of *AtRad1* but not *AtRAD51* and *AtKu70*), so that DNA repair genes are induced in the leaves of the non-irradiated progeny by the transmitted parental DNA damages.

4. CONCLUSION

Based on discussed data could draw conclusion, that radiation-induced changes in plants can be transmitted to the next generation. The transgenerational transmission of modified transcriptional rates of key DNA repair genes correlates with phenotypic effects in the non-irradiated offspring of the irradiated plants. Dose fractionation with 24 h time interval cause correlation between a larger number of phenotypic features of irradiated plants and their progeny than after acute irradiation. It is unclear whether this is a manifestation of the hypothetical inheritance of the altered transcriptional activity or reflects the transfer of DNA lesions from parent to offspring via gametogenic cell line. But there are indirect evidences in favor of the second possibility meaning transgenerational transmission of the unrepaired ssDNA lesions.

In general, these results shed light on the fundamental biological problem of inheritance of acquired characteristics under influence adverse stress factors.

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Effect of Phytohormones of Kinetin and Epibrassinolide on Content and Intracellular Localization of Glucosides and Free Amino Acids in Pea Plants Cells (*Pisum sativum L.*)

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Abstract: During injection of kinetin and epibrassinolide in pea seedlings (*Pisum sativum L.*) an increase of content of specific for this compound isosuccinimide-β-glucoside (IS-glucoside) for 15-30%, ethyl-β-glucoside for 40-80%, GABA free amino acids, glutamate and alanine only under kinetin for 15-20% was observed. The field experiments showed an increase of aglycone content but the amount of IS-glucoside decreased at first and then increased 14 days after plant treatment with phytohormones. It was shown that effect of phytohormones on content of IS-glucosides and free amino acids was dependent on action time and age of plant. By using membrane trophic compound of DMSO it was discovered that in pea seedlings cells the vacuolar fund of IS-glucoside was 70% of initial and aglycone with γ-aminobutyric acid as its precursor were localized in cytoplasm. Aspartate and glutamate were almost equally distributed between cytoplasmic and vacuolar cell funds. It is suggested that reactions of pyrrolidone structure of IS-glucoside aglycone formation and synthesis of the glucoside were processed in cytoplasm. Further IS-glucoside was transferred and stored in vacuoles of pea seedlings cells by contrast with ethyl-β-glucoside mainly localized in cytoplasm.

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Vacuole

1. INTRODUCTION

Along with proteins, carbohydrates, lipids and vitamins in plants there are the substances usually so-called of secondary metabolites. One of the most important groups of secondary metabolites are the glycosides. In natural glycosides an O-glycosidic bond connects residuals of monosaccharides with non-hydrocarbon components (aglycones). Relative ease of formation and cleavage of all types' glycosidic bonds provides metabolic versatility of relevant compounds in living cell and explains wide occurrence of this structural unit in living systems [1]. Glycosides perform different roles in plants including transport, protective [1] and detoxicative such as transformation of toxic ethanol accumulated under hypoxia into inert compound of ethyl-β-glucoside [3]. Chemical nature of glucosidic donor is limited to relatively small group of compounds. Most frequently the content of glucosides has glucose. The nature

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of acceptor is diverse. The environment in the form of glucosides possesses alcohol and phenols which have mainly β-configuration but also saponins and alkaloids. Therefore, the process of phenol compounds glucosidation is an exclusive characteristic of higher plants [4]. Various types of glucosides are mainly localized in vacuolar compartment of plant cells [5].

Discovered that pea plants (*Pisum sativum L.*) have a specific glucoside which was extracted, purified and identified as 5-oxy-2-pyrrolidone-β-glucopyranoside and matched with isosuccinimide-β-glucoside (IS-glucoside) also found in this plant per UV- and IR-spectrophotometry, melting temperature and hydrolysates [5,6]. Using different C^{14} -amino acids we determined that aglycone of IS-glucoside is a cyclic derivative of nonproteinogenic γ-aminobutyric acid [7]. The significant fluctuation of IS-glucoside content was dependent on the kind of pea plants and ontogenesis stage [8]. Found that IS-glucoside is generated during seed germination and plays and important role in young, growing organs and tissues. Maximum amounts of this glucoside present in green parts of seedlings. Etiolated plants contain its' smaller quantities as well as aglycone [8].

It is well known that origin of all ontogenesis stages is under hormone control and a possibility of regulation by exogenous plant growth regulators of special exchange processes in plant cells is also shown for various classes of secondary metabolites [2]. An effect phytohormones of cytokinin and epibrassinolide (EBL) have on IS-glucoside content and related to its metabolism compounds including amino acids was investigated. The volumes of some intracellular funds of these compounds were analyzed.

2. MATERIAL AND METHODS

The experiments were conducted with 10-15 days old pea seedlings "Ramonskiy 77" grown in laboratory conditions by hydroponics method under 12h photoperiod. The plant material was obtained from the Department of Plant Selection and Seed Breeding of the Voronezh State Agrarian University (Russia). In some experiments the plants in bud formation stage grown under field conditions were used. In all experiments performed the plants were subjected to treatment with phytohormone solutions of cytokinin and EBL of 10mg/l concentration. Under laboratory conditions the phytohormone solutions were injected by transpiration stream into over ground part of seedlings during 12-24hr. In the field experiments the plants were sprayed with phytohormone solutions and the leaves were analyzed after 7-14 days. The content of IS-glucoside and its aglycone in plant cells was determined by our method after chromatographic separation of uncharged compound by absorption spectrophotometry of 208nm and 212nm [7]. Amount of ethyl-β-glucoside was calculated after hydrolysis by using glucooxidase method per generated glucose [8]. Content of certain amino acids was determined after ninhydrin reaction [9].

Isolation of cytoplasmatic and vacuolar cell funds was investigated using membrane trophic compound of dimethyl sulfoxide (DMSO) previously used for cell funds analysis [6]. During preliminary experiments, we found the DMSO concentrations which can selectively change permeability of only plasmalemma or tonoplast in pea seedlings leaf cells. Disruption of tonoplast permeability was estimated by neutral red output used for treatment of hewn leaves for 20-180 min. Output of colorant from cells was calculated according to change of concentration under 530 nm and damage level of tonoplast was controlled by microscopic display. The experiments showed that disruption of plasmalemma happens under DMSO concentration of 7.5-15.0% and of tonoplast – around 50%. To analyze an intracellular localization of glucosides and amino acids a quantity of leaves (0.5g) was shred and placed into DMSO solution of matched concentrations. The yield of analyzed compounds was studied through the analysis of solutions after chromatographic separation on plates with silica gel G and calculated on mg of protein.

All experiments were conducted in 3 biological and 2 chemical replications and were processed by mathematical statistics methods with $p>0.05$. The presented data is the arithmetical mean plus the error. Data of one of the typical experiments is presented in current paper.

3. RESULTS AND DISCUSSION

We investigated an impact of kinetin and EBL on content of IS-glucoside and its aglycone and on ethyl-β-glucoside, glucose and free amino acids - GABA, glutamate, alanine and aspartate. The data shown (Figure 1. and Figure 2) suggests that growth regulators influenced the content of investigated compounds to different extent. It was discovered that in 2-week-old seedlings kinetin and EBL after 12hr of injection stimulated reactions of IS-glucoside synthesis followed by increase of IS-glucoside for 30% and 11% respectively. At the same time the content of aglycone and endogenous glucose in plant cells was decreasing. At the same time the level of free amino acids – GABA, glutamate, aspartate and alanine – was increasing. But after 24hr of injection both kinetin and EBL were decreasing the content of GABA and glutamate. With that the content of IS-glucoside and glucose was also decreasing in the presence of high speed of aglycone and ethyl-β-glucoside formation which suggests about the speed change of these compounds synthesis under various duration term of phytohormones on pea seedlings.

When the plants were treated with kinetin on latter ontogenesis stages i.e. during flower bud formation then 7 days after the aglycone content in pea seedlings leaves was increasing for 20% relative to control and GABA for 28% respectively. In the meantime, the synthesis of IS-glucoside was suppressed since its content was decreased for 40%. The decrease of free amino acids content in average for 7-8% was noted. Our data shows (Figure 1.) that kinetin did not have an impact on synthesis of ethyl-β-glucoside and glucose content. In 14 days after the treatment the content of aglycone increased 1.5-fold while the GABA content did not change.

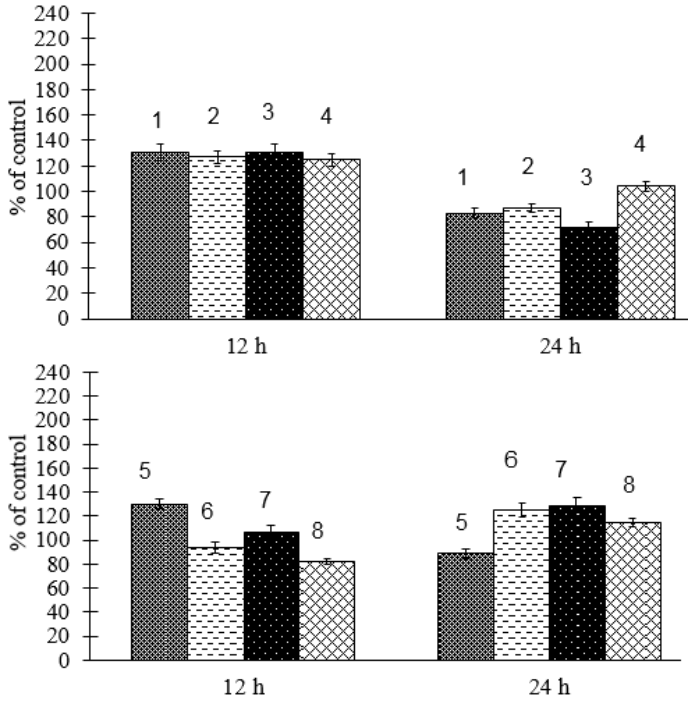

Figure 1. Impact of kinetin on content of amino acids and IS -glucoside in pea plants under 12 and 24h of injection: 1 – aspartate, 2 – glutamate, 3 – alanine, 4 – GABA, 5 – IS-glucoside, 6 – aglycone, 7 – ethyl-β-glucoside, 8 – glucose

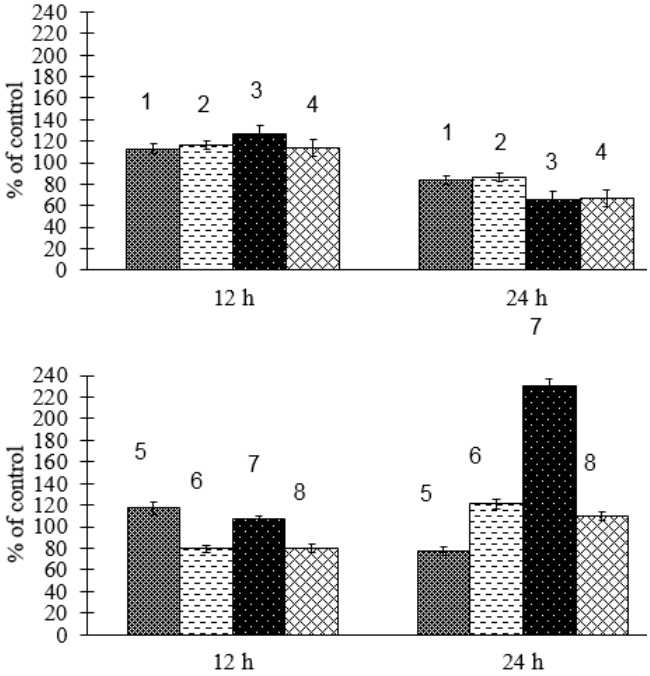

Figure 2. Effect of EBL on content of amino acids and IS-glucoside in pea plants under 12 and 24h of injection: 1 – aspartate, 2 – glutamate, 3 – alanine, 4 – GABA, 5 – IS-glucoside, 6 – aglycone, 7 – ethyl-β-glucoside, 8 - glucose

It can be assumed that the pea plants have relatively active enzyme systems participating in cyclization of GABA with the formation of pyrrolidone structure of IS-glucoside aglycone as showed before [7] and systems able to synthetize the glucoside itself. This was evidenced by increase on 20% of the aglycone content in pea plants cells after 14 days since treatment compare to the control plants. Beyond that kinetin contributed to glucose accumulation. The phytohormone stimulated also alanine formation but decreased the amounts of aspartate and glutamate.

Special aspect of cell structure is a complexity of its dimensional organization. Specificity in demonstration of metabolic qualities of the same compounds is a result of their spatial isolation i.e. compartmentation. In plant cells there are reserve vacuolar and active cytoplasmic funds [10]. Diverse compartmentation of compounds and enzymes of their metabolism determines speed of exchange processes of cells. Vacuole plays an important role in storage of not only primary but also secondary metabolites [5,10]. However vacuolar funds of low molecular compounds such as amino acids and glucosides are investigated only for a small number of plants. For this reason, further, we analyzed the allocation of isosuccinimide-β-glucoside, its aglycone and several free amino acids related to their metabolism between cytoplasmic and vacuolar cell funds of pea seedlings. Extraction of the funds was conducted by method using a membrane trophic compound DMSO.

The data presented in Table 1 and Table 2 show results of studying compounds distribution between vacuolar and cytoplasmic cell funds of pea seedlings. In Table 1 we can see that most quantity of IS-glucoside, up to 70% of total cell content, is accumulated in vacuole. As it was discovered [1] most of plant glucosides have vacuolar localization. The vacuole being a multifunctional organelle can reserve relatively large number of compounds including glucosides thus isolating from enzymes of their catabolism – β-glucosidases. Such spatial designation allows an itself existence of glucosides in plant cells and perform functions of defense and detoxication. Therefore, an IS-glucoside aglycone was mainly localized in

cytoplasm, where 83% of total cell fund of this compound was accumulated as it was suggested by the experiment results.

Table 1. Content of IS-glucoside and aglycone in vacuolar compartment and cytoplasm (a – μmol/gr of fresh weight, b - % of total cell content)

Compounds	cell		cytoplasm		vacuole	
	a	b	a	b	a	b
IS-glucoside	3.00±0.10	100	0.96±0.04	32	2.04±0.08	68
Aglycone	4.10±0.20	100	3.41±0.08	83	0.70±0.02	17
Ethyl-β-glucoside	0.26±0.005	100	0.16±0.1	61	0.10±0.005	39

It was discovered that primary spot of GABA formation in cells is cytoplasm [9]. At the same time, it was shown [11] that vacuolar fund can contain significant amounts of this amino acid. However, such researches are occasional. As shown in Table 2, we analyzed allocation of GABA and several free amino acids between cytoplasmic and vacuolar cell funds of pea seedlings using membrane trophic DMSO compound.

Table 2. Intracellular allocation of amino acids in pea seedlings (a – μmol/gr of fresh weight, b - % of total cell content)

Compounds	cell		cytosol		vacuole	
	a	b	a	b	a	b
GABA	3.93±0.05	100	2.57±0.10	65	1.30±0.02	33
Glutamate	6.72±0.31	100	3.56±0.16	53	3.00±0.13	46
Alanine	4.04±0.20	100	2.91±0.09	72	1.13±0.04	26
Aspartate	8.57±0.13	100	4.30±0.10	50	4.28±0.15	50

The results showed that GABA and alanine had preferential cytoplasmic localization with the content of 65% and 72% of total cell pool respectively. With that the contents of aspartate and glutamate were approximately equally distributed between cytoplasmic and vacuolar cell funds. Obtained results are corresponded to other works [9] suggest that alanine, glutamate and GABA in cells of some plants are concentrated substantially in cytoplasmic cell funds.

It was shown before that GABA in synthetized in cytoplasm where the enzyme of glutamic acid decarboxylase (GAD) is localized. In that context, it can be assumed that reactions of cyclization of carbon skeleton of GABA accompanied by pyrrolidone structure formation of IS-glucoside are particularly processed in cytoplasm. Probably the reactions of IS-glucoside formation are also processed in cytoplasm [7]. At the same time the generated glucoside later could be stored in vacuolar fund of pea seedlings cells which is suggested by our results.

4. CONCLUSION

Our research showed that growth regulators such as kinetin and epibrassinolide have an impact on content of isosuccinimide-β-glucoside - a pea seedlings specific glucoside. Interconnection of content change for glucoside, its aglycone, free glucose and GABA in pea seedlings under the influence of phytohormones was noted. Therefore, the phytohormones' effect on content of studied glucoside and aglycone was determined as by activity period of phytohormones as by age of the treated plants. Mechanism of change of IS-glucoside

biosynthesis under influence of growth regulators can be discussed. Capability of phytohormones to affect the activity of the number of enzymes and increase permeability of membrane structure which contribute to better conditions of intracellular metabolite transport were shown [12]. In this case stimulation of glucoside formation after plant treatment with kinetin can be considered as an outcome of significant improvement of supply of enzyme biosynthesis by substrates due to reduction of membrane barriers preventing entry of precursor molecules to the points of use.

While investigating intracellular localization of IS-glucoside, its aglycone and number of free amino acids related to their exchange while using membrane trophic DMSO compound it was discovered that in pea seedlings cells there are at least two IS-glucoside funds – cytoplasmic and vacuolar ones. In this case, vacuolar fund of IS-glucoside was about 70% of its cell pool. At the same time aglycone and its precursor – γ-amino butyric acid- were localized mainly in cytoplasm. This proves the hypothesis [7] that the reactions processed right in the cytoplasm were: formation of pyrrolidone structure of aglycone and synthesis of the glucoside itself further to be transferred to and stored in vacuoles of seedlings cells. The vacuole can reserve relatively large number of glucosides thus isolating from enzymes of their catabolism – β-glucosidases. Such spatial designation allows an itself existence of glucosides in plant cells and perform functions of defense and detoxication.

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Mineral composition of some wild mushrooms from Eastern Anatolia, Turkey

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Abstract: Within this study 40 samples including sequential extracts, water extracts and drug samples obtained from five mushroom samples wild grown from Turkey were evaluated for their mineral composition analysis. All samples were found as rich sources of minerals notably Ca, K, Si, Mg, Se and Si which might contribute health enhancing properties. The levels of heavy metals were detected in low amounts in the extracts of mushroom species (except *T. populinum*). Acetone and ethyl acetate were detected as the most efficient solvents in the isolation of minerals from mushroom samples. Our findings showed that extracting of mineral compounds were varied due to the type of solvents applied and mycochemical diversity and the reported mineral compounds profiles suggest that these wild-edible mushrooms might be potential sources of therapeutic nutraceuticals.

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1. INTRODUCTION

Mushrooms have been utilized for centuries by humankind for various purposes particularly for food and medicine. They are utilized as fresh material, drugs, infusions, decoctions or crude extracts [1]. *Ganoderma lucidum* (Reishi), *Lentinus edodes* (Shiitake), *Inonotus obliquus* (Chaga) are among the most used mushroom samples as therapeutic based nutraceuticals [2].

Extracts obtained from several mushroom species have been reported for their pronounced health enhancing activities which are associated to the presence of active chemical compounds such as phenolics, glycosides, tocopherols, polysaccharides, carotenoids, ergothioneine, vitamins, ascorbic acid and minerals [3-5].

Among these mycochemical compounds, minerals have specific properties due to their essential roles in biological reactions as catalyst. They are essential compounds in all cognitive and physiological processes in several tissues. For instance, iron, copper, selenium and zinc enrolling to the antioxidant enzymes as cofactor. Na and K are constituents of acid-base balance and nerve stimulation. Fe joins to the structure of haemoglobin. Zn present in some enzymes structure and plays significant roles in protein synthesis. Moreover, mineral compounds are

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necessary in the absorption of vitamins; Ca for Vitamin C, Zn for Vitamin A, Mg is for Vitamin B and Se for Vitamin E [6].

Addition to the health benefits of mineral compounds, there are also some hazardous effects of some mineral compounds called heavy metals such as Cd, Pb and Ni. Pb is well known for its harmful effects on cardiovascular system. Cd has side effects on kidney, urinary and skeleton system [7]. The accumulation of heavy metals particularly mercury, cadmium, copper and arsenic in mushroom species collected from wild such as *Boletus badius*, *Suillus variegatus* and *Rozites caperata* was reported previously [8].

Tricholoma scalpturatum (Fr.) Quél., *Tricholoma populinum* J.E. Lange, *Neolentinus cyathiformis* (Schaeff.) Della Maggiora & Trassinelli, *Chlorophyllum agaricoides* (Czern.) Vellinga and *Lycoperdon utriforme* Bull. are among mushroom species that most utilized for nutritional and medicinal purposes in Turkey. However, studies regards to chemical analysis particularly mineral components are limited in scientific literature. Therefore, this study focused on evaluating mineral composition of these mushroom species comprehensively by using various organic solvents. For this purpose, three different extraction methods including sequential, water and drug extracts were prepared for analysis.

Sequential extracts were used in order to understand the effect of polarity on mineral extraction capability of various organic solvents including n-hexane, chloroform, ethyl acetate, acetone, ethanol and pure water. Water extract was applied in order to reveal the traditional utilization of these species. Moreover, drug samples were directly analysed for mineral compound analysis.

2. MATERIAL AND METHODS

2.1. Mushroom Material

Fruiting bodies of mushroom samples (*Tricholoma scalpturatum* (Fr.) Quél. (GPS coordinates 38º34ʾ09.81ʾʾN, 43º16ʾ53.23"E), *Tricholoma populinum* J.E. Lange (GPS coordinates 37º17ʾ24.35ʾʾN, 44º35ʾ47.56"E), *Neolentinus cyathiformis* (Schaeff.) Della Maggiora & Trassinelli (GPS coordinates 38º17ʾ31.69ʾʾN, 43º 05ʾ25.12"E), *Chlorophyllum agaricoides* (Czern.) Vellinga (GPS coordinates 37º23ʾ59.2ʾʾN, 44º29ʾ49.02"E) and *Lycoperdon utriforme* Bull. (GPS coordinates 37º23ʾ59.92ʾʾN, 44º29ʾ49.02"E)) were harvested from Eastern Anatolia Region of Turkey, on 5-19 May 2016. Mushroom materials were isolated in clean polythene bags and transferred to the laboratory within a maximum of 3 h after harvest. The identities of mushroom materials were confirmed by Yusuf Uzun, PhD at Mcygology Research Fungarium, Science Faculty, Van Yuzuncu Yil University, Turkey and a voucher specimen was stored at the university's fungarium (Fungarium codes: 7484, Acar 481, 7485, Acar 636 and 7486 respectively). The mushroom materials were properly cleaned from dust and contaminants by minimizing the loss of chemical components and left at room temperature in the dark until dry. The dried mushroom materials were subsequently ground for a fine powder and stored at -20 °C until analysed.

2.2. Reagents

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Istanbul, Turkey) and were of analytical or HPLC grade.

2.3. Preparation of sequential lyophilized extract

The ground mushroom materials were extracted sequentially using a range of organic solvents with increasing degrees of polarity; n-hexane, chloroform, ethyl acetate, acetone, ethanol and pure water respectively, as recommended by Dai and co-authors [9]. Firstly, the ground air-dried mushroom samples were mixed with a 10-fold volume of n-hexane (gr/ml) shaken for 2 h at room temperature (22°C) and centrifuged for 20 min at 15320g (10000 rpm)

at 4°C (Sorvall RC-5B; DuPont, Wilmington, DE, USA; rotor Beckman JA14 (137 mm) serial no. 02U8152, USA) with the supernatant collected.

The same extraction procedures were applied to the pellet using aqueous 10-fold those of chloroform, ethyl acetate, acetone (80%), ethanol (80%) and pure water (gr/ml) respectively, with the supernatants collected. The supernatants from n-hexane, chloroform, ethyl acetate, acetone, ethanol and pure water fractions were evaporated individually under reduced pressure at 37°C using a rotary evaporator (Rotavapor R-205; Buchi, Switzerland). The derived concentrated fractions were dissolved in a minimum amount of purified water and freeze-dried under a vacuum at -51°C to obtain fine lyophilized powders.

2.4. Preparation of water extract

The water extracts were prepared as described previously [10] using pure water as solvent. Briefly, the ground mushroom material was mixed with a 10-fold volume of pure water, shaken for 2 h at room temperature (22°C) and centrifuged for 20 min at 15320g (10000 rpm) at 4°C with the supernatant collected. The extraction was repeated one more time. The supernatants from the consecutive extractions were combined and the solvent evaporated under reduced pressure at 37°C using a rotary evaporator (Rotavapor R-205; Buchi, Switzerland). The derived fraction was dissolved in purified water and freeze-dried under a vacuum at -51°C to obtain a fine lyophilized powder.

2.5. Mineral Composition Analysis

The mineral composition of the extracts were prepared as described previously [11]. The analysis were conducted using AAS (Thermo Scientific, ICE-3000 series, USA), ICP-MS (Thermo Scientific, ICAP RQ series, USA) and ICP-OS (Thermo Scientific, ICAP-7200 series, USA). The analysis solutions were prepared by dissolving the lyophilized extracts and drug material in HNO_3 individually. Subsequently, the solution was subjected to microwave assisted extraction procedure and identities of mineral compounds were confirmed by comparison of authentic standards.

3. RESULTS

Tricholoma scalpturatum had a rich mixture of mineral compounds as shown in Table 1. Na, Ca, K, Fe, Si, S, Zn and Cu were the dominant mineral compounds of the extracts and drug. Acetone was more efficient than other apolar and polar solvents used in sequential fractions in terms of mineral compounds extraction, followed by ethyl acetate and ethanol. Interestingly hexane fraction was found as efficient in the extraction of Ag, B and Mo. No significant difference was detected between water extract and drug as shown in Table 1. The levels of heavy metals such as Cd, Ni and were detected in low levels. As and Cr were not detected in the extracts. Among heavy metals Pb was detected in high levels particularly in drug and acetone fraction (Table 1).

Table 2 presents mineral composition of *Neolentinus cyathiformis* extracts and drug. The results showed that water was the most efficient solvent in terms of mineral extraction. The levels of heavy metals such as Cd, Ni and Pb were detected in low levels. As and Cr were not detected in the extracts. The highest levels of Ag, B, Mo, Na, Se and Si were found in hexane fraction.

As shown in Table 3, sequential fractions were more efficient than those of water extract and drug in terms of mineral extraction. n-Hexane extracted B and Mo effectively than those of organic solvents. Relatively high amounts of minerals were extracted by chloroform, followed by ethyl acetate, water and acetone. Drug was the less efficient solution compare to water and sequential extracts except of Pb. Heavy metals including, Ni, As and Cr were not found, while the amount of Cd was detected at low level (Table 3).

Table 1. Mineral Composition of *Tricholoma scalpturatum* (µg/g extract)*

	Sequential Fractions						Water Extract	Drug
	n-Hexane	Chloroform	Ethyl Acetate	Acetone	Ethanol	Residue		
Ag	**48.2**	ND	ND	ND	ND	ND	ND	ND
B	**2151**	371.3	ND	ND	195.3	ND	ND	ND
Ba	27.3	1.3	8.3	**54.1**	ND	ND	ND	ND
Be	**26.3**	ND	6.4	ND	ND	ND	ND	ND
Ca	1966.8	795.7	8488.9	**36800**	289.5	1983.4	2190.5	2612.8
Cd	8.9	1.5	18.6	**120.2**	T	T	T	T
Co	6.6	1.3	6.2	**58.2**	T	T	T	T
Cu	43.9	5.5	41.5	**464.6**	7	T	1.4	1.9
Fe	459.7	56.5	441.5	**5264**	11.5	1.8	244.9	785.3
K	25807	5736.9	ND	**34960**	**35197**	18.95	6294.2	3256.1
Mg	1368	357.9	429	**3336**	153.8	ND	184.1	180.6
Mn	40.1	3.9	12.5	**78.9**	T	T	T	T
Mo	**404**.7	T	8.1	18.4	ND	ND	ND	ND
Na	2607.2	1337.5	5896.3	**45616**	412.3	2.8	494.4	249.1
Ni	ND	T	3.4	**43.2**	T	T	T	ND
Pb	5.9	1.4	99.4	245	1	T	287.9	**406.7**
Sb	21.6	3	6.9	**223.5**	T	T	ND	T
Se	9.1	ND	46.8	**108.2**	ND	T	ND	ND
Si	2296.9	187.8	1376	**8718.4**	134.4	ND	213.6	401.6
Ti	**53.2**	ND	ND	ND	T	ND	T	2.1
V	51.6	26.7	419	**2088**	6	5.3	39.6	39.9
Zn	157.4	65.8	192.1	**1985.6**	23.8	ND	62.3	67

ND: Not detected. T: Traces. * Bold written values indicate the highest content of mineral compounds.

Table 2. Mineral Composition of *Neolentinus cyathiformis* (µg/g extract)*

	Sequential Fractions						Water Extract	Drug
	n-Hexane	Chloroform	Ethyl Acetate	Acetone	Ethanol	Residue		
Ag	**20**	3.4	ND	ND	ND	ND	2.3	ND
B	**2588**	ND	ND	ND	ND	ND	ND	ND
Ba	**7.2**	T	3.5	4.1	T	1.9	ND	ND
Be	1190.3	547.6	804.4	883.6	232.4	**1109.4**	**1517.9**	731.3
Ca	**3.7**	1.2	**3.7**	3,4	T	T	T	T
Cd	2.4	T	1.2	**3.2**	T	1.3	T	0.5
Co	11.9	1.7	**14.3**	5.9	1	7.5	T	T
Cu	60.6	40.8	**230.3**	168.4	9.2	90.4	71.8	**367.1**
Fe	10947.9	1625.6	ND	691.4	11787.8	**52487.5**	2046.9	1868
K	835.9	559.3	198	123.2	109.2	**3170.3**	166.7	156.5
Mg	4.3	5.1	3.5	4.5	T	**15.7**	ND	T
Mn	**138**	T	7.5	3.8	T	1.5	ND	ND
Mo	**4128**	574.8	1143.4	1559	431.1	1142.4	528.5	295.4
Na	ND	T	**5.7**	ND	T	T	ND	ND
Ni	5.1	T	4.8	2.1	T	1	233.1	**491**
Pb	3.4	1.9	**12.3**	T	1	T	ND	0.1
Sb	**8.8**	ND	ND	2	T	T	ND	ND
Se	**865.9**	120.2	380.8	446.2	65.9	237.9	142.4	465.9
Si	**7**	1	ND	ND	ND	2	ND	ND
Ti	26	26	**49**	44	6	13	40.2	40
V	32.9	23.7	**86.4**	82	5.3	49.1	43.2	46.4
Zn	**20**	3.4	ND	ND	ND	ND	2.3	ND

ND: Not detected. T: Traces. * Bold written values indicate the highest content of mineral compounds.

Table 3. Mineral Composition of *Chlorophyllum agaricoides* (µg/g extract)*

	Sequential Fractions						Water Extract	Drug
	n-Hexane	Chloroform	Ethyl Acetate	Acetone	Ethanol	Residue		
Ag	ND	ND	ND	ND	ND	ND	T	T
B	**1169**	ND	ND	ND	ND	14.2	ND	ND
Ba	3.4	3.5	8.6	**236**	T	1.3	ND	ND
Be	614.9	**3281.4**	1644.7	1363.6	385.4	1915.1	2102.9	1079.7
Ca	2.7	**10.9**	4.1	4.8	T	T	T	T
Cd	T	6.5	ND	1.9	T	T	**11.1**	T
Co	4.8	3.1	18.3	**27.9**	13.7	27.2	T	6.4
Cu	13.1	**282**	187.4	153.3	16.6	30.9	56.4	193.4
Fe	5155.2	11600	6131.6	7812.1	23180	**76582**	5023	3988.1
K	302.4	675.5	934.2	446	46.3	**2306.6**	176.5	185.7
Mg	3.4	7.6	**11.9**	3.1	T	8.1	11.5	T
Mn	**48**	36	9.1	2.6	T	T	ND	1.1
Mo	1696.1	3258.3	**3494.7**	2925.5	387.6	1133.9	486.5	215.3
Na	ND	ND	ND	ND	T	T	ND	ND
Ni	6.6	4.9	13.4	3.5	T	T	179	**469**
Pb	7.2	**27.1**	5.5	ND	T	T	ND	ND
Sb	8.1	**21**	8.4	ND	6.5	3.8	2.2	3.5
Se	770	**1006**	667	870	66	84	82.2	438
Si	ND	ND	ND	**1.8**	ND	ND	ND	T
Ti	17	**136**	61	88	6	14	40.1	39
V	22	119	**161**	79	10	76	57.6	100
Zn	ND	ND	ND	ND	ND	ND	T	T

ND: Not detected. T: Traces. * Bold written values indicate the highest content of mineral compounds.

Table 4. Mineral Composition of *Tricholoma populinum* (µg/g extract)*

	Sequential Fractions						Water Extract	Drug
	n-Hexane	Chloroform	Ethyl Acetate	Acetone	Ethanol	Residue		
Ag	ND	9.7	**11.3**	ND	0.9	ND	ND	2.1
B	**451**	ND	ND	ND	ND	ND	ND	ND
Ba	4	1.4	2.1	**171.8**	11.7	1.1	ND	ND
Be	42.2	21.1	59.2	**96.3**	6.5	95.5	ND	ND
Ca	ND	T	ND	**18.1**	ND	T	**1528.5**	**1285.6**
Cd	1009.2	803.9	1890.4	**32400**	618.1	1362.8	1.8	3
Co	3.4	1.7	8	**67.6**	T	2	T	T
Cu	T	T	3.5	**25**	T	T	T	1.5
Fe	6.7	6.4	**18.7**	ND	T	14.8	92.3	325.9
K	132.9	211.5	200.6	3530	3.9	183	**5389.2**	5614
Mg	4739.8	1084.4	3686.6	14060	29820.7	**74975.3**	109.3	120.7
Mn	460.6	160.8	227.3	654.1	88.9	**1422.8**	T	T
Mo	10.4	5.2	4	**45.8**	T	14	ND	ND
Na	34.1	2.3	ND	ND	ND	T	**216.6**	94.6
Ni	1392.8	620.3	2919.5	**23170**	193.6	388.3	ND	ND
Pb	T	18.4	ND	16.3	ND	ND	**153**	**444.9**
Sb	6.3	2.5	7.8	ND	T	1.7	T	T
Se	7.5	1.7	16.6	**98.9**	T	T	T	1.1
Si	5.1	1.2	32.9	**168.5**	1.6	1.4	53.5	415.7
Ti	279.6	269	440.7	**3871**	98.1	386.5	ND	ND
V	ND	1.6	ND	ND	ND	4.45	**39.6**	38.5
Zn	21.7	31.6	141.5	**1295**	6.3	7.9	50.6	51.4

ND: Not detected. T: Traces. * Bold written values indicate the highest content of mineral compounds.

Mineral composition of *Tricholoma populinum* was presented in Table 4. Relatively higher levels of minerals were detected in water-based fraction and extracts and drug compare to organic solvents-based fractions. Similar to other mushroom samples, drug contained the highest level of Pb. Interestingly, Cd and Ni levels of acetone-based fraction, water extract and drug were detected in pronounced levels. Mg, K, Ca and Zn were detected as the major mineral compounds. Additionally, Ag, B, As, Cr were not detected in all extracts (Table 4).

Table 5. Mineral Composition of *Lycoperdon utriforme* (µg/g extract)*

	Sequential Fractions						Water Extract	Drug
	n-Hexane	Chloroform	Ethyl Acetate	Acetone	Ethanol	Residue		
Ag	**12.2**	ND	ND	7.4	T	T	ND	T
B	**271**	ND	ND	ND	ND	ND	ND	ND
Ba	24.9	T	22.6	**322**	T	1.44	ND	ND
Be	5540	4566.9	**6720**	2609.1	214.3	1350.4	2127.6	1353.3
Ca	24.3	3.8	**28.2**	6.8	0.4	0.5	T	0.7
Cd	**10.3**	1.8	7.7	1.3	T	T	T	T
Co	**54**	13	5	4	9	35	4.2	9
Cu	692	253.2	**764**	262.7	25.3	143.3	244.2	224.6
Fe	5496	3403	ND	3623	9722	**72560**	3368.7	1640
K	1136	364	611	796	30	**2144**	124.6	126
Mg	11	4.2	ND	7.4	T	**15**	ND	T
Mn	**266**	3.17	41.7	6.13	T	2.3	ND	ND
Mo	**11060**	1865	9608	5649	284	740	651.5	117
Na	ND	ND	**16**	ND	ND	ND	ND	ND
Ni	75.4	2.8	ND	ND	T	1.2	**532.5**	418
Pb	4.2	9.4	**53**	6.2	T	1.07	ND	ND
Sb	**132**	9	47	10	1.1	T	ND	T
Se	**3870**	380	1936	936	218	176	104	337
Si	ND	6	**11**	ND	ND	1.6	ND	T
Ti	317	65	**478**	130	6.6	9.3	148.5	38
V	161	159	**1412**	133	8	44	54.3	96
Zn	**12.2**	ND	ND	7.4	T	T	ND	T

ND: Not detected. T: Traces. * Bold written values indicate the highest content of mineral compounds.

The levels of mineral compounds present in *Lycoperdon utriforme* were presented in Table 5. K, Na, Mg, Si and Zn were the dominant compounds of mineral composition. With regards to heavy metal contents; As and Cr were not detected, while Ni and Cd were at low levels except Pb (Table 5). n-Hexane based fraction was the most efficient in the context of extracting Ag, B, Cu, Mo, Na, Se and Si. Water-based extracts were found as more efficient in extraction of K, Mg and Pb than those of organic solvents (Table 5).

4. DISCUSSION AND CONCLUSION

Mushrooms have been used as important dietary supplements because of their pleasant tastes, nutritional and pharmaceutical constituents such as rich protein, low-fat content, secondary metabolites, vitamins and minerals. Minerals are vital chemical compounds for humans which have crucial functions such as maintaining acid-base balance, the osmotic regulation of fluid and oxygen transport in the body and also playing significant roles in the catalytic processes [12].

Wild grown edible mushroom species are able to cumulate significant amounts of mineral constituent's specifically K, P, Ca, Mg, Na and Fe which are essential to fungi and its consumers. Mushrooms can also be enriched with toxic elements such as As, Hg and Cd [13], which can cause several health risks and have no any significant biological roles. The toxic effects of these hazardous elements are harmful for the human body and its proper functions.

For instance, Cd is the seventh most toxic heavy metal which has adverse effects on the enzymatic systems of cells and oxidative stress [14].

Previous analysis carried out on mineral composition of mushroom species were generally focused only on drug materials which have directly treated with HNO_3 [11, 15, 16]. The utilization of a single solvent (HNO_3) restricted the extraction of mineral compounds from mushroom tissues and therefore various extractions including different solvent systems with different polarities should be applied in order to analyse the mineral composition comprehensively.

Our findings showed that extracting of mineral compounds were varied due to the type of solvents applied and mycochemical diversity. For instance, hexane is a proper solvent in the extraction of Ag, B, Be, Mo, Ti, while ethyl acetate is more appropriate in the extraction of Zn and Fe. Deductively it can be suggested that, acetone and/or ethyl acetate were relatively more efficient solvents than those of other organic solvents.

Mushrooms rich in K and Mg and poor in Na are recommended for the treatment of hypertension [17]. Our findings were in accordant with this aspect and it can be suggested that all mushrooms analysed in this study (except *T. populinum*) might be helpful in the management of hypertension. Moreover, the extracts contained high levels of Ca and Mg, which might help for the maintenance of nerve transmission, glandular secretion, muscle contraction and hinder the biochemical abnormalities and clinical manifestations [18].

All samples were found as rich sources of minerals notably Ca, K, Si, Na and Mg. The levels of heavy metals were generally in low amounts except Pb. However, *Tricholoma populinum* extracts were accumulated significant amounts of Cd. With regards to Pb accumulation, organic solvent fractions were found as more selective than those of the drug and water extract which indicate that polarity of solvents are a significant factor of Pb isolation from mushroom tissues. Although *T. populinum* were detected as a rich source of mineral compounds, it also contained an excessive amount of Cd and therefore care should be taken in the consuming of this mushroom species.

This study confirms the presence of some important bioactive mineral compounds, including K, Mg, Si, Se and Fe. The reported mineral compounds profiles propose the use of wild mushrooms as potential sources of curative nutraceuticals.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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