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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Evaluation of Fatty Acid Compositions of Some Important Wild and Domestic Turkish Mustard Genotypes (*Brassica* spp.)

Fatma Kayaçetin [*,1], **Banu Efeoğlu** [1], **Gül Sarıoğlu** [1]

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Abstract: The seed oil samples of 57 mustard genotypes belonging to six species of mustard (*Brassica* spp.) *Brassica juncea* (31 genotypes), *B. rapa* (6 genotypes), *B. napus* (2 genotypes), *B. nigra* (6 genotypes), *B. arvensis* (10 genotypes) and *B. alba* (2 genotypes) collected from USA gene bank and diverse ecologies of Turkey were used as research material and evaluated for their fatty acid composition by gas-liquid chromatography (GLC). The aim of this study was to evaluation of fatty acid compositions of some important wild and domestic Turkish mustard genotypes (*Brassica* spp.). The results showed significant variability among all genotypes for fatty acid composition. Erusic acid (C22:1; 20.63-47.87%), oleic acid (C18:1; 7.42-24.54%) and linoleic acid (C18:2; 9.61-25.11%) were determined to be the dominant fatty acids among all genotypes. It has been observed that the results in wild mustards have too many resemblances with the composition of fatty acid of mustard which is used by people in alternative medical science, paint, food industry and biodisel production. The great variability in seed oil contents in *Brassica* genotypes showed their potential for use in future breeding programs.

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1. INTRODUCTION

Brassicas are members of the *Brassicaceae (Cruciferae)* family. *Brassicaeae* comprise a diverse family of plants and provide one of the most extensive and varied range of end products used by man from a single plant genus [1]. *Brassica rapa* (rapeseed or rape mustard), *B. juncea* (Asian mustard or brown mustard), *B. arvensis* (charlock), *B. nigra* (black mustard) and *B. alba* (white or yellow mustard) are some of the important species of *Brassicaceae* family. Turkey has a rich flora; more than 9000 flowering plant species. The family *Brassicaceae* contains 84 genera with 441 species [2]. *Brassica alba* L., *B. arvensis* L., *B. nigra* L. and *B. juncea* L. family *Cruciferae*, grow in Turkey under natural conditions as weed (3, 4).

Brassica species are cultivated extensively to produce edible and industrial oils throughout the world. They occupy a unique position in world agriculture as a source of

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vegetables, oilseed, forage and fodder, green manure and condiments [5-7]. *Brassica* seed oil is used for the edible purpose, as industrial lubricants and as a base for polymer synthesis. Oilseed brassica cake is used as a source of protein in animal feeds [8, 9]. The oil is commonly used for cooking and to add a hot and spicy flavor to food [10]. As a crop, they are also one of the maximum oil yielding and high protein containing oilseed species [1, 11].

Mustard oil contains the major saturated fatty acids like palmitic and stearic acids along with mono and polyunsaturated fatty acids like oleic, eicosenoic, erucic, linoleic and linolenic acids. The fatty acid composition of the seed oil depends on the genetic, ecological, morphological, physiological and cultural factors. There may be big or small differences among *Brassica* species in terms of fatty acid composition and it may be changeable to a small and large scale depending on this factors [12, 13, 14, 15]. To know the fatty acid composition of *Brassica* species oil makes oil production possible for special using purposes. Thus, it would be possible to produce suitable oils by cultivating the desired genotypes.

The purpose of the study was to evaluate seed fatty compositions of 57 mustard genotypes *Brassica* belong to six species which selected from different mustard genotypes as a potential material for starting mustard breeding programs in Turkey.

2. MATERIALS and METHODS

The study made use of 57 mustard genotypes as research material including six *Brassica* species which were selected from different mustard genotypes obtained from the USA gene bank and collected locally from diverse ecologies in Turkey. All genotypes were planted during the growing seasons of 2016-17 at the experimental fields located at Yenimahalle (fall sowing) 39°12′ - 43°6′ N, 35°58′ - 37°44′ E, and 925 m altitude, with semiarid climatic characteristics ecological dry conditions. The station belongs to the Central Research Institute for Field Crops, Ankara, Turkey. Table 1 shows long term and monthly meteorological data of experimantal station where *Brassica* field experiment was carried out. There was a total of 400.2 and 393.6 mm precipitation, 12.2 and 12.1 °C average temperature, and 56.5% and 60.3% average humidity, respectively at Yenimahalle. It was determined that more arid conditions persisted during 2016-2017 growing season compaired to the averages of long years.

Table 1. Growing season and long term monthly meteorological data of the experimental area, where the *Brassica* genotypes were sown for experimantal purposes

Climatic factors	Years	Months												Total or average
		S	O	N	D	J	F	Mr	A	M	Jn	Jl	A	
Precipitation	Long years	17.5	31.8	34.2	42.0	40.2	33.0	36.7	46.7	49.9	34.2	14.3	13.1	393.6
(mm)	2016-17	11.1	4.0	23.7	47.9	28.1	7.5	46.1	19.8	96.2	102.8	0.0	13.0	400.2
Relative humidity	Long years	49.1	60.5	69.7	76.5	76.4	70.7	63.2	59.0	56.5	52.1	45.1	45.3	60.3
(%)	2016-17	45.4	53.9	53.8	74.1	76.4	66.5	59.6	49.8	55.7	58.3	38.4	45.8	56.5
Average	Long years	19.0	13.1	6.8	2.3	0.4	2.3	6.4	11.5	16.2	20.3	23.8	23.5	12.1
temperature (ºC)	2016-17	19.2	13.7	6.9	-0.3	-1.3	3.1	8.1	11.2	15.7	20.3	25.5	24.7	12.2
Maximum	Long years	32.6	27.6	19.7	13.9	11.9	14.7	21.4	25.7	29.3	33.6	36.2	35.8	36.2
Temperature(ºC)	2016-17	32.9	28.1	21.5	10.1	9.2	18.7	19.9	27.2	29.2	35.8	38.3	37.8	38.3
Minimum	Long years	6.6	1.1	-3.8	-8.2	-11.5	-9.9	-5.9	-0.8	4.1	8.1	11.4	11.5	-11.5
temperature (ºC)	2016-17	5.3	-0.1	-4.9	-9.7	-10.5	-12.0	-1.5	-1.0	5.0	9.0	14.2	13.3	-10.5

S: September,O: October,N: November, D: December, J: January, F: February, Mr: March, A: April, M: May, Jn: June, Jl: July, A: August

Data were obtained from the General Directorated of Meteorology at Ankara, Turkey

Table 2 shows soil samples features belonging to the experimental area. The soil analysis during 2016, performed out of the soil taken at a depth of 0-20 and 21-40 cm showed low organic matter (1.26%), in alkaline (pH 7.99), lime (7.3%), and clay-loamy soils at Yenimahalle.

Table 2. Soil samples features belonging to the experimental area

Location	Year	Depth (cm)	Texture	Saturation percentage (%)	Total salt (%)	pH (%)	Lime (%)	Phosphorus (P)	Potassium (K)	Organic Substance (%)
Ankara	2016	0-20	Clay loamy	56.0	0.014	8.00	7.5	8.3	174.7	1.15
		20-40	Clay loamy	62.0	0.015	7.97	7.0	9.1	187.8	1.36
		Average		59.0	0.015	7.99	7.3	8.7	181.2	1.26

Data were ontained from the Soil Fertilizer and Water Resources Institute

Each genotype was planted as two row, 3 m plots with 30 cm row spacing unreplicated. Gas chromatgraphy (GC) analysis: The fatty acid methyl esters (FAME) were performed with Shimadzu AOC-20i gas chromatgraphy equipped with a flame ionization detector. Analyses were conducted using Teknokroma capillary calumn 100 m x 0.25 mm x 0.2 µm. The column temperature was programmed to waiting 140 °C for 5 min., increase 4 °C/min and was kept at 240 °C for 20 min. The injector and detector temperature were 250 °C using helium, air and hydrogen. 15 major fatty acids palmitic (C16:0), stearic (C18:0), arachidic (C20:0), behenic (C22:0), lignoceric (C24:0), palmitoleic (C16:1), oleic (C18:1n9c), eicosenoic (C20:1), erusic (C22:1n9t), nervonic (C24:1), linoleic (C18:2n6c), linolenic (C18:3n6), Cis-11,14-eicosadienoic acid (C20:2), Cis-11, 14, 17-eicosatrienoic acid (C20:3n3) and cis-13, 16-docosadienoic acid (C22:2) were identified as percentage of total fatty acids.

3. RESULTS AND DISCUSSION

The results indicated that the major saturated fatty acids like palmitic and stearic acids along with mono and polyunsaturated fatty acids like oleic, eicosenoic, erucic, linoleic and linolenic acids were found in the oil. Number of genotypes of mustard were taken for fatty acid analysis. The fatty acid composition was variable among 57 different genotypes. Overall a total of 15 fatty acid components were detected (Table 3). The saturated fatty acids mainly include palmitic acid (C16:0; 2.11% in BNa-14-Turkey, Tekirdağ of *B. napus*-4.38 % in BA2-Turkey, Tokat of *B. arvensis*); stearic acid (C18:0; 1.17 % in BR-A48-Turkey, Tekirdağ of *B. arvensis*-2.68 % in BA7-Turkey, Şereflikoçhisar of *B. arvensis*), arachidic acid (C20:0; 0.48 % in BAI-B58-Israel of *B. alba*-1.53 % in BN-A21-Turkey, Ankara of *B. nigra*), behenic acid (C22:0; 0.00% in BAI-Russia of *B. alba*-1.65% in BN-B53-Italy of *B. nigra*) and lignoceric acid (C24:0; 0.25 in BAI-Russia of *B. alba*-1.00% in BN-A16-Turkey of *B. nigra*). Monounsaturated fatty acids included palmitoleic acid (C16:1; 0.10% in BR-A48-Turkey, Tekirdağ of *B. rapa*-0.49% in BA2-Turkey, Tokat of *B. arvensis*), oleic acid (C18:1; 7.42% in BJ-B18-United States, California of *B. juncea*-41.58% in BAI-Russia of *B. alba*), eicosenoic acid (C20:1; 5.30% in BAI-Russia of *B.alba*-17.11 % in BA7-Turkey, Şereflihoçhisar of *B. arvensis*), erusic acid (C22:1; 20.63% in BAI-Russia of *B. alba* -47.87 % in BNa-A14-Turkey, Tekirdağ of *B. napus*) and nervonic acid (C24:1; 1.06% in BN-Ucraina of *B. nigra*-2.37% in BJ-B20-Russian of *B. juncea*). Polyunsaturated fatty acids included linoleic acid (C18:2; 9.61% in BJ-C2-India of *B. juncea*-25.11% in BJ-A20-India of *B. juncea*), linoleic acid (C18:3; 4.94 % in BA7-Turkey, Şereflikoçhisar of *B. arvensis*-14.70 % in BJ-A20-India of *B. juncea*), cis-11, 14 eicosadienoic acid (C20:2; 0.31 % in BJ-C2-India of *B. juncea*-1,15 % in BN-B53-Italy of *B. nigra*), cis 11, 14, 17 eicosatrienoic acid (C20:3; 0.00 % in BJ-B18-United States, California, BJ-A20-India, BJ-A99-India of *B. juncea;* BR-A48-Turkey, Tekirdağ of *B.rapa*; BA3-Turkey, Isparta of *B.arvensis*; BAI-B58-Israel, BAI-Russian of *B.alba*; BA7-Turkey, Şereflikoçhisar of *B. arvensis*-1.68 % in BA-Turkey, Tokat of *B. arvensis*) and cis-13, 16-docosadienoic acid (C22:2;

0.00% in BA7-Turkey-Şereflikoçhisar of *B. arvensis*; BAI-Russian of *B. alba*-1.92% in BJ-B18-United States, California *B. juncea*) (Table 3). Erusic acid (C22:1), oleic acid (C18:1) and linoleic acid (C18:2) were determined to be dominant fatty acids among all genotypes. The maximum total saturated fatty acids percent was observed in BA7-Turkey, Şereflikoçhisar of *B. arvensis* (10.18%) while the minimum oil percent was noted in BR-A48-Turkey, Tekirdağ of *B. rapa* genotype (5.25%). The maximum total monounsaturated fatty acid percent was obtained in BA7-Turkey, Şereflikoçhisar of *B. arvensis* (75.23%) while the minimum fatty oil percent was noted in BJ-A20-India of *B. juncea* (50.90%). The maximum total polyunsaturated fatty acid percent was obtained in BJ-A20-India of *B.juncea* (41.56%) while the minimum in BA7-Turkey, Şereflikoçhisar of *B. arvensis* (15.27%). Seed oil quality and utility largely depend on fatty acid composition. Fatty acids exhibit rich variety, exhibiting variable fatty acid composition and content across species (and even across varieties). Therefore, fatty acid composition and content profiles can be used as fingerprints to identify useful biological resources, in addition to their current use for oil authentication [16, 17]. The differences between fatty acids among genotypes were very obvious. Especially oleic acid (7.42 to 24.54%), linoleic acid (5.81 to 23.97%) and erusic acid (20.87 to 50.25%) were the most prominent. These differences in the fatty acid profile may be due to the variations in phenotypic or environmental conditions as well as the genetic background of the experimental material [18]. Both genotypic and phenotypic conditions (environmental parameters) determine the amount and quality of fatty acids in plant [15]. Maturation and harvest time of plants drought ere found to have significant effects on the fatty acid composition of the seeds [19- 23]. It is effective to choose the accessions with high monounsaturated fatty acid (MUFA) content as optimal germplasm resources for biodiesel production [17, 24].

The significant variability in seed oil contents among *Brassica* genotypes showed their potential for use in future breeding programs and supported the findings of [25-27] who also recorded great variations in seed oil contents among different *Brassica* species [28].

Table 3. Fatty acid composition of mustard genotypes belong to *Brassica species*

Genotypes	Palmitic asit (C16:0)	Stearic asit (C18:0)	Arachidic asit (C20:0)	Behenic acid (C22:0)	Lignoceric acid (C24:0)	ΣSFA	Palmitoleic acid (C16:1)	Oleic asit (C18:1n9c)	Eicosenoic asit (C20:1)	Erusic asit (C22:1n9t)	Nervonic asit (C24:1)	ΣMUFA	Linoleic acid (C18:2n6c)	Linolenic acid (C18:3n6)	Cis-11,14-eicosadienoic acid (C20:2)	Cis-11,14,17-eicosatrienoic acid (C20:3n3)	cis-13,16-docosadienoic acid (C22:2)	ΣPUFA
Brassica juncea																		
BJ-A2-Turkey, İzmir	2.97	1.97	0.95	0.51	0.33	7.02	0.18	22.24	11.52	21.69	1.15	56.78	21.65	12.77	0.88	0.36	0.42	36.21
BJ-A3-Turkey	2.85	1.89	0.94	0.55	0.32	6.67	0.18	20.79	11.09	23.89	1.22	57.17	20.87	13.31	0.92	0.38	0.47	36.16
BJ-A4-Turkey	3.25	1.79	0.99	0.55	0.34	7.08	0.18	22.08	11.73	22.50	1.09	57.57	21.15	12.70	0.88	0.37	0.40	35.36
BJ-A5-Turkey, Tekirdağ	3.16	1.79	0.94	0.49	0.29	6.74	0.18	20.90	11.91	23.21	1.21	57.40	20.83	13.41	0.96	0.35	0.44	35.80
BJ-A6-Turkey, Kayseri	3.20	1.85	0.93	0.51	0.32	6.99	0.16	20.52	11.26	23.52	1.27	56.73	21.02	13.64	0.96	0.41	0.47	36.29
BJ-A7-Turkey, Tekirdağ	3.20	1.92	0.95	0.52	0.34	7.01	0.18	21.01	11.09	23.30	1.32	56.89	21.31	13.17	0.93	0.38	0.45	36.11
BJ-A8-Turkey	2.24	1.20	0.81	1.33	0.74	6.32	0.12	11.06	6.01	46.23	1.96	65.37	14.31	10.85	0.69	1.12	1.34	28.31
BJ-A9-Turkey, Tekirdağ	2.86	1.72	0.98	0.77	0.43	6.83	0.16	18.11	10.39	30.11	1.33	60.10	18.92	12.28	0.87	0.55	0.64	33.07
BJ-A10-Turkey, Kırklareli	3.21	1.87	1.03	0.80	0.50	7.40	0.19	17.32	10.28	29.81	1.41	59.00	19.92	11.54	0.89	0.53	0.73	33.61
BJ-A11-Turkey, Edirne	3.51	2.10	0.97	0.55	0.35	7.79	0.22	19.55	11.04	22.53	1.29	54.63	22.17	13.38	0.96	0.43	0.52	37.58
BJ-B5-Turkey, Tekirdağ	3.28	1.96	0.98	0.53	0.35	7.10	0.19	20.56	11.66	22.13	1.22	55.75	21.92	13.46	0.90	0.44	0.44	37.15
BJ-B6-India	2.69	1.32	0.92	0.99	0.58	6.49	0.16	15.41	9.36	36.21	1.54	62.68	17.72	11.38	0.84	0.00	0.89	30.83
BJ-B7-India, Rajasthan	2.68	1.42	0.96	1.19	0.64	6.88	0.17	14.27	8.13	39.19	1.62	63.36	17.81	9.30	0.81	0.79	1.07	29.77
BJ-B8-Pakistan, Punjab	2.65	1.46	0.90	1.38	0.72	7.11	0.19	12.84	6.71	41.06	1.82	62.61	17.88	9.28	0.74	1.03	1.35	30.28
BJ-B13-China	3.78	1.87	1.00	0.84	0.48	7.98	0.28	14.47	9.09	27.79	1.59	53.23	22.64	13.35	1.09	0.73	0.99	38.80
BJ-B15-Pakistan	2.65	1.63	0.91	1.32	0.69	7.18	0.17	13.56	9.91	38.64	1.73	64.01	15.41	10.96	0.83	0.69	0.91	28.80
BJ-B16-Canada	3.10	1.87	1.02	0.74	0.38	7.11	0.19	18.11	11.69	29.09	1.22	60.31	18.87	12.01	0.93	0.11	0.56	32.48
BJ-B17-Canada	3.42	1.59	0.91	1.03	0.54	7.49	0.25	13.66	8.27	33.83	1.61	57.62	20.08	11.89	0.99	0.80	1.13	34.88
BJ-B18-United States, California	3.79	1.37	0.98	1.19	0.99	8.30	0.15	7.42	5.33	42.06	2.37	57.33	18.04	13.43	0.98	0.00	1.92	34.37
BJ-B20-Russian Federation	2.70	1.57	0.90	0.84	0.42	6.42	0.15	19.46	9.57	31.56	1.40	61.73	19.58	10.23	0.91	0.47	0.69	31.86
BJ-B22-China, Xizang	2.83	1.83	0.97	1.15	0.52	7.39	0.19	14.59	8.41	36.15	1.62	60.97	19.22	9.79	0.95	0.73	0.95	31.64
BJ-B23-Pakistan	2.76	1.22	0.93	1.06	0.61	6.58	0.17	14.80	8.85	36.33	1.72	61.88	18.58	10.22	0.88	0.77	1.10	31.55
BJ-B24-Germany	3.31	1.65	0.92	1.00	0.49	7.36	0.22	15.29	8.22	31.84	1.57	57.14	21.24	11.54	0.98	0.74	1.03	35.51
BJ-B25-Germany	2.78	1.61	1.01	0.72	0.42	6.53	0.15	19.81	11.55	28.65	1.33	61.50	18.44	11.81	0.85	0.47	0.56	31.97
BJ-B27-United States, Minnesota	3.63	1.90	1.00	0.63	0.41	7.61	0.20	20.02	12.22	23.95	1.24	57.63	21.47	11.34	0.99	0.43	0.55	34.77
BJ-B28-United States, Minnesota	3.33	1.68	0.96	0.68	0.39	7.09	0.19	18.78	10.67	26.23	1.27	57.15	21.21	12.50	0.94	0.49	0.63	35.77
BJ-C1-India	2.49	1.32	0.95	1.13	0.55	6.44	0.16	11.13	6.92	44.09	1.73	64.03	15.10	11.52	0.80	0.95	1.16	29.53
BJ-C2-India	2.71	1.40	0.98	0.54	0.28	5.91	0.11	24.54	9.27	37.41	2.27	73.61	9.61	9.56	0.31	0.63	0.37	20.48
BJ-A20-India	4.07	2.24	0.92	0.47	0.33	8.03	0.22	16.16	9.78	23.00	1.74	50.90	25.11	14.70	1.08	0.00	0.67	41.56
BJ-A99-India	3.27	1.81	0.90	0.43	0.30	6.71	0.14	21.03	11.09	24.21	1.38	57.85	19.95	14.07	1.01	0.00	0.42	35.45
BJ-AK-Turkey, Konya	2.95	1.57	0.95	0.45	0.30	6.22	0.19	21.66	11.75	21.16	1.36	56.11	21.97	11.85	0.94	0.37	0.39	35.39
Maximum	4.07	2.24	1.02	1.32	0.99	8.30	0.28	24.54	12.22	44.09	2.37	73.61	25.11	14.70	1.09	0.95	1.92	41.56
Minimum	2.49	1.22	0.90	0.43	0.28	5.91	0.11	7.42	5.33	21.16	1.22	50.90	9.61	9.56	0.31	0.00	0.37	20.48
Average	3.15	1.65	0.95	0.84	0.47	7.08	0.18	16.73	9.56	31.53	1.60	59.59	19.21	11.81	0.91	0.49	0.83	33.22
Brassica rapa																		
BR-A23-Turkey	2.50	1.31	0.92	0.94	0.59	6.35	0.14	14.39	9.29	37.42	1.82	63.06	16.13	11.76	0.91	0.80	0.98	30.59
BR-A24-Turkey, Balıkesir	2.65	1.32	0.88	0.90	0.47	6.23	0.14	15.34	8.95	36.84	1.58	62.84	17.28	11.24	0.82	0.74	0.85	30.93
BR-A32-Turkey, Balikesir	2.52	1.24	0.87	1.34	0.76	6.73	0.20	11.08	5.99	44.42	2.01	63.70	16.40	9.77	0.72	1.28	1.40	29.57
BR-A47-Turkey, Bursa	3.14	2.08	1.02	0.56	0.36	7.17	0.19	21.77	11.48	21.59	1.11	56.15	21.34	13.72	0.85	0.39	0.39	36.68
BR-A48-Turkey, Tekirdağ	2.13	1.17	0.80	0.83	0.33	5.25	0.10	12.78	8.23	47.54	1.50	70.15	13.16	10.01	0.69	0.00	0.73	24.60
BR-B30-India	2.31	1.52	0.95	1.20	0.74	6.73	0.15	12.04	7.44	43.67	1.80	65.10	15.03	10.44	0.78	0.77	1.14	28.17
Maximum	3.14	2.08	1.02	1.34	0.76	7.17	0.20	21.77	11.48	47.54	2.01	70.15	21.34	13.72	0.91	1.28	1.40	36.68
Minimum	2.13	1.17	0.80	0.56	0.33	5.25	0.10	11.08	5.99	21.59	1.11	56.15	13.16	9.77	0.69	0.00	0.39	24.60
Average	2.59	1.42	0.90	0.91	0.50	6.35	0.15	15.07	8.79	37.56	1.60	63.18	16.86	11.30	0.80	0.64	0.87	30.47

Table 3. Continues

Brassica napus																	
BNa-A13-Turkey, Samsun	*3.29*	*1.55*	*0.97*	*0.70*	*0.40*	*7.31*	*0.17*	*17.99*	*11.09*	*28.33*	*1.35*	*58.93*	*20.16*	*11.85*	*1.01*	*0*	*0.61*	*33.76*
BNa-A14-Turkey, Tekirdağ	*2.11*	*1.36*	*0.79*	*0.82*	*0.33*	*5.40*	*0.12*	*11.65*	*7.99*	*47.87*	*1.61*	*69.24*	*12.79*	*10.43*	*0.66*	*0.67*	*0.81*	*25.36*
Maximum	*3.29*	*1.55*	*0.97*	*0.82*	*0.40*	*7.31*	*0.17*	*17.99*	*11.09*	*47.87*	*1.61*	*69.24*	*20.16*	*11.85*	*1.01*	*0.67*	*0.81*	*33.76*
Minimum	*2.11*	*1.36*	*0.79*	*0.70*	*0.33*	*5.40*	*0.12*	*11.65*	*7.99*	*28.33*	*1.35*	*58.93*	*12.79*	*10.43*	*0.66*	*0.00*	*0.61*	*25.36*
Average	*2.70*	*1.46*	*0.88*	*0.76*	*0.37*	*6.36*	*0.15*	*14.82*	*9.54*	*38.10*	*1.48*	*64.09*	*16.48*	*11.14*	*0.84*	*0.34*	*0.71*	*29.56*
Brassica nigra																		
BN-A16-Turkey	2.96	1.60	1.42	1.44	1.00	8.50	0.16	13.43	10.07	35.89	1.77	61.32	16.09	12.17	0.99	0.11	0.82	30.18
BN-A17-Turkey	3.08	1.54	1.31	1.31	0.85	8.17	0.17	11.99	9.52	36.61	1.80	60.09	15.63	13.58	1.07	0.58	0.88	31.74
BN-A18-Turkey	3.07	1.50	1.40	1.35	0.92	8.24	0.17	12.35	9.02	37.59	1.81	60.93	15.95	12.51	0.94	0.51	0.92	30.83
BN-A21-Turkey, Ankara	3.28	1.80	1.53	1.27	0.84	8.82	0.19	14.56	9.92	32.90	1.49	59.07	17.16	12.87	0.89	0.51	0.68	32.11
BN-B53-Italy	2.95	1.42	0.82	1.65	0.89	7.73	0.15	10.40	12.07	37.90	1.89	62.50	12.81	14.43	1.15	0.81	0.58	29.77
BN-Ukraine	3.44	2.01	0.58	0.45	0.30	6.78	0.14	21.03	10.61	21.13	1.06	53.97	24.99	13.27	0.65	0.00	0.34	39.25
Maximum	3.44	2.01	1.53	1.65	1.00	8.82	0.19	21.03	12.07	37.90	1.89	62.50	24.99	14.43	1.15	0.81	0.92	39.25
Minimum	2.95	1.42	0.58	0.45	0.30	6.78	0.14	10.40	9.02	21.13	1.06	53.97	12.81	12.17	0.65	0.00	0.34	29.77
Average	3.13	1.65	1.18	1.25	0.80	8.04	0.16	13.96	10.20	33.67	1.64	59.65	17.11	13.14	0.95	0.42	0.70	32.31
Brassica arvensis																		
BA1-Turkey, Ankara	3.16	1.73	0.88	1.84	0.86	8.47	0.16	13.34	12.50	35.65	1.67	63.32	14.41	11.58	0.99	0.72	0.51	28.21
BA2-Turkey, Tokat	4.38	2.54	0.89	1.34	0.70	9.86	0.49	13.67	12.62	28.68	1.71	57.16	17.28	12.36	1.11	1.68	0.54	32.98
BA3-Turkey, Isparta	2.73	1.69	0.85	1.62	0.93	7.81	0.12	11.26	13.60	38.60	1.90	65.48	12.68	12.40	1.13	0.00	0.49	26.70
BA4-Turkey, Haymana	2.79	2.34	0.99	0.45	0.27	6.95	0.15	23.48	12.95	21.56	1.23	59.37	19.44	12.75	0.87	0.31	0.30	33.68
BA5-Turkey, Kazan	3.08	2.06	0.82	1.23	0.66	8.03	0.11	15.06	15.91	31.47	1.48	64.02	14.26	12.25	1.02	0.10	0.31	27.95
BA6-Turkey, Şanlıurfa	2.25	1.45	0.86	1.23	0.71	6.51	0.17	11.79	6.61	44.83	1.99	65.38	14.51	10.63	0.72	0.99	1.26	28.11
BA7-Turkey, Şereflikoçhisar	4.37	2.68	1.02	1.29	0.82	10.18	0.16	16.70	17.11	39.72	1.54	75.23	9.80	4.94	0.53	0.00	0.00	15.27
BA-B72-Israel	3.13	1.99	0.97	0.54	0.35	6.98	0.21	22.34	11.52	20.98	1.09	56.14	22.20	13.04	0.85	0.38	0.41	36.88
BA-B73-Israel	3.11	1.60	0.96	0.81	0.44	6.91	0.22	16.12	10.70	30.36	1.45	58.85	19.68	12.36	0.98	0.54	0.68	34.24
BA-B76-Israel	3.12	1.80	0.97	0.68	0.40	6.95	0.22	19.23	11.11	25.67	1.27	57.50	20.94	12.70	0.92	0.46	0.55	35.56
Maximum	4.38	2.68	1.02	1.84	0.93	10.18	0.49	23.48	17.11	44.83	1.99	75.23	22.20	13.04	1.13	1.68	1.26	36.88
Minimum	2.25	1.45	0.82	0.45	0.27	6.51	0.11	11.26	6.61	20.98	1.09	56.14	9.80	4.94	0.53	0.00	0.00	15.27
Average	3.22	2.00	0.92	1.11	0.61	7.95	0.22	16.48	12.36	31.94	1.53	62.82	16.43	11.08	0.90	0.57	0.53	29.31
Brassica alba																		
BAl-B58-Israel	3.23	1.67	0.98	0.51	0.35	6.90	0.16	23.15	11.99	21.84	1.18	58.31	21.29	12.24	0.88	0.00	0.38	34.79
BAl-Russia	3.45	2.02	0.48	0.00	0.25	6.20	0.16	41.58	5.30	20.63	1.30	68.97	12.89	11.96	0.00	0.00	0.00	24.85
Maximum	3.45	2.02	0.98	0.51	0.35	6.90	0.16	41.58	11.99	21.84	1.30	68.97	21.29	12.24	0.88	0.00	0.38	34.79
Minimum	3.23	1.67	0.48	0.00	0.25	6.20	0.16	23.15	5.30	20.63	1.18	58.31	12.89	11.96	0.00	0.00	0.00	24.85
Average	3.34	1.85	0.73	0.26	0.30	6.55	0.16	32.37	8.65	21.24	1.24	63.64	17.09	12.10	0.44	0.00	0.19	29.82

4. CONCLUSION

To know the fatty acid composition of *Brassica* species oil makes oil production possible for special using purposes. Genotypes belonging to six species of mustard evaluated in our study exhibited an important level of diversity for fatty acid compositions. Oleic acid (7.42 to 24.54%), linoleic acid (5.81 to 23.97%) and erusic acid (20.87 to 50.25%) were the most prominent. The result of our study showed that *Brassica* genotypes have significant potential for use in future breeding programs due to their significantly differed fatty acid compositions. While BJ-A20-India of *Brassica juncea* has maximum percent of total polyunsaturated fatty acid; BA7-Turkey, Şereflikoçhisar of *Brassica arvensis* was selected as promising genotype in terms of saturated and monounsaturated fatty acid contents. Especially the high erucic acid genotypes can be used for industrial applications. Furthermore these species are crossed to cultivated species for special using purposes and can be used as a new source. Therefore, an extensive work on these genotypes could suggest them in future breeding programs under Turkish conditions.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Evaluation of Antioxidant, Antimicrobial and Antimutagenic Activity with Irritation Effects of *Ceramium rubrum* (Red Algae) Extract

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Abstract: This study was conducted to evaluate antioxidant, antimutagenic and antimicrobial activities as well as irritation/toxicity efficacy of n-hexane, chloroform and methanol extracts of the red algae *Ceramium rubrum* C. Agardh (1881). While spectrophotometric methods were used for the determination of total phenol and flavonoid content, antioxidant activity was evaluated by DPPH assay. Antimicrobial and antimutagenic efficacy of extracts were determined by MIC method and Ames test, respectively. Also, HET-CAM test was used for irritation/toxicity of extracts. While methanol extract was found to have higher total flavonoid and phenolic contents than the other extracts and hexane showed higher antioxidant activity than other extracts. All extracts exerted moderate antimicrobial activity against tested microorganisms as 64–256 µg/mL. However, extracts of the algae did not show any mutagenicity or irritation effect even at the highest concentration.

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1. INTRODUCTION

Plant secondary metabolites or natural products are active chemical compounds that are not directly involved in the vital activities of an organism other than the primary metabolites, often produced for protection during environmental interactions. Secondary metabolites play important roles in defense against microbial pathogens, predators and abiotic stresses (drought, salinity and UV exposure), communication with other organisms and other ecological functions. This active compounds have been intensively studied since the 19th century, and especially macroalgae represents for about 20% of the compounds reported from the marine area [1].

Unlike other species, macroalgae which have dispersions in almost every part of the world have developed a superior protection mechanism to survive against many factors, particularly coastal algae, UV, wind, tides, sudden temperature changes, marine pollution and aquatic life thanks to their secondary metabolites. These active chemicals have shown many useful

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biological activities such as antioxidant, antiviral, antimicrobial, antifouling, anticoagulant and anticancer for human health [2].

Especially, antioxidant studies have been one of the most popular research areas of algae. Numerous studies have shown that algae can contribute to the system in different ways by preventing, delaying or eliminating of free radicals as an antioxidant resource. Algae are rich in metabolites such as carotenoids (α- and β-carotene, fucoxanthin, astaxanthin), catechins, gallate, florotannins and tocopherols as well as reactive antioxidant molecules such as glutathione and ascorbate [3]. Previous studies have shown that while pireniltoluqinones obtained from *Cystoseira crinita* brown algae have potent radical scavenging activity [4], fluorotannins from *Ecklonia stolonifera* have this effect [5].

Commonly used antibiotics are less effective against certain diseases because of their different chemical structures and mechanism of action, toxin production and resistance to drugs. For this reason, the discovery of new antibiotics has a vital role. Algae extracts have been the subject of many studies in terms of antimicrobial activity and have been shown to inhibit the growth of many gram-negative and gram-positive bacterial pathogens [6,7]. Consequently, antimicrobial compounds such as oil, lipophilic compound, phenolics, alkaloids, polyphenols, halogenates and isophorone have been described [8].

The genomes of all living organisms are continuously exposed to damage by endogenous and exogenous processes. This damage leads to many diseases in the organism. The antimutagenic properties of algae phytochemicals have not been studied as intensively as in many other degenerative diseases, but effective results have been obtained. Photosynthetic pigments isolated from some algae species have been identified as chemical protection agents. In a study of the antimutagenic effects of different algae species, it was found that *Caulerpa sertularioides* and *Spyridia filamentosa* exhibit high antimutagenic activity against *Salmonella typhimurium* TA98 and TA100 strains [9].

In the current study, *in vivo* irritation/toxicity effects as well as the effects of antioxidant, antimutagenic and antimicrobial activities of the n-hexane (Hex), chloroform (Chl) and methanol (Met) extracts of *Ceramium rubrum* (Hudson) (red algae) were first evaluated.

2. MATERIALS and METHODS

2.1. Seaweed material

C. rubrum was collected at a depth of 1-2 m, in a region of high light intensity, from the coastline of Urla, Izmir, in April 2013. Voucher specimen (*C.rubrum*) (number: 41324) were deposited in the Hydrobiology Laboratory of Ege University, Faculty of Science, Department of Biology. The samples were washed three times with tap water to remove salt, epiphytes and sand attached to the surface, then carefully rinsed with fresh water, and maintained in a refrigerator at -20°C.

2.2. Preparation of extracts

Algal samples were dried at 45°C. Powdered material (100 g) was extracted subsequently with n-hexane (Hex) (purity ≥ 99.9%, Merck, Darmstadt, Germany), chloroform (Chl) (purity 99.0-99.4%, Merck, Darmstadt, Germany) and methanol (Met) (purity ≥ 99.9%, Merck, Darmstadt, Germany) at room temperature in an ultrasonic bath (3 x 1 L of each solvent, 40°C, 24 h). The combined extracts were evaporated separately for each solvent under reduced pressure by using Rotary evaporator (Heidolph300 LabroRota, Germany) to dryness and were obtained 102 mg, 108 mg and 2104 mg, respectively, from the Hex, Chl and Met extracts.

2.3. Determination of total phenolic and flavonoid contents

Total phenolic content was determined by Folin-Ciocalteu method [10]. Briefly, 0.1 mL of extracts (0.5 mg/mL and 1 mg/mL) were mixed with 2.8 mL deionized water. This solution was mixed with 2 mL 2% sodium carbonate and 0.1 mL of 0.1 N Folin-Ciocalteu reagent. After incubation at room temperature for 30 min, the absorbance of the mixture was measured at 750 nm against a deionized water blank on a UNICAM 8625 UV/Vis spectrophotometer. Gallic acid was chosen as a standard. The data expressed as milligram gallic acid equivalents.

Total flavonoid content was determined by the aluminum chloride colorimetric method described by [11]. 0.5 mL of the extracts (0.5 mg/mL and 1 mg/mL) were mixed with 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride and 2.8 mL of distilled water. The mixture was kept at room temperature for 30 min and the absorbance was recorded at 415 nm with the help of UNICAM 8625 UV/Vis spectrophotometer. Quercetin equivalent (QE) was chosen as a standard. The amount of flavonoid was expressed as QE.

2.4. DPPH radical scavenging activity

The capacity of the *C.rubrum* extracts to scavenge the DPPH radical was measured according to Blois method with a slight modification [12]. 1 mL of the extracts (0.5 mg/mL and 1 mg/mL) were added to a 4 mL of a 0.004% methanol solution of DPPH. After 30 min of incubation in dark at room temperature, the absorbance was read against a blank at 517 nm. Inhibition (I) of a free radical by DPPH in percent I (%) was calculated as follows:

$$\text{Inbition \%} = 100 \times (A_{blank} - A_{sample} / A_{blank})$$

Where, A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. Tocopherol was used for comparison.

2.5. Determination of antimicrobial activity

In vitro antimicrobial studies were carried out against eight bacteria strains, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538/P, *Streptococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, *Klebsiella pneumonie* ATCC 13883, *Bacillus cereus* ATCC 7064, *Pseudomonas aeruginosa* ATCC 9027, which were obtained from the Microbiology Department of the Faculty of Science, Ege University.

Determination of the minimum inhibitory concentration (MIC) was carried out according to the method described by NCCLS [13] with some modifications. Dilution series of the extracts were prepared in test tubes and then transferred to the broth in 96-well microtiter plates. Final concentrations in the medium were 512–0.5 μg/mL. Before inoculation of the test organisms, the bacteria strains were adjusted to 0.5 McFarland standards (Thermo Oxoid Remel, Basingstoke, Hampshire, UK) and diluted 1:100 (v/v) in Mueller–Hinton broth (Oxoid, Basingstoke, Hampshire, UK). Plates were incubated at 35 °C for 18–24 h. All tests were performed in broth and repeated twice. The MIC was defined as the lowest concentration that showed clear against a black background (no visible growth). Samples from clear wells were subcultured by plotting on to Mueller–Hinton agar. Gentamycin and Ampicillin were used as standard antibacterial agents. All antibiotics were purchased from Sigma-Aldrich Co. and dilutions were prepared at concentrations ranging from 128 to 0.25 μg/mL in microtiter plates.

2.6. Bacterial reverse mutation assay (Ames test)

The assay was performed in two histidine-requiring strains of Salmonella typhimurium, tester strains TA98 and TA100 according to Maron and Ames [14]. Two separate experiments

were performed, using triplicate plates, in the presence and absence of metabolic activation by an Aroclor 1254 (Sigma-Aldrich Co.)-induced (500 mg/kg body weight) Swiss albino mice liver post-mitochondrial fraction (S9). The post mitochondrial fraction was used at a concentration of 10 % v/v in the S9 mixture (metabolic activation). Negative controls and positive controls were tested in all strains in both experiments. Dimethyl sulfoxide (DMSO) was used as a reference negative control. Benzo[a]pyrene (Sigma-Aldrich Co.) (5 µg/plate), 2-nitrofluorene (Sigma-Aldrich Co.) (5 µg/plate) and sodium azide (Sigma-Aldrich Co.) (10 µg/plate) were used as positive controls. Fresh cultures of tester strains were grown to approximately 109 cell/mL in 5 mL Oxoid nutrient broth (Oxoid). The cultures were incubated for 10–12 h at 37 °C in a gyratory incubator in order to insure adequate aeration. The strains were periodically raised from a single colony to check the genetic markers.

The extracts with the amounts of 1, 2.5 and 5 mg per plate were tested on TA98 and TA100. All positive and negative controls as well as the extracts were prepared in the absence (0.5 mL/plate) and presence of S9 mixture (0.5 mL/plate). The mixtures containing positive and negative controls and extracts and 0.1 mL of overnight bacterial cultures with or without S9 was vortexed and pre-incubated at 37 °C for 30 min. It was then plated in 2 mL of top agar on glucose-supplemented minimal agar. After 48 h of incubation at 37 °C, revertant colonies (his^+) were counted. The results of the test were presented as the mean ± SEM. Comparisons were made between control and treatment groups using one-way analysis of variance (ANOVA) followed by Dunnett's test. Values of $p \leq 0.05$ were regarded as statistically significant.

2.7. HET-CAM (Hen's egg test chorioallantoic membrane) irritation test

The toxicity and/or irritation effects of each algal extracts revealed by using a chorioallantoic membrane model on fertilized hen eggs. *In vivo* irritation effects of samples was carried out on fertile Leghron chicken eggs weighing 50-60 gr obtained from commercial sources (Lezita, İzmir, TURKEY) by using HET-CAM method modified of [15]. Fertilised hens' eggs were placed into an incubator with conveyor rotation system at 37±1°C and 80±2% humidity for 7 days. On day 7, the eggs were opened on the snub side sucked off through a hole on the pointed side and then a round piece of shell (3-4 cm diameter) was removed carefully with forceps. Then, the inner membrane carefully removed with forceps, without injury to the blood vessel. After that, 300 µl of the freshly prepared sample at 0.5 and 1 mg/ml concentration that dissolved in DMSO (0.05%) (0.5 and 1 mg/ml) was applied to the CAM. The irritation severity (IS) for a period of up to 5 min was scored as:

$$IS = [(301\text{-}h) \times 5]/300 + [(301\text{-}l) \times 7]/300 + [(301\text{-}c) \times 9]/300$$

Where, h is the time of vascular hemorrhage occurred; l is the time of first vascular lysis occurred; and c is the time of first vascular coagulation occurred. Irritation classification based on IS: 0.0–0.9, non-irritation; 1.0–4.9, slight irritation; 5.0–8.9, moderate irritation; and 9.0–21.0, severe irritation. Also, 0.9% NaCl as negative control and 0.1N NaOH as positive control at the concentration of 300 µL were also tested. For every test compound, 5 eggs were utilized. All samples were tested in triplicate at different times.

3. RESULTS and DISCUSSION

Algae which constitute the primary source of marine and freshwater ecosystems have been intensively studied in many pharmacological research as well as consumption as a food supplement. This study was concluded to reveal the biological activities as well as *in vivo* toxicity of *Ceramium rubrum* extracts collected from the Aegean coast.

Antioxidant agents play a vital role in a wide spectrum against various diseases. However, the worrying side effects of synthetic originated products have increased interest in natural origin sources. Previous studies revealed that algae metabolites were effective via metal chelation, scavenging and inhibition of free radical. This activity of the algae is provided by different metabolites such as polysaccharides, polyunsaturated fatty acids, fluorotannins and other phenolic compounds and carotenoids. Similarly, in studies evaluating the antioxidant capacity of algae samples, it has been reported that phenolic contents have significant contributions to antioxidant activity [16,17]. Our results showed that Met extract of *C. rubrum* had higher total phenolic and flavonoid contents than the other extracts as 5.42 mg GAE/g and 0.541 mg QE/g, respectively (Table 1). However, according to antioxidant activity in Table 1, exhibited that Hex extract showed higher antioxidant effective than other extracts. It has been suggested that the increase in DPPH activity may be due to the presence of lipophilic antioxidants such as tocopherol, color pigment and unsaturated fatty rather than the activity of the phenol and flavonoid compounds in the polar solvent. Horincar et al., [18] also declared that while the methanol extracts of *C.rubrum* had higher phenolic content than hexane similar to our results, methanol had higher efficacy in terms of antioxidant activity unlike our results. These differences can be explained by seasonal differences.

Table 1. Antioxidant activity and content of flavonoid and total phenolic compounds of C.rubrum extract expressed as quercetin (QEmg/g) and gallic acid (GA) equivalents (GAEs; mg of GA/g of extract). (Hex: n-Hexane, Chl: Chloroform, Met: Methanol)

Sample	Concentration (mg/mL)	DPPH Inhibition (%)	α-tocopherol equivalent antioxidant activity values (µg/mL)	IC_{50} (mg/ml)	Flavonoid content (QEmg/g)	Phenolic content (GAE mg /g)
Hex	0.25	3.07±0.02	1.76±0.02	4.83± 0.01	0.08± 0.01	1.28± 0.04
	0.5	2.12±0.01	3.24±0.02			
	1	9.46±0.03	9.32±0.03			
Chl	0.25	4.56±0.01	4.87±0.05	6.72 ± 2.58	0.121± 0.02	2.14± 0.03
	0.5	9.11±0.03	8.14±0.05			
	1	10.26±0.02	10.23±0.06			
Met	0.25	5.21±0.04	4.12±0.02	11.52 ±1.92	0.541± 0.02	5.42± 0.05
	0.5	6.32±0.02	6.43±0.01			
	1	8.21±0.01	10.32±0.02			

Each value is the average of three analyses ± standard deviation. IC 50 values for standart compound equivalent to the Hex, Chl and Met extract were 4.94± 0.02, 6.81±0.02 and 5.83± 0.01, respectively.

Marine algae have developed resistance to the continuous exposure to pathogenic agents such as bacteria, fungi and yeast in the ecosystem. These features of algae have become a focus of attention for use in human health and previously study showed that marine macro algae can prevent the development of some bacteria [19, 20]. As seen Table 2, *C. rubrum* extracts showed moderate antimicrobial activity against assayed Gram (+) and Gram (−) microorganisms by MIC method. Tuney et al. [21] revealed that methanol extracts of the *C.rubrum* has moderate antibacterial activity against *E. coli* and *P. aeruginosa.* Several authors have reported that antimicrobial activity of algae is significantly changed by factors such as seasonality, extraction methods and location [22, 23]. Also, given the suggestions of the Amico et al. [24], presence of different compounds in polar and apolar solvent significantly affect antimicrobial activity.

Table 2. Minimum inhibitory concentration (MIC) results of C. rubrum extracts.

	MIC (µg/ml)			
Microorganisms	Hex	Chl	Met	Gentamycin
Escherichia coli ATCC 23999	256	256	256	1.0
Klebsiella pneumonie CCM 2318	256	256	128	1.0
Pseudomonas aeroginosa ATCC 27853	256	256	256	1.0
Salmonella typhimurium CCM 5445	256	128	256	1.0
Bacillus cereus ATCC 7064	64	128	64	4.0
Staphylococcus aureus ATCC 6538/P	64	256	128	4.0
Enterococcus faecalis ATCC 29212	128	128	64	16.0
Staphylococcus epidermidis ATCC 12228	256	256	128	2.0

The antimutagenic effects of *C. rubrum* were also assessed using *Salmonella typhimurium* TA 98 and TA 100 strains in the absence and presence of S9. Our result reported that the chemical concentrations and his + revertants of the extracts demonstrated no mutagenicity compared to the controls, even 5 mg/mL concentration (Table 3). Similarly, Syad and Kasi [25] reported that red algae species did not lead to any mutagenicity in TA 98, TA 100 and TA 1538 strains.

Table 3. His + revertants in the Bacterial reverse mutation assay of C. rubrum extracts and control groups. (Hex: n-Hexane, Chl: Chloroform, Met: Methanol)

Extracts	Concentration (µg/mL)	his + revertants/plate (X ± SEM)			
		TA98		TA 100	
		- S9	+S9	-S9	+S9
DMSO	10	31±2	42±3	112±6	104±8
	1	42±1	15±1	138±10	112±9
Hex	2.5	54±3	22±4	121±8	142±10
	5	56±2	34±3	110±9	160±12
	1	20±1	20±1	114±7	122±8
Chl	2.5	22±1	42±1	122±8	121±9
	5	38±3	54±2	124±8	158±14
	1	50±2	60±3	191±9	108±8
Met	2.5	41±4	17±2	196±8	107±7
	5	32±1	16±2	121±10	146±9

Chemicals	Concentration/plate (µg)	Strain	S9	his + revertants/plate (X ± SEM)
Benzo[a]pyrene	5	TA98	+	742±22
	5	TA100	+	658±46
2-Nitrofluorene	5	TA 98	-	926±34
Sodium azide	10	TA 100	-	1026±48

HET-CAM irritation/toxicity method was performed to prove toxicity such as lysis, coagulation and haemorrhage in response to the application of algae extracts, and the scores obtained were calculated separately for each algae extract (Figure1). Extracts had no lead to any irritation even at the highest concentrations for a period of up to 5 min. However, while the DMSO and negative group no irritation were determined, the positive control immediately interacting with the CAM resulted in hemorrhage, lysis and coagulation, respectively and cause severe irritation with the IS score of 17.8±0.1 for a period of up to 5 min.

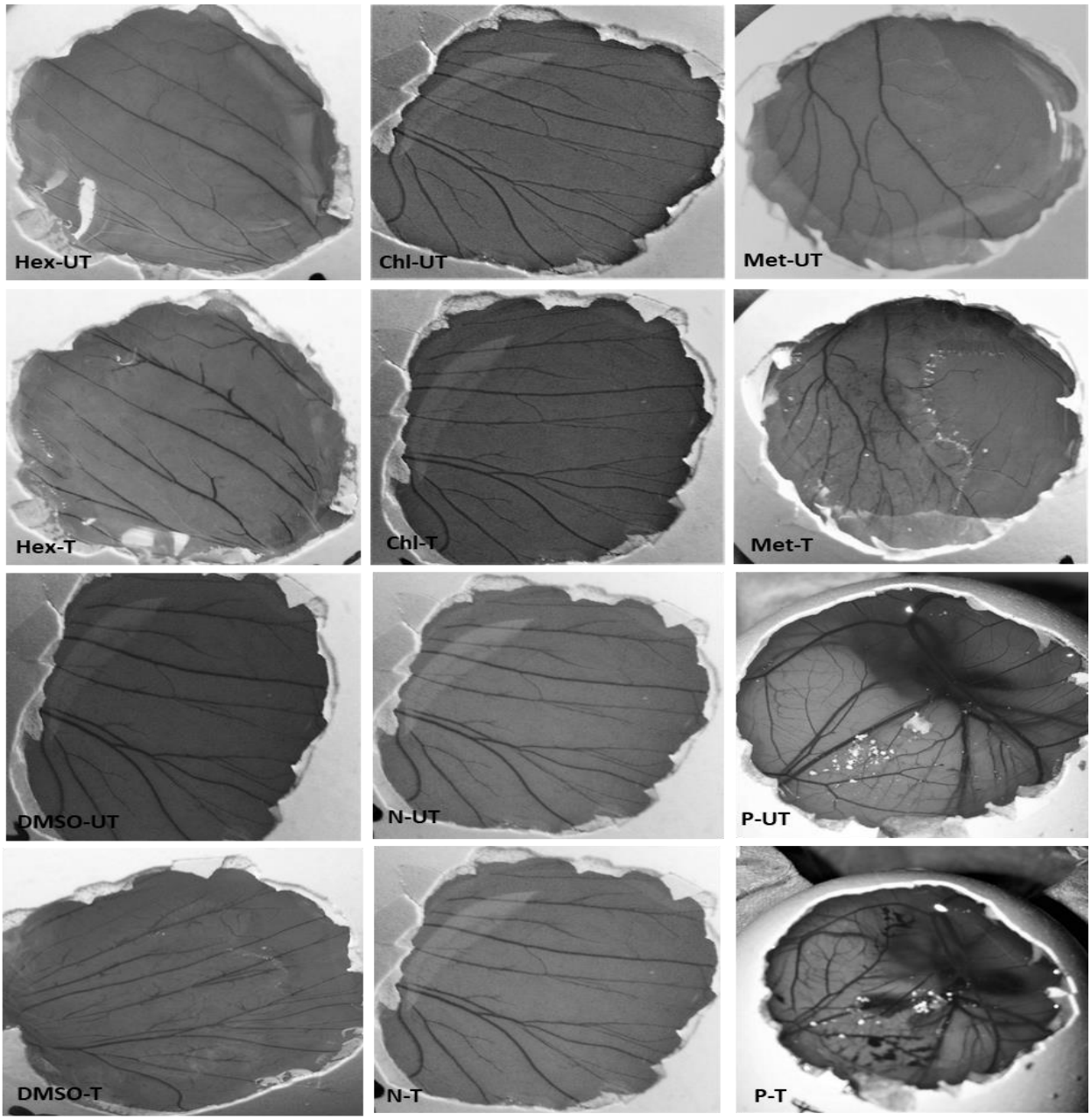

Figure 1. Photographs illustrating of the potential irritation or toxicity on vascularization before exposure to the test samples (0 min) and after the exposure for up to 5 min by HET-CAM assay. Hex: *n*-Hexane, Chl: Chloroform, Met: Methanol, UT: before traetment, T: after treatment, N: Negative control (0.9% NaCl), P: Positive control (0.1 N NaOH)

In present study, antioxidant, antimicrobial and antimutagenic activity as well as irritation/toxicity of *C.rubrum* collected from Urla of Aegean Sea were investigated. Given the increasing interest in studies related to the discovery and potential use of new compounds from marine algae, our results is a preliminary opinion for the synthesis of new active compounds from common algal species in our country. This result may also supported that *C. rubrum* which extracts does not cause any irritation/toxicity can be used as a reliable natural antioxidant compound.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Review Article**

Brief Review on Fungal Endophytes

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Abstract: Fungal endophytes are the critical organisms in the environment residing in the plant tissues without showing any harmful effects on its host life cycle. The finding of fungal endophytes in natural habitat has been insufficient due to some non-sporulating and non-culturable fungal endophytes by traditional method. Several investigations on fungal endophytes in plants have resulted in an excessive knowledge of the group. This review emphasis on the biology of fungal endophytes, their discovery, isolation, identification by morphological and molecular methods, production, purification and structure elucidation of the bioactive compounds.

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1. INTRODUCTION

In present scenario, we are dealing with different emerging diseases in our society due to rehabilitated environment and life style. Several researchers are working on the different emerging diseases to understand and cure them by using different natural and chemical formulations however still many areas are untouched due to poor knowledge, techniques and myths [1,9,12,31,47,57]. One such area is fungal endophytes. As far as fungal endophytes are concerned, researchers failed to explain their potential medicinal applications in emerging disease [19,37,47,57,61]. Some attempts are made to purify fungal endophytes from natural habitats though not effective as compared to other microbes [41,42]. Researchers are trying to isolate new bioactive compounds from newer species of fungal endophytes for medicinal, agricultural and industrial applications though they are unable to explain their origin, pathways

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and mechanism of actions [4,17,27,46,48]. The need of the hour is to encourage researchers to isolate novel bioactive compounds [1,2,4,5,6,7,28,34,45,61].

The finding of novel bioactive compounds involves isolation, structural elucidation and knowing the biosynthetic pathway of the secondary metabolites. This is an area of significant curiosity to scientists due to the structural diversity, complexity and various bioactivities of isolated compounds [7,16,42,59]. Crude natural products have been used unswervingly in drugs manufacturing which forms the basis for deriving semi-synthetic natural products [13,16,50,51].

The objective of this review is to acquaint the researcher with information about the biology of fungal endophytes, and in particular, on the aspects of endophyte research.

2. HISTORY

Ecosystem has fungi as significant module. Certain critical practices in ecosystems such as transportation of nutrients from one environment to other, decomposition, recycling etc. are carried out by fungi. Diverse evaluations suggest that the earth may be hosting more than a million fungal species and approximately 3% have been truly identified [10,46,50] which constitute the second largest cluster of tropical ecosystems of the world. Since they are heterotrophic, they have saprophytic or parasitic association with the hosts. However, in the path of evolution, the fungi developed various types of relationships with them. One of these associations is ‘endophytes’ [10,12,37]. The fossil archives shows that plants are linked with endophytic and mycorrhiza fungi for approximately 400 million years and most doubtless they were associated when plants started colonizing the land, thus they played a very lengthy and vital role in the evolution [10,42,50,51].

In 1866, De Barry introduced the term "endophyte" for those microbes that reside inside the healthy tissues of plants without causing any apparent diseases [12]. Carroll (1986) coined the term 'endophyte' for those organisms that causes asymptomatic infections within the plants [12]. Petrini (1979) elucidated Carroll's definition as a commensalism in plants [37]. Wilson (1995), further expanded endophytes and included both fungi and bacteria as a commensals [39,53]. Stone et al. (2004) have defined endophytes as “infections are inconspicuous, the infected host tissues do not show symptoms at least for transient period and the microbial colonisation can be demonstrated to be internal.” This definition attributes the tenure endophyte to a short-lived grade. It may therefore include an accumulation of microorganisms with dissimilar life history approaches as well as latent pathogens and virulent pathogens in the premature stages of infection [16,47].

Inappropriately, if we take the correct definition, it can include all pathogens at some stage of their development. Therefore, additional characteristic of “not causing apparent harm” as described by Petrini is important as it refers to reflect the lack of macroscopically visible symptoms. Nowadays this term is prolonged as endophytic microorganisms, which includes fungi and bacteria with Actinomycetes, resides intra or intracellularly for their whole or at least part of life cycle [3,4,5,6,7,9].

In 20th century, extensive work has been carried out for the isolation of fungal endophytes from temperate and rainforest region plants because of their largest tropical biodiversity [10,11,13,17,58]. Although most studies are on flowers, leaves and stem as compared to bark, fruits, roots and seeds [5,30,31,41]. In Indian natural therapy and Ayurveda, seeds, bark, fruits and roots have been proved safe and natural remedies for different aliments therefore one can go for bark, fruits, roots and seeds in the view of fungal endophytes purification [2,6,7].

3. ENDOPHYTISM

Darwin proposed the idea that diversity can have tough effects on ecosystem practices. Many hypothetical models and investigational test revealed important functions for diversity including the improvement of primary efficiency, nutrient holding, nutrient flow, water availability and resistance to pathogen invasion [8,9,21]. The diversity of fungal endophyte is confirmed not only in the specificity of the hosts and their morphology, but also in the types of assistances that they offer to the host. Today fungal endophytes have been isolated from plants ranging from large trees, palms, sea grasses and even from lichens. The numbers of strains and species of endophytes vary drastically and generally depend on the concentration of the research study [11,12,13,28,46]**.**

In general, very little information is available about distributions and factors affecting the shape of fungal endophytes at local and regional level [14,15,43,44]. However research has been focussed on relative importance of host and habitat features in shaping local level. As far as tropical endophytes are considered different abiotic factors such as humidity, ultra violet radiation, desiccation and density of leaf litter play a major role [16,18].

It is expected, that the collection of endophytes from a selected plant species is an association of diverse ecological groups of fungal endophytes. For occurrence, fantasy is that at least some of the fungal endophytes endure to survive in the dead leaf tissue as leaf litter decomposers. Long ago, it is reported that some of the foliar endophytes of the mangrove *Rhizophora apiculata* continue to rise in dropped leaves and produce extracellular enzymes, which act on wall polymers of plant cells. Several research endorse that some fungal endophytic species are also litter decomposers [19,21,22,60] and they have capacity to remain as saprotrophs in dropped leaves. Endophytism represents a stage of fungal endophytes which surrogates between a saprotrophic and an endophytic lifestyle. Spores of such biphasic fungal endophytes in forests undergoing periodic ground fires unveil constitutive heat tolerance proving this hypothesis [20,51].

Expression of unambiguous genes during endophytic and saprotrophic phases have been observed for a root endophyte [23,24,58,61]. Comparable genomic studies are required to question whether multi-host endophytes, which were not subjected to host-guided specialization, have also evolved as an endophyte saprotroph biphasic directly by expressions of specific genes [8,25,59,61].

4. ISOLATION OF FUNGAL ENDOPHYTES

Isolation of fungal endophytes is the critical procedure as far as plant selection and impingement methods are concerned. One should understand the criteria used for the plant selection and methods.

4.1. Criteria for selection and identification of plants

The criteria used for the selection and identification of plants by the local peoples have been investigated by researcher [25,26,]. Thus, currently there are mainly two hypotheses for the selection of plants namely apparent and non-apparent. Apparent hypothesis involves shrubs and trees while non- apparent deals with only herbs. Apparent plant species produces high molecular weight organic compounds with low toxicity while non-apparent plants harvests low molecular weight organic compounds with high toxicity and bioactivity [27,29,32].

Criteria used for selection and identification of plants are as follows [33,37,41,42,4831].

1. Plants from a distinctive ecological environmental niche, and growing in unusual habitats and holding novel strategies for survival should be considered.

2. Plants with an ethno botanical antiquity, and used for traditional medicines should be designated for study, as squatting endophytes may be the source of the medicinal stuffs of this plant.
3. Plants that are widespread, having an unusual longevity, or have occupied a certain prehistoric land mass, are suitable for study.
4. Plants growing in areas of great biodiversity have the prospective for housing endophytes with prodigious multiplicity.
5. Plants enclosed by pathogen-infected and showing no symptoms are gatehouse for endophytes.
6. Fresh plant tissue is fit for isolation than older tissues, which often contain many other fungi that make isolation of slow growing fungi easy.

Which part of plants is effective for fungal endophytic research? Somehow, the answer for this question is under debate. According to literature, not only leaves, flowers and fruits are effective but also stem and roots are effective in the view of ethnobotany and pharmacognosy.

The collected plant samples are stored at 4°C. Isolation should be carried out as soon as possible after collection to avoid contamination by air microspore (Bacon & White 1994).

After the selection of plants, one can go for authentication with consultation with experts and voucher specimens to be deposited in a herbarium [41,42,48,58].

4.2 Surface sterilization of selected plant parts.

In laboratory**, p**lant materials are surface sterilized with surface sterilants as shown in Table 1, 2, 3 and 4. After sterilization, samples are dried and used for isolation process. The species of host plant, and host tissue type sampled and surface sterilization procedures vary according to the investigator. Some investigators have compared carefully the effects of different surface-sterilization procedures [36,40,43], isolation medium and sample-unit size [10] on isolation frequencies. We recommend that investigators experiment with these factors prior to initiating detailed investigations so that protocols optimal for recovery of endophytes from individual host species or specific organs and tissues can be established. For root tissues, serial washing may be preferable to surface sterilization to obtain demonstrative frequencies of fungal colonists [34,35,46].

Table 1. Surface sterilization method used for selected plant samples (1,2,3,4,30,37,41,53].

Plant samples	Sterilants immersion duration (seconds)			
	Running tap water	70% Ethanol	4% Sodium Hypochlorite	Distilled water
Leaves	300	30	60	60
Flowers	300	30	60	60
Roots	300	30	60	60
Stem	300	30	60	60
Fruits	300	30	60	60

Table 2. Surface sterilization method used for selected plant samples [7,24,27,28,61].

Plant samples	Sterilants immersion duration (seconds)		
	70% Ethanol	0.5 % Sodium Hypochlorite	Distilled water
Leaves	180	60	60
Stem	180	60	60
Seeds	180	60	60

Table 3. Surface sterilization method used for selected plant samples [25,31,34,45].

Plant samples	Sterilants immersion duration (seconds)		
	Running tap water	75% Ethanol	Distilled water
Leaves	300	30	60
Flowers	300	30	60
Roots	300	30	60
Stem	300	30	60
Fruits	300	30	60

Table 4. Surface sterilization method used for selected plant samples [50,51,55,59].

Plant samples	Sterilants immersion duration (seconds)			
	Running tap water	70% Ethanol	10% Commercial bleach	95% Ethanol
Leaves	300	120	120	30
Flowers	300	120	120	30
Roots	300	120	120	30
Stem	300	120	120	30
Fruits	300	120	120	30

4.3 Methods for isolation of fungal endophytes

4.3.1 Isolation by cutting of selected plant parts

Surface sterilized plant samples are dissected under sterilized conditions into small pieces (0.2cm X 0.21cm) and placed on selected artificial media at room temperature [27,31,37,34,38,40].

4.3.2 Isolation by Blender shaft

Surface sterilized plant samples were added to 250 ml of distilled water in beaker and shredded with blender shaft with solvent. Such shafted extract further spread on artificial media for the growth of endophytic fungi [21,38,45,55].

4.3.3 Isolation by Mortar and Pestle

To the surface sterilized plant sample, add 1ml of distilled water and 1g of sterile sand into mortar and crushed with a pestle. Resulting suspension were spread on artificial media for further growth of endophytic fungi [54,55,56].

Table 5. Media used for isolation of fungal endophytes

Media Name	Composition (g/L)	References
Wickerham medium	Malt extract 3, Peptone 5, Yeast extract 3, Glucose 10 pH- 7.2	[41,45,60]
SAB	Peptone 10, Dextrose 20, agar 15	[3,11]
YM agar	Malt extract 10, Yeast extract 2, Agar 20.	[48,55]
CYA	Czapek 10, Yeast extract 5, Sucrose 30, K_2HPO_4, Agar 15	[21,24,46]
YES	Sucrose 150, Yeast extract 20, $MgSO_4.7H_2O$ 0.5, $CuSO_4.5H_2O$ 0.005, $ZnSO_4$. $7H_2O$ 0.01.	[31,54]
MEA	Malt extract 30, Peptone 5, agar 15, Chloramphenicol 0.1	[33,43,53]
PDA	Potato 200, Dextrose 20, agar 15	[2,3,4,5,112,13 41,42,60]

5. IDENTIFICATION OF FUNGAL ENDOPHYTES

Morphological identification of endophytic fungi by mycologists is a very critical step [26,48,54]. It includes development of fungus on standard media, examination of sexual reproduction and determination of growth characteristics. In recent years, according to taxonomical groups, few tests were added such as sub culturing, quick test for purity, examination of sexual reproduction, conidiogenesis and estimation of radial growth and media temperature [2,3,26,43].

Further reference sequences can be created from this identified culture [47,48,49,53]. The axenic culture is disinfected and Sequencing the ITS barcode and a subsequent NCBI Gen Bank BLAST search provide a match with an accessioned sequence. Further, fungal DNA extracted from the needles and ITS barcodes are amplified using different platforms such as NGS (e.g: Illumina MiSeq platform). A bioinformatics pipeline processes raw sequence reads and designates OTUs. Sequence does not result in an identification; e.g: 100% match with unidentified endophyte sequence or no similar sequences present in database [27,28,54,58].

Briefly, **m**olecular characterization of isolated fungal endophytes can be carried out by isolation of genome DNA, PCR amplification of ITS regions, DNA sequencing and sequences analysis [43,46,53,57,59].

Table 6. Fungal endophytes isolated from various plants

Host plant	Identified Endophytic fungus	References
Oryza sativa	*Alternaria alternata, Cladosporium tenuissimum, Epicoccum purpurescens, Fusarium equiseti, F. oxysporum, Hymenula cerealis, Phoma sorghina, Pleospora herbarum, Pythium* sp., *Trematosphaeria sp., Fusarium* sp. *Penicillium* sp. *Aspergillus* sp. *Paecilomyces* sp. *Pyricularia Sacc, Helminthosporium* sp. *Yeast, Sterile mycelium.*	[15,31,37]
Manilkara bidentata	*Xylaria* sp., *Colletotrichum crassipes, Pestalotiopsis versicolor*	[,4,6,27]
Lycopersicon esculentum	*Alternaria alternata, Colletotrichum gloeosporioides, Cladosporium* sp., *Penicillium* sp., *Arthrinium* sp., *Chaetomium globosum, Colletotrichum coccodes, Nigrospora sphaerica, Phomopsis* sp., *Ulocladium alternariae, Stemphylium botryosum*	[25]
Taxus cuspidate	*Alternaria* sp.	[31]
Nothapodytes foetida	*Neurospora* sp.	[2]
Camellia sinensis (Tea)	*Fusarium* sp., *Penicillium* sp., *Diporthe* sp., *Schizophillum* sp.	[2,6,31,56]
Coffee	*Aspergillus, Bipolaris, Cladosporium, Clonostachys, Colletotrichum, Epicoccum, Fusarium,Guignardia, Mycospharella, Phomopsis, Rosellinia, Talaromyces, Trichoderma, Xylaria*	[42,46]
Quercus variabilis	*Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp., *Rhizoctonia* sp.	[56,59]
Azadirachta indica	*Phomopsis oblonga, Cladosporium cladorsporioides, Pestalotiopsis* sp, *Trochoderma* sp., *Aspergillus* sp., *Periconia, Stenella, Drechslera*	[30,48,51]
Huperzia serrata	*Acremonium* sp.	[26,27]
Ananas ananassoides	*Muscodor crispans*	[32,58]
Jatropha curcas	*Leptosphaeria* sp.	[44,52]

Table 6. Continues.

Paris polyphylla var. Yunnanensis	*Fusarium, Gliocladiopsis irregularis, Gliomastix murorum var. murorum, Aspergillus fumigatus, Cylindrocarpon, Podospora* sp., *Plectosphaerella cucumerina, Pichia guilliermondii, Neonectria radicicola*	[6,36,44]
Foeniculum vulgare , Lactuca sativa, Cichorium intybus, Apium graveolens	*Acremonium, Alternaria, Fusarium, Plectosporium*	[14,17]
Antiaris toxicaria	*Trichothecium, acremonium, Rhizoctonia*	[11]
Iris germanica	*Rhizopus oryzae*	[61]
Saussurea involucrate	*Cylindrocarpan* sp. *Phoma* sp., *Fusarium* sp.	[29]
Dendrobium devonianum	*Fusarium sp., Phoma sp., Epicoccum nigrum*	[58]
Podocarpus species	*Aspergillus fumigates*	[59]
Hemionitis ariflora	*Several endophytic fungi*	[21,22]
Oryza granulate	*Dothideomycetes, Arthrinium* sp., *Magnaporthe* sp., *Muscador* sp.	[31,41,46]
Actinidia macrosperma	*Acremonium furcatum, Cylindrocarpon pauciseptatum, Trichoderma citrinoviride, Paecilomyces marquandii, Chaetomium globosum*	[28]
Solanum cernuum Vell.	*Arthrobotrys foliicola, Colletotrichum gloeosporioides, Coprinellus radians, Glomerella acutata, Diatrypella frostii, Phoma glomerata, Mucor* sp., *Phlebia subserialis, Phoma moricola, Phanerochaete sordida, Colletotrichum sp.*	[55]

6. PRODUCTION AND OPTIMIZATION OF ENDOPHYTE DERIVED BIOACTIVE COMPOUNDS

6.1 Production of bioactive compounds from fungal endophytes

The symbiotic relationship among endophytic fungi and plants gives powerful ability to produce new bioactive compounds. However, there are main two substrate-based methods for the production of bioactive compounds such as Solid state fermentation and submerged state fermentation [14,21,56,32,44].

6.1.1 Solid state fermentation (SSF)

Solid State fermentation is widely used for the bioactive compounds production from the fungal endophytes [21,31,41,51,55]. This biomolecules are mostly metabolites generated by endophytic fungi grown on solid support selected for this purpose. In this fermentation process, different solid substrates such as Wheat bran, Rice bran, coconut oil cake, vegetable waste, gram husk, orange peel, sugarcane bagasse etc were used with pure cultures of endophytic fungi [14,21,56,32,44,50,61].

In environment, fungal endophytes breed on the ground; decomposing vegetables combinations under naturally ventilated conditions, Therefore SSF enables the optimal growth of endophytic fungi, permitting the mycelium to spread on the surface of solid compounds through which air can flow [14,32,59,61]. SSF uses culture substratum with low water levels. The solid medium contains both the substrates and solid support [21,56]. After fermentation, fermented media are mixed with effective solvent and further used for purification and analysis [14,32,56,59,61].

6.1.2 Submerged fermentation

In submerged fermentation, enzymes and other reactive compounds are submerged in a liquid such as alcohol, oil, or nutrient broth [21,42,24]. Endophytic fungi are sited in a small closed flask containing the rich nutrient broth with high volume of oxygen. The in situ production of enzymes results in production of bioactive molecule [27,28,35,49,58,60]. Batch Fed fermentation method is used commonly which utilizes the sterilised nutrients under optimized conditions along with fungal endophytes which increase in density [23,36]. The growth rate of fungal endophytes are maintained by the addition of nutrients, also reduces risk of overflow of metabolism [9,23,36].

6.2 Optimization of production of bioactive compounds from fungal endophytes

Optimization of both fermentation processes depends on considerations of carbon homes and nitrogen homes, inoculums, phosphorus, organic acids, surfactants, incubation period, temperature, moisture level and pH level under optimized conditions to achieve greatest production of bioactive compounds from fungal endophytes [13,21,31,33,36,59].

6.2.1. Effect of different medium

Effect of the different medium on the production of bioactive compounds were observed [13,21,31,33,36,59].

6.2.2 Effect of carbon sources

In order to determine the effect of various carbon sources on the production of bioactive compounds, main sugar was replaced by different carbon sources like cellulose, fructose, lactose, galactose, malt dextrin, mannitol and sucrose in the production media [13,33,36,59].

6.2.3 Effect of nitrogen sources

Effect of different organic (beef extract, casein, peptone, malt extract, tryptone, soybean meal) and inorganic (KNO_3, $NaNO_3$ and NH_2CONH_2) at 1% (w/v) as additional nitrogen source were studied [21,31,36,59].

6.2.4 Effect of inoculum amount

To study the effect of inoculum on bioactive compounds production, different concentrations (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 % (v/v)) of activated culture were used [13,21,31,33,36,59].

Table 7. Media used for the production of bioactive compounds by fungal endophytes

Sr. No	Medium	Conditions	References
1	Liquid Wickerham medium	26^0C, 21 days	[21]
2	S_7 medium	26^0C, 21 days	[36]
3	Minimal medium	28^0C, 10-14 days	[15,16]
4	Lactose & Starch Casein broth	37^0C, 120rpm, 18 days	[24,31]
5	M_2 medium	28^0C, 124rpm, 7 days	[40,43]
6	C2 broth, Sabourauds broth, PDB, MEB	28^0C, 10 days	[21]
7	Nutrient Broth	30^0C, 120rpm, 5 days	[2,4,6,8,11,13,21,22,30]
8	Liquid fermentation	37^0C, 120rpm, 18 days	[30.31]
9	Nutrient Broth	30^0C, 124rpm, 24 hrs	[58]
10	Corn meal medium	26^0C, 21 days	[30,31]

6.2.5 Effect of inoculum time

10-24 h old culture at the interval of 2 h used as inoculum in order to determine the best suitable inoculum age for maximum production of bioactive compounds [13,21,31,33,36,59].

6.2.6 Effect of pH and temperature

The effect of different pH ranging from 4.0 to 10.0 observed on of bioactive compounds production. However to study the effect of different temperature, flasks were incubated at different temperatures (20-45°C) ([13,21,31,33,36,59].

6.3. Phytochemical analysis

The endophytic fungal extract can further be screened by phytochemical analysis, which serves as a key reserve for evidence on analytical and tangential methodology for the presence of bioactive compounds in the extract.

6.3.1. Test for Flavonoids

Test-tube containing 1-2 ml of fungal crude extract, 5-10 drops of dilute HCl, a piece of Magnesium strips were added and the solution was boiled for few minutes. A reddish pink or dirty brown coloration of solution indicates the presences of flavonoids in the extract [13,16,23,25,55].

6.3.2. Test for Alkaloids

The fungal crude extract is evaporated to dryness in boiling water bath. The residue obtained is dissolved in 2N HCl. The mixture treated with equal amount of Wagner's reagent. The reaction shows the appearance of brown precipitate, indicates presences of alkaloids [13,16,23,25,55].

6.3.3. Test for Terpenoids

1 ml of fungal crude extract is mixed in 1ml of chloroform and 3-4 drops of concentrated H_2SO_4 were added. A cherry red colour or reddish-brown precipitate at the interface indicates the presence of terpenoids [13,16,23,25,55].

6.3.4. Test for Cardiac glycosides

To 1 ml of fungal crude extract, add 1ml of $FeCl_3$ reagent. To this solution, add few drops of concentrated H_2SO_4 were added. Appearance of greenish blue colour within a few minutes indicates the presence of cardiac glycosides [13,16].

6.3.5. Test for Steroids

To 1 ml of fungal crude extract, add 1ml of chloroform and 1 ml of acetic anhydride and little concentrated H_2SO_4. A blue green ring indicates the presence of steroids [13,16,23,25,55].

6.3.6. Test for Saponins

The crude dry powder of fungal extract was energetically shaken with distilled water and was allowed to stand for 10 min. No froth indicates lack of saponins and stable forth more than 1.5cm indicated the presence of saponins [13,25,55].

6.3.7. Test for Phenols

The fungal crude extract is dissolved in 5ml of distilled water. Few drops of neutral 5% $FeCl_3$ solution was added. A dark green indicates the presence of phenolic compounds [13,16,23,25,55].

6.3.8 Test for Tannins

The fungal crude extract treated with alcoholic FeCl3 reagent. A bluish black colour, which disappears on adding little dilute H_2SO_4 followed by the configuration of yellowish brown precipitate, indicates the presence of tannins [13,16,23,25,55].

7. PURIFICATION AND STRUCTURE ELUCIDATION ENDOPHYTE DERIVED BIOACTIVE COMPOUNDS

Endophyte derived bioactive compounds include a broad diversity of structures and functionalities that provide a greatest pool of molecules for the production of different in-house products [6,9,13,16,23,35,41,55]. Many of these compounds can be found at very high concentration in nature while some found to be very low in concentration so that extraction is required to obtain sufficient amounts and their structural diversity and complexity create chemical synthesis unbeneficial [3,5,7,11,13,14,16,13,23,35,55]. From long time, Liquid-liquid extraction or solid- liquid extraction are used for the extraction purpose but nowadays pressurized liquid extraction, Subcritical & Supercritical extraction, and ultrasound assisted extraction are highlighted [3,5,7,11,13,14]. Fermented media is exposed to different solvent which takes up compounds of interest which is further centrifuged and filtered to obtain crude extract. Mostly, Hexane, ether, chloroform, acetonitrile, benzene, ethanol and distilled water is used in different proportion [3,5,7,11,13,14,16,13,23,35,55].

Purification is the physical separation of specific substances from contaminating substances. Purification includes mainly filtration, centrifugation, crystallization, distillation, chromatography; electrophoresis etc. While structure elucidation of endophyte derived bioactive compounds determination of chemical composition by NMR, mass spectroscopy, Crystallography, UV-visible spectroscopy etc. [1,2,3,5,7,11,13,14,16,13,17,23,35,55].

Table 8. The use of different solvents extraction for the recovery of endophyte derived bioactive compounds.

Solvents used	Raw Material	Compound of interest	References
Ethanol, methanol, n- hexane	Liquid Wickerham medium, solid rice medium	Alternariol, altearіol methyl ether, stemphylperylenol, bostrycin, Tenuazonic acid, indole-3, carbaldehyde, Cyclo (Threonylisoleucinyl), Aloesol, Deoxybostrycin, Equisetin, Citrinin.	[21]
Chloroform, methanol, n- hexane	Mycological medium	Taxol	[36]
n- hexane, chloroform, ethyl acetate, ethanol, methanol, butanol, petroleum ether	L.B & S.C broth	Alkaloids, phenolic compounds	[24,42]
Water	Plant extract	Triethylene glycol	[22]
Ethanol	PDB	Naphthoquinones	[2,44,45]
Chloroform, ethyl acetate	Minimal medium	Flavonoids, Saponins, Alkaloids,	[43]
n- hexane, chloroform, ethyl acetate, ethanol, methanol	NB	Pyrrolo, methyl-2-o-methyla-arabino pyranoside, Propionylfilicinic, benzene, Carboxylic acid.	[30,59,60]
Ethanol, methanol	Plant extract	Flavonoids, Saponins, Tannins, Terpenoids	[35,57]

Table 9. Different purification and structural elucidation techniques for the recovery of endophyte derived bioactive compounds

Methodology		Compounds of interest	References
Purification	Structure elucidation		
TLC, HPLC, SGC	NMR, Crystallography	Steriods, ergosterol, cerevisterol	[31,44]
TLC, HPLC	GC-MS, NMR	Taxol, Taxane III	[45,61]
TLC, VLC, LC, HPLC	Ms, ESI-MS, LC-MS, HRMS, NMR	Alternariol, altearial methyl ether, stemphylperylenol, bostrycin, tenuazonic acid, indole-3, carbaldehyde, cyclo (Threonylisoleucinyl), aloesol, deoxybostrycin, equisetin, citrinin	[21]
TLC, VLC, HPLC	GC-MS, NMR	Shamiminol	[15]
TLC, CC, HPLC	NMR, ESI-MS	Cytochalasin J, cytochalasin H, 5- epialtenuene alternariol monomethylether alternariol, cytosporone C	[4,5]
TLC, HPLC	NMR	Resaveratrol	[2,58]
HPLC	ESI-MS, NMR, TOF-MS	Gold nanoparticles	[34,42]
TLC, CC, HPLC	GC-MS, NMR	Teadenol A	[61]
TLC,UV-VS	GC-MS, NMR	Alkaloids	[22,41]

8. NOVEL FUNGAL ENDOPHYTES VERSES NOVEL BIOACTIVE COMPOUNDS

Discovering novel bioactive compounds from undiscovered endophytes is current trend. Not all endophytes are culturable [47,48] and these may produce useful bioactive metabolites. There are numerous techniques to detect unculturable fungi and these include whole DNA analysis monitored by DNA cloning, DGGE or T-RLFP [34,41,42,43]. Therefore, apart from isolating culturable endophytes from different taxonomic groups of plants and plants growing in different habitats, shotgun metagenomics for endophyte community analysis and function-based screening of their metagenomics libraries could be used to harness the unculturable and truly cryptic endophytes from environmental samples for drug production. Such a metagenomics approach has been quite rewarding with soil samples [4,5,24,50]. Metabolomics of endophyte infected and endophyte free plant hosts could reveal intersections in secondary metabolite paths that may be pushed into synthesizing novel chemical species or lead compounds another possibility of manipulating these chemo diverse organisms [50,51,60]. Additionally, other novel techniques such as radiochemical labelling can be used for detecting products of genes with low expressions among endophytes (Lodge et al. 1996). In addition, the biological potential of fungal secondary metabolites could also be fully realized by the application of combinatorial techniques [23,46,47,51].

In fungal endophytes, genes coding for enzymes of secondary metabolic pathways usually occur as gene clusters being positioned in the same locus and co-expressed [23]. These gene clusters are known to evolve swiftly through multiple rearrangements, duplication and losses, and are capable of interspecific feast through horizontal gene transfer [25,27,40]. It is important to screen fungal species for their secondary metabolite assortment under different growing conditions; culture parameters such as composition of growth medium, aeration, pH and the

presence of certain enzyme inhibitors change vividly the secondary metabolite profile and even induce the synthesis of several new metabolites [8,9,21,31,35,44].

As far as drug discovery is concerned, screening of libraries created by combinatorial combination once appeared to be more hopeful than natural products screening [13,58]. Though combinatorial synthesis can churn out molecules in enormous numbers, endophytic fungi can still be a good source of novel drugs and natural product-based scaffolds for combinatorial synthesis and libraries [56]. This is because the synthetic capability of endophytes, like in other organisms, has been fine tuned by natural selection over millions of years. Smith et al. (2008) united sequence analysis with bioassay procedures to explore the endophyte diversity of the tropics. Their results suggest that tropical plants harbour a substantial portion of undiscovered endophytes that may be vested with novel biochemical diversity. Hence, the need for the inclusion of fungal endophytes in natural products discovery programmes. Testing endophytes isolated from different tissues of plant hosts and from plants, growing in unusual and less studied habitats will be more productive. We suggest a global initiative involving fungal taxonomists, ecologists, and natural product chemists to evolve systematic and rapid screens for endophytic fungi by scheming considered bioassays that would indicate the production of novel bioactive compounds [11-14,16,23,25,35,46,55].

9. CONCLUSION

Isolation of fungal endophytes from medicinal and other plants may result in methods to produce biologically active agents for biological exploitation on a large commercial scale, as they are easily cultured in laboratory and fermenter instead of harvesting plants and affecting the eco-friendly biodiversity.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Phenolic Constituents of *Vaccinium* Species from Both Natural Resources and Micropropagated Plantlets

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Abstract: Fruits and leaves of *Vaccinium* species have rich bioactive phytochemicals. These bioactive phytochemicals make these plants particularly valuable for the medical and food industry. This novel approach was designed to determine the phenolic content of *Vaccinium* species obtained from both micropropagated and naturally growing leaves. An efficient micropropagation protocol was developed to produce tree *Vaccinium* species plantlets via direct organogenesis. Lateral buds containing one or two leaves were cultured in McCown woody plant medium (WPM), supplemented with zeatin/indole-3-butyric acid (IBA) (1.0/0.1 mg L^{-1}). In conclusion, Protocatechuic acid, Chlorogenic acid, Syringic acid and Routine phenolic compounds were determined in significant amounts. It has been determined that the phenolic compounds of leaves produced in tissue cultures is higher than the phenolic compounds obtained from naturally growing leaves.

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1. INTRODUCTION

Secondary metabolites are important compounds that human beings frequently use especially in the medical field. The development of new and effective production methods leading to these valuable compounds has gained momentum in recent years [1, 2]. *Vaccinium* species L., belonging the family Ericaceae, is an economically most important wild plant species growing in indigenous population of Turkey. The genus *Vaccinium* is represented by four species in the Turkish flora, namely *Vaccinium uliginosum* L., *Vaccinium arctostaphylos* L., *Vaccinium myrtillus* L., and *Vaccinium vitis-idaea* L., and their fruits are consumed by local people as dry or fresh fruit, marmalade, jam, compote, etc. The leaves of *Vaccinium* species

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have antioxidants and antibacterial properties as well as rich phenolic content. The leaves of these plant species have also been consumed as a herbal tea in the past [3, 4].

Because of these biological benefits, there is a growing interest in *Vaccinium* species, and many researchers study the commercial cultivation of these species. Due to the many advantages, including a mass effective clonal propagation, a fully controlled environment, a shortened growth cycle, an uninterrupted production process, and production of disease-free plants, plant tissue culture techniques have recently been preferred for the propagation of these species [5-8].

Collection of such valuable plants directly from natural population causes negative effects on natural habitats of plants and serious reductions in plant numbers; however, with plant tissue cultures, it is possible to prevent the use of natural plants for that purpose and keep nature's beauty intact [9, 10]. An also the micropropagation technique is useful for the production of active compounds during formation of secondary metabolites in medicinal and aromatic plants and in particular, to increase the levels of some important metabolites [11, 12]. The purpose of this novel study was to develop an efficient and rapid micropropagation protocol for producing *Vaccinium* plantlets and thereafter compared their phenolics contents with naturally growing plants.

2. MATERIALS and METHODS

2.1.Plant Material

Actively growing young *Vaccinium* nodal segments were collected from the natural populations of different regions of Trabzon in 2013. Lateral buds were washed with tap water for 1 h, and then surface sterilized with 70% (v/v) ethanol for 1 min, followed by 15 (10 min for *V. uliginosum* explants) min of incubation in 3% sodium hypochlorite (NaOCl). Finally, they were washed with sterile distilled deionized water 3 times for 15 min. Sterilized explants dried on sterile whatman filter paper and cultured on approximately 50 mL of nutrient media in 98.5 × 59 mm glass containers. Each treatment was performed in triplicates and 30 explants were used for each treatment.

2.2. Experimental

For shoot multiplication, McCown woody plant medium including vitamins (WPM) [13], suplemented with zeatin/ indole-3-butyric acid (IBA) (1.0/0.1 mg L^{-1}), was used to obtain in large quantities number of plants. WPM basal medium containing 2% sucrose and 0.8% agar. Zeatin and IBA used in the study were sterilized with 0.22-µm filters and added to the cooled media after autoclaving. The medium pH was adjusted to 5.0 before autoclaving. Cultures were incubated in a growth chamber maintained at 24 ± 2 °C under a 16/8-h day and light conditions. A subculturing protocol was performed every 8 weeks. The regeneration ability of cultures was then evaluated on the basis of mean number of shoots per explant, length of shoots emerged from each explant, mean number of nodes, fresh and dry weight. Each experiment was repeated in triplicate**.**

2.2. Extraction

In vitro grown biomass was extracted as reported elsewhere [14, 15]. Briefly, 200 mg of each sample was macerated in n-hexane (HE) and dichloromethane (DCM) (10 mL_each) for 10 min, solvent was filtered out and residue was removed via methanol (MeOH) extraction for 30 min, and methanol was evaporated in vacuo. The obtained methanol extract was then dipped in pH 2.0 ± 0.1 water and shaken vigorously followed by extraction three times with 5 mL diethyl ether and ethyl acetate. Organic phases were combined, evaporated and made up in methanol (2.0 mL) for HPLC analysis after passing through 0.45 µm filter. Determination of

total phenolic contents were determined with previously reported method [16]. Briefly, 750 µL of Folin-Ciocalteu's/water mixture (1:14) were added to 50 µL sample and after 3 min, 200 µL of 20 % Na2CO3 was added. Then the reaction mixture was incubated in dark for 30 min. Absorbance was measured on an ultraviolet-visible (UV–Vis) spectrophotometer (Unicam UV2-100) at 760 nm and methanol was used as blank. Gallic acid was used as standard and total phenol contents in extracts were calculated as mg gallic acid equivalent total phenolic in mg Gallic Acid Equivalent/100 (GAEq mg/100 gm) gm dry weight of plant.

2.3. HPLC analysis of phenolic compounds

A previously developed and validated HPLC method [16] was used in the quantification of phenolics. The analysis was carried out on an ELITE LaChrome (Hitachi HPLC system), quaternary pumps (L-2130 model), auto injector (model L-2200) and variable wavelength PDA detector (photo diode array (L-2455 Model). A C-18 reverse phase column (250 mm × 4.6 mm id, 5 µm particle size, Agilent (USA)) was used in the analysis which was fixed in column oven (Model-2300). Mobile phase was a mixture of solvent A (2 % acetic acid in water) and solvent B (70:30, acetonitrile/water). The injection volume was 20 µL and column was kept at 30 °C. The flow rate was kept constant at 1 mL min^{-1} using gradient programming; starting the flow of mobile phase as B (5 %) to three min, gradual increase (up-to 15, 20, 25, 40 and 80 % at 8, 10, 18, 25 and 35 min, respectively) and drop back to 5 % at 40 min and left for 10 min to equilibrate in column. Eluent was continuously monitored through PDA by measuring at three different wavelengths, i.e., 280, 315 and 350 nm.

2.4. Statistical Analysis

Each treatment included 6 Magenta B-caps (each containing 5 explants) and each was carried out in triplicate for shoot multiplication. All data were analyzed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). The data collected for mean shoot length, mean number of shoots, mean number of nodes for shoot multiplication, fresh and dry weight were analyzed using analysis of variance (ANOVA) with Pearson's correlation. Values are means ± standard deviation**.**

3. RESULTS and DISCUSSION

3.1. Shoot Multiplication

Three different assessed *Vaccinium* species also improved in the WMP medium supplemented with zeatin/IBA. All *Vaccinium* species have higher shoot regeneretion frequency (90%). The highest shoot multiplication was obtained in *V. arctostaphylos* with 5.08 shoots per explant. Although *V. myrtillus* and *V. uliginosum* gave the close shoot multiplication values, there was also a statistically significant difference between these species ($P \leq 0.05$, Table 1). In recent years, the production of secondary metabolites has been accelerated by tissue culture methods, and many researchers have reported many studies on the production of these valuable metabolites [1, 2, 17, 18]. In addition, although there are many studies to determine the secondary metabolite content of *Vaccinium* species fruits naturally growing in our country [19-22], there isn't any study to compare phenolic contents of *Vaccinium* species from both natural resources and micropropagated plantlets. Although researchers have found different results depending on the species studied with different media and plant growth regulators [23, 24] in terms of shoot number, the most effective results have been obtained from studies using zeatin [8, 25].

Our preliminary studies show us, multiplication depends not only on the presence or absence of the growth regulators but the basal medium and the plant species to be reproduced also has a great impact on micropropagation. The highest shoot length was again obtained from *V.arctostaphylos* with 44.60 mm. This value is higher than the shoot length obtained from *V.*

myrtillus and *V. uliginosum*, with 12.31 % and 21.57 %, respectively. Moreover, remarkable statistical differences was also established between these three *Vaccinium* species in terms of the shoot length ($P \leq 0.05$, Table 1). Unlike the shoot length, a different result was obtained between the three species in terms of the number of nodes. No significant difference was found between *V. arctostaphylos* and *V. myrtillus* in regard with the node number. The highest node number was obtained from *V. arctostaphylos* and *V. myrtillus* with 12.38 and 12.12 per shoot, respectively. This value was determined at 11.00 in *V. uliginosum* (Table 1). Well-developed plantlets production are needed in the production with micropropagation. Because of this, many researchers have applied different plant growth regulators on the micropropagation of depending on the plant species [26, 27]. Some of these researchers were reported that zeatin is more effective than other cytokinins in terms of the shoot length of *Vaccinium* species [6]. Also some other researchers were also reported that zeatin was more effective on shoot length than other cytokinins on *V. uliginosum* and *V. arctostaphylos* in terms of the shoot length [8, 28]. The findings obtained from previous reports support the our findings.

In such biomass studies where the secondary compounds are sought to be obtained, fresh and dry weight findings have also great significance. In this context, the fresh and dry weights of the plant leaves obtained by natural and micropropagation were calculated. The higher fresh and dry weight parameters were achieved from collected natural plant leaves with 1.88 and 0.38, respectively. These findings were higher than *V. arctostaphylos* with 12.23% and 18.42%, *V. myrtillus* with 25% and 34.21%, and 33.51% and 50.00%, respectively. Above mentioned, natural collection methods are likely to impact negatively on the natural habitat of these plants and lead to a dramatic decrease in plant populations. Therefore, it is important to carry out studies to obtain valuable secondary products using the advantages of plant biotechnology. This study is the first report to investigate the phenolic content of *Vaccinium* leaves produced in tissue cultures. Naturally collected *Vaccinium* leaves were evaluated as control groups.

3.2. Determination of Phenolic Contents

Free phenolics were analyzed by HPLC and the findings were summarized in Table 2. Protocatechuic acid, Chlorogenic acid, Syringic acid and Rutin were calculated significant levels. Protocatechuic acid was highest in micropropagated *V. myrtillus* followed by *V. arctostaphylos* with 2.27 and 1.868. One of the dramatic results was showed from Protocatechuic acid content of natural *V. myrtillus* and *V. uliginosum* leaves and no statistically significant data could be obtained ($P \leq 0.05$, Table 2). In the Chlorogenic acid contents, a significant increase was observed in the leaves of *V. arctostaphylos* and *V. myrtillus* obtained by micropropagation. Micropropagated *V. arctostaphylos* leaves had 164.09% more Chlorogenic acid content than natural *V. arctostaphylos* leaves and there was a significant statistical difference between them ($P \leq 0.05$). A similar condition has also occurred for *V. myrtillus* and this ratio was calculated as 180.98% (Table 2). The lowest Chlorogenic acid content was achieved from micropropagated *V. uliginosum* with 1.62.

Table 1. Effect of zeatin in the presence of IBA (0.1 mg L^{-1}) on shoot multiplication of three *Vaccinium* species.

	PGRs Concentration (mg L^{-1})	Number of Shoots/ per Explant	Shoot Length (mm)	Node Numbers/ per shoot	Fresh Weight (mg)	Dry Weight (mg)
Natural	-	-	-	-	1.88 ± 0.21 a	0.38 ± 0.032 a
V. arctostaphylos	1.0/0.1	5.08 ± 0.48 a	44.60 ± 2.47 a	12.38 ± 0.91 a	1.65 ± 0.20 b	0.31 ± 0.039 b
V. myrtillus	1.0/0.1	3.86 ± 0.51 b	39.11 ± 1.62 b	12.12 ± 0.67 a	1.41 ± 0.07 c	0.25 ± 0.036 c
V. uliginosum	1.0/0.1	3.46 ± 0.54 c	34.98 ± 1.51 c	11.00 ± 0.99 b	1.25 ± 0.16 d	0.19 ± 0.011 d

Data were recorded 8 weeks after the culture with a total of 3 replicates of 20 plants per treatment for shoot regeneration. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at $P \leq 0.05$. PGRs: Plant Growth Regulators

Table 2. Phenolics identified of *Vaccinium* species from both natural resources and micropropagated plantlets though HPLC (mg/100 g)

	NATURAL			MICROPROPAGATED		
	V. arctostaphylos	*V. myrtillus*	*V. uliginosum*	*V. arctostaphylos*	*V. myrtillus*	*V. uliginosum*
Protocatechuic acid	1.752 ± 0.097 b	-	-	1.868 ± 0.017 b	2.27 ± 0.17 a	1.29 ± 0.0 c
Chlorogenic acid	7.52 ± 0.61 d	47.15 ± 1.70 b	133.85 ± 5.16 a	19.86 ± 0.45 c	132.48 ± 0.88 a	1.62 ± 0.18 e
Syringic acid	0.608 ± 0.077 d	0.63 ± 0.038 d	0.97 ± 0.091 c	1.72 ± 0.14 a	1.42 ± 0.08 b	0.66 ± 0.07 d
Rutin	2.998 ± 0.012 a	-	-	-	-	-

Data were recorded 8 with a total of 3 replicates. Values having the same letter(s) in the same line are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

The importance of the study has emerged with the identified secondary compounds, which are predominantly high in micropropagated plants. Syringic acid content was also found to be higher in micropropagated plantlets than in natural ones except for again *V. uliginosum*. Rutin was calculated only natural sample of *V. artostaphylos* and amount of it was determined as 2.998. Although many researchers have mostly studied the biologically active components of *Vaccinium* fruits [29-31], very few researchers have studied on natural leaves of these valuable plants [32, 33]. In one of the these studies researchers performed such a study on *Vaccinium angustifolium* and reported that tissue culture techniques enhance the total phenolic contents of *V. angustifoliumum* [34]. The same researchers have reported that tissue culture technique used in the study can affect the phenolic content. Similarly, the other some researchers have reported that chlorogenic acid myricetin, syringic acid and rutin are main phenolic components of micropropagated blueberry seedlings [35]. These researchers have specified that micropropagated *Vaccinium* seedlings are a good source of antioxidant compounds in terms of the above mentioned phenolic compounds. These results also support our findings.

4. CONCLUSION

This study emphasized to determined efficient *in vitro* micropropagation method and to compare of phenolic compounds of micropropagated *Vaccinium* plantlets and natural ones. At this occasion, phenolic contents of natural *Vaccinium* leaves and micropropagated seedlings of three *Vaccinium* species growing in the Turkish flora compared for the first time. According to the results, there were significant increases in some phenolic compounds in micropropagated seedlings of some species. Some factors such as the type of plant species, the type of medium and the type and combination of plant growth regulator can affect these desired outcomes. The efficient results of these valuable plant species may be a pioneer 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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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Agrobacterium – caused transformation of cultivars *Amaranthus caudatus* L. and hybrids of *A. caudatus* L. x *A. paniculatus* L.

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Abstract: The procedure for vacuum infiltration of cultivars *A. caudatus* L. and hybrids of *A. caudatus* L. x *A. paniculatus* L. was optimized**.** The functioning of gene construction pCBv19 in the *Amaranthus* leaves was evaluated by the transient expression after vacuum infiltration with *Agrobacterium rhizogenes* A4. After hypocotyl transformation of the varieties of amaranth species *A. caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, and hybrids *A. caudatus* x *A. paniculatus* L. – cv. *Sterkh*, *A. caudatus* x *Sterkh*- cv. *Zhaivir* with the wild strain *A. rhizogenes* A4, the culture of "hairy roots" was obtained. Embedding and transcription of genes in the roots are confirmed by the results of the PCR analysis.

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transgenic roots.

1. INTRODUCTION

Amaranth plants are used in the food industry, medicine, cosmetics and agriculture, they are also a source of biologically active compounds, the most valuable of them are squalene and amarantin. Squalene has anticancer and wound healing properties while amarantin – has an antioxidant activity [1, 2]. The seeds of *Amaranthus* species are rich in methionin and lysine amino acids. Biologically valuable substances can be obtained by using biotechnological methods.

Plants that synthesize alien substances may well be received through genetic transformation by using *Agrobacterium* bacteria. Members of this genus are pathogens. Due to Ti- and Ri - plasmids these bacteria can cause the formation of plants «hairy roots» (*A. rhizogenes*) or tumors (*A. tumefaciens)*. The hormonal balance of plants changes (after insertion and expression of bacterial gene), which results in specific phenotype in infected plants [3].

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Initial experiments connected with the transformation of amaranths species with *Agrobacterium* strains were unsuccessful [4]. By then, it was proved that transgenic amaranths plants with *A. rhizogenes* and *A. tumefaciens* could be obtained. But still there are only several works devoted to the transformation of amaranth.

The transgenic roots were obtained for *Amaranthus tricolor* [5] and *A. spinosus* L. [6]. The transgenic plants were obtained for *A. hypochondriacus* L. and *A. tricolor* L. [7, 8], *A. retroflexus*, *A. viridis*, *A. cruentus* [9]. There is no information about transformation of *A. caudatus*, the varieties of which is also used in agriculture.

The aim of this work was to evaluate the functioning of gene construction pCBv19 of *Agrobacterium rhizogenes* A4 in amaranth tissues and obtain transgenic roots of amaranth after genetic transformation with using the wild strain of *A. rhizogenes* A4.

2. MATERIALS and METHODS

2.1. The transient expression of transferred genes in the leaves of *Amaranthus*

The objects of the research were cultivars of species of *Amaranthus caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, hybrids: *A. caudatus* x *A. paniculatus* L. – cv. *Sterkh*, *A. caudatus* x *Sterkh* - cv. *Zhaivir*.

Our objective was to check and evaluate the functioning of gene construction pCBv19 of *A. rhizogenes* A4 (Fig. 1) in transiently transformed amaranth leaves.

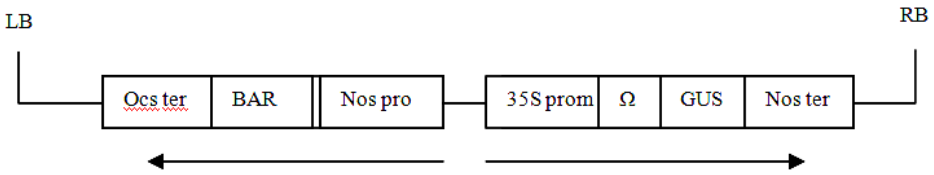

Fig. 1. Schematic representation of the T-DNA site of the pCBv19 construction LB – left border sequence, RB – right border sequence; Nos pro – nopaline synthase promoter, Nos ter - nopaline synthase terminator; 35S prom – 35S promoter; BAR – *bar* gene, GUS – *gus* gene; Ocs - octopine synthase; Ω - regulatory sequence enhancer

For this purpose we used the method of vacuum infiltration [10] and detection of GUS activity [11]. For infiltration of the 2-month-old plant leaves, the varieties and hybrids mentioned above, were used. As an infection agent we used *A. rhizogenes* A4 gene construction pCBv19, which contained *bar* and *gus* genes.

First, *A. rhizogenes* A4 was sown in the liquid LB medium (for 24 h. mixing on shaker). We added 1 ml of *A. rhizogenes* into 50 ml of the medium with 0,2 mM of acetosiringone. Next, *Agrobacterium* was centrifuged during 12 min, 5000 rpm. Then, *Agrobacterium* was resuspended into the medium with sucrose (50 g/l + 0,2 % super wetting agent Silwet).

The next stage was dipping the leaves of amaranth into the flask with the medium containing *A. rhizogenes* (for 5-10 min, 22 - 24C^0) in the vacuum chamber. After this operation, leaves were put on the wet filter paper in Petri plates (Fig. 4).

After the leaves had been lying on the wet filter paper for 4 days, β – glucuronidase fluorometric assay (GUS activity) was conducted at 37 ^{0}C according to Jefferson [11]. GUS reactions were stopped in 24 h. of incubation at 37^{0}C. Specific activities were detected visually by the appearance of staining the plant tissues in blue color.

2.2. Transgenic roots obtaining

Seeds of amaranth germinated on the sterile nutrient agar medium Murasige and Skoog (MS_{30}) [12] with 30 g/l sucrose. For transformation we used the hypokotyl segments of 14-day-old seedlings of cultivars of the following species *A. caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, and hybrids: *A. caudatus* x *A. paniculatus* L. - cultivar *Sterkh*, *A. caudatus* x *Sterkh* - cultivar *Zhaivir*, the seeds were obtained from the Botanical Garden of M.M. Grishko NAS of Ukraine. Transformation was carried out by co-cultivating the hypokotyls with the agropine strain of *A. rhizogenes* A4. Transformation of amaranths was carried out according to the modification of techniques proposed by Jofre-Garfias and colleagues [13].

First, *A. rhizogenes* A4 was sown in the liquid LB medium (for 24 h. mixing on shaker). We used 1 ml of *A. rhizogenes* into 50 ml of the medium with 0,2 mM of acetosiringone. Next, *Agrobacterium* was centrifuged during 12 min, 5000 rpm. Then, *Agrobacterium* was resuspended into the medium liquid ½MS_{15}. In this medium explants were soaked for 2 hours.

After 2 hours of incubation, explants were transferred on the solid growth medium ½MS_{15} without antibiotics. Co-cultivation on this medium lasted 1 day and then the hypocotyls transferred on the ½MS_{15} medium with the addition of 500 mg/l of cefotaxime ("Darnitsa", Ukraine).

Every 2 weeks hypocotyls were transferred to the ½MS_{15} medium with a reduced content of cefotaxime (400 mg/l, 300 mg/l, 200 mg/l). In the last transfer we used ½MS_{15} medium without adding of cefotaxime.

Hypocotyls of 14-day-old seedlings of the same varieties were used as a control, which weren't cocultured with *A. rhizogenes* A4, first, they were laid out on ½MS_{15} medium, then on ½MS_{15} with 500 mg/l of cefotaxime. The following sub-cultivation for control samples were not carried out, because after 15 days the hypocotyls died.

2.3. Polymerase chain reaction

Genomic DNA isolated by CTAB method [14]. For the PCR analysis we used the reaction mixture of the following composition: 2 µl single PCR buffer with ammonium sulphate (Dream Taq Green Buf.), 2µl primers, 2µl deoxyribonucleotide triphosphate (dNTP), 0.15µl Dream Taq-polymerase, 2µl DNA (20-30 ng/ml DNA). The volume of the reaction mixture is 20µl).

To identify the gene *rolB*, the primers were used: *5'*-CTCACTCCAGCATGGAGCCA-*3'* as well as *5'*-ATTGTGTGGTGCCGCAAGCTA-*3'*. The expected size of the amplification product for *rolB* gene was 592 bp. Amplification conditions: initial denaturation at 94 ^{0}C for 3 min, annealing at 60 ^{0}C for 30 s, extension at 72 ^{0}C for 30 s for the first cycle followed by 32 cycles each. The duration of the synthesis of the *rolB* was 40 s, at 72 ^{0}C, final polymerization was at 72 ^{0}C for 5 min.

3. RESULTS

For evaluation of functioning of *Agrobacterium rhizogenes* A4 gene construction pCBv19 in *Amaranthus* tissues, we carried out the histochemical reaction [11] (Fig.2, Fig. 3).

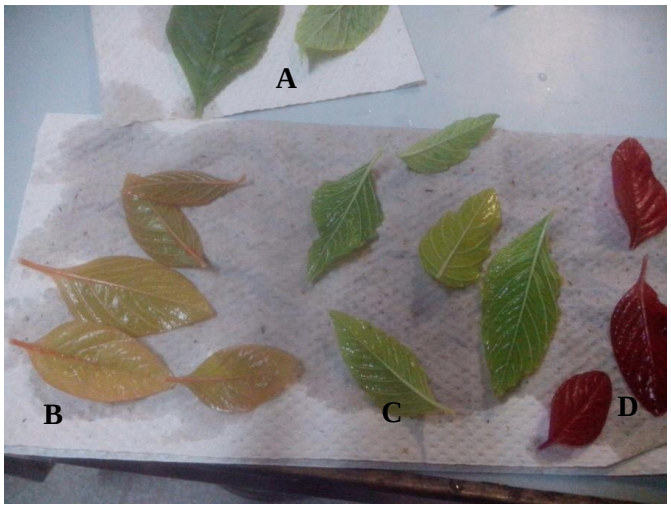

Fig. 2. Leaves of varieties A - *Kremovii ranii*, B – *Helios*, C – *Rushnichok*, D - *Karmin* after infiltration under vacuum.

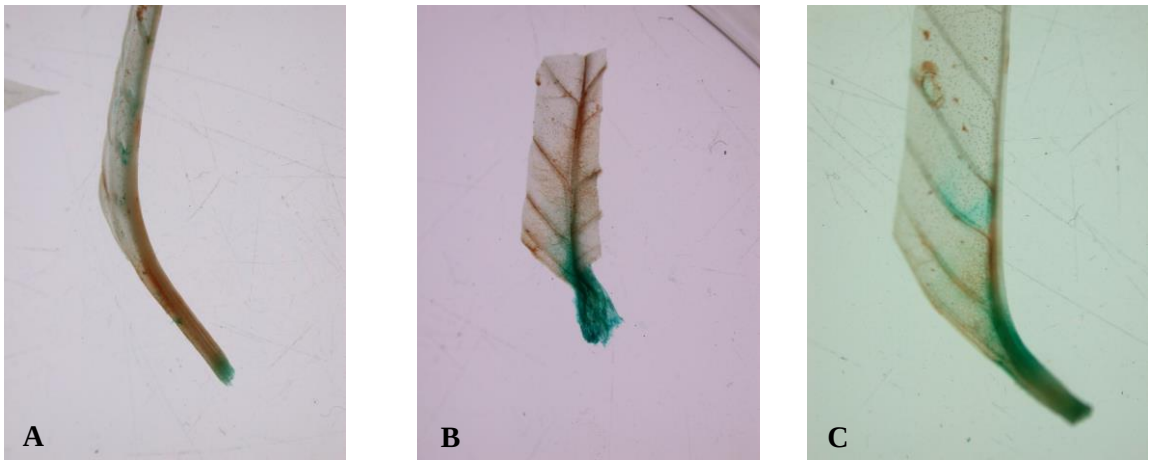

Fig. 3. Leaves of varieties A - *Kremovii ranii*, B – *Helios*, C – *Rushnichok*, D - *Karmin* after a histochemical reaction (detection the activity of GUS).

The GUS activity result was positive for 2 varieties (*Karmin* and *Helios*) (sites that are of blue color). Positive sites were in the area of the midrib (Fig.3). At the next stage transgenic roots were obtained. The growth of “hairy roots” of *Amaranthus* on the hypocotyl explants were observed in 20-25 days after *A. rhizogenes* transformation on the ½MS_{15} medium with cefotaxime. Eight lines of roots were received. When transferred the parts of the roots (~10 mm) on the hormone free medium without growth regulators, their intensive growth was observed. The roots outwardly resembled "hairy roots" (Ri- roots) form (Fig. 4), due to transfer of TL- fragment of the T-DNA of pRi plasmid of the agropine type with gene *rolB*.

Fig. 4. Formation of "hairy roots", after transformating the hypocotyls of *A. caudatus Helios* with the strain *A. rhizogenes* A4

To confirm the presence in the transformed roots TL-fragment of the T-DNA pRi plasmid, the amplification of total DNA with primers, specific to *rolB* gene, was carried out. During the analysis of 8 samples of tested cultivars of species *A. caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, and hybrids: *A. caudatus* x *A. paniculatus* L.- cv. *Sterkh*, *A. caudatus* x *Sterkh* – cv. *Zhaivir*, the presence of the DNA fragment with 592 bp size for 3 samples (№3 – *Helios*; №6, №8 - *Karmin*), was discovered, this confirms the presence of the *rolB* gene in the transformed roots (Fig. 5).

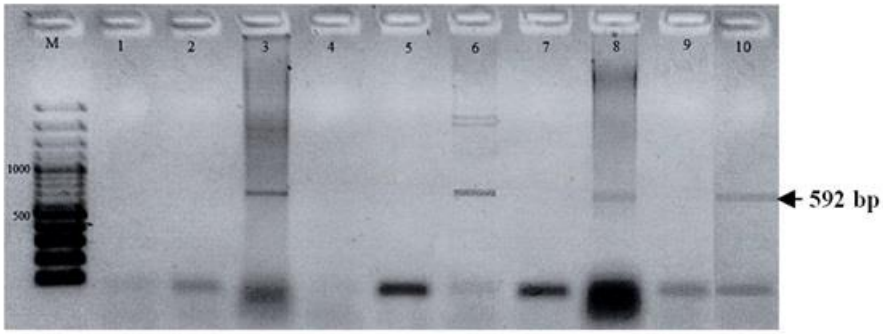

Fig. 5. PCR analysis of amaranth plants using primers for *rolB* gene: M - DNA marker (O'GeneRuler^{TM}1kb DNA Ladder, "Fermentas"), 1-8 total DNA of plants transformed with *A. rhizogenes* A4 (1 - *Sterkh*; 2, 3, 4 - *Helios*; 5- *Kremovyi rannii*; 6, 8 - *Karmin*; 7 - *Zhaivir*), 9 - negative control, DNA non-transformed plants, 10 - positive control, the plasmid DNA of *A. rhizogenes* A4 (592 bp).

4. DISCUSSION

Positive results were obtained in the transformation of *Amaranth tricolor* L. - Swain with colleges [5] and *Amaranth spinosus* L. - Pal and colleges [6] with the wild strains of *Agrobacterium rhizogenesis* A 4. The authors get transgenic roots. Positive results were obtained in the transformation of amaranth species with strains of *Agrobacterium tumefaciens.* Jofre-Garfias with co-authors – *Amaranthus hypochondriacus* L., cv. "Azteca". They used *Agrobacterium* construction with marker genes [13]. Transgenic *Amaranthus tricolor* L. was obtained by two different groups of scientists - Swain with colleges and Pal with co-authors [7, 8]. Pal with co-authors used the construction with marker genes. Also there are 2 works dedicated to the transformation through amaranth inflorescence - Umaiyal Munusamy with co-authors. They used the construction with selective genes [15].

Prof. Taipova conducted experiments with *Amaranthus retroflexus*, *A. viridis*, *A. cruentus* [9]. In both works it is said that positive and promising results were obtained and they get transgenic seeds. The results obtained don't seem convincing. Since in the work by Umaiyal Munusamy it isn't indicated with what kind of amaranth they worked. Prof. Taipova didn't indicate with what kind of bacteria they worked. The results of the biochemical and genetic analysis aren't shown, referring to which it would be possible to state with accuracy that they received transgenic seeds.

We have for the first time obtained transgenic plant parts for the varieties of *Amaranthus caudatus* L. Since, there are still no reports on the transformation of *A. caudatus*. The PCR analysis shows that not all the "hairy root" samples carried the genes of the *Agrobacterium*, although all the variants were phenotypically identical and were similar to typical "hairy roots", grew on the hormone-free MS_{30} medium. The results of the GUS activity were also not positive for all explants. Only 25% of the leaf samples showed a positive result. Positive results for the GUS activity were obtained for the *Karmin* and *Helios* varieties. Transgenic roots were obtained for the same varieties. The reasons for that are still not clear. Perhaps these varieties are more susceptible to *Agrobacterium rhizogenesis* than the other varieties which we tested. To clarify these reasons, further researches will be required.

5. CONCLUSION

So, after the vacuum infiltration of leaves, the GUS activity result was positive for 2 varieties of *A. caudatus*: *Karmin* and *Helios*. After the transformation of hypocotyls of cultivars of amaranth species of *Amaranthus caudatus* L.: *Helios*, *Karmin*, *Kremovyi rannii*, and hybrids: *A. caudatus* x *A. paniculatus* L.- cultivar *Sterkh*, *A. caudatus* x *Sterkh* – cultivar *Zhaivir*, using *A. rhizogenes* A4, were obtained transgenic roots of cultivars *Helios* and *Karmin*. The analyzed samples had gene *rolB* of *A. rhizogenes*.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Photoprotective Properties of Natural Pulvinic Acid Derivatives toward Ultraviolet-Induced Damages

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Abstract: Pulvinic acid derivatives are considered as worthy to be evaluated as skin protection factor toward ultraviolet-induced damages because of their colors and locations in lichens. Due to the lack of literature about photo-protective features of pulvinic acid derivatives, their cosmetic potentials for skin protection were evaluated *in silico*, for the first time. Computational chemistry, biology and pharmacology platforms such as Gaussian, GAMESS, PASS, PaDEL-DDPredictor and VEGA QSAR platforms were employed to determine the activities of pulvinic acid derivatives. Pulvinic acid derivatives were divided into three groups as the most promising, promising and unpromising compounds according to the calculated *p*-values. Although leprapinic acid, demethylleprapinic acid, pinastric acid, leprapinic acid methyl ether, 4-hydroxyvulpinic acid and vulpinic acid were determined as the most promising compounds, epanorin and rhizocarpic acid were identified as promising compounds. The proposed model seems to be reliable because the calculated *p*-value for vulpinic acid was found to be compatible with previously obtained experimental results. The pulvinic acid derivatives that were identified as the most promising ones should be therefore further studied by *in vitro* and *in vivo* multiple experiments.

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1. INTRODUCTION

Lichens are known as ecologically obligate associations between photobionts that transfer and release necessary carbohydrates such as ribitols, erythritols or sorbitols, and mycobionts that provide a humid and safe environment [1,2]. Lichens have adapted to live in almost every habitat on the planet including the extreme ones such as high mountains, dry deserts or the Polar Regions, where the depletion of the ozone layer is dramatically higher [3]. Moreover, lichens are considered as the pioneer organisms on rock surfaces, dead woods, living barks of trees, animal bones, and even on the man-made surfaces such as rusty metals or the surfaces of the medieval buildings where are also dramatically exposed to ultraviolet rays [4,1]. Lichens have achieved to live under the intense ultraviolet rays by using a series of adaptation mechanisms

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such as the membrane and macromolecule repair systems, antioxidant defense, thermal dissipation, light scattering and radiation screening [5]. The adaptation of lichens to the extreme conditions seems to be also connected to their secondary metabolites (ca. 1000) that are mainly synthesized by the fungal partner of lichen association and produced by depending on the environmental stress factors such as temperature fluctuations, drought, excess light or ultraviolet rays [6-8]. Pulvinic acid derivatives are considered as potential photoprotective substances due to their chemical structure, having an oxolane-carbonyl chromophore unit, present ultraviolet (UV) profiles characterized by two absorption bands with a high absorption coefficient, and their locality in the lichen thalli where are the most exposed parts of lichens to ultraviolet rays [5]. Moreover, it is known that biosynthesis rates of pulvinic acid derivatives, e.g., calycin or rhizocarpic acid, are increased with the increasing levels of ultraviolet radiation [9,10]. Although the similarity is a fundamental concept that has been used for more than two centuries in the sciences, perspective of structure–activity relationships in medicinal chemistry has been started to emerge as a central paradigm and distinct discipline in the late 1990s [11-13]. According to the perspective of structure–activity relationships that can be defined as the application of mathematical and computer models to predict biological activity depending on its chemical structure, similar molecules should also display similar biological activity [13]. The perspective of structure–activity relationships is a rapidly developing and widely accepted discipline because it has provided research and development (R&D) budget- and labor-saving feature for the screening of the vast variety of compounds [14]. In this research, we would like to investigate the photoprotective potential of pulvinic acid derivatives by using a combination of computational models because there is an increasing interest of scientist and great need to discover new natural photoprotective substances from certain organisms, including lichens, and the photoprotective capacity of pulvinic acid derivatives is considered as worthy [15-17,5].

2. MATERIAL and METHODS

Chemical structures of pulvinic acid derivatives that are composed by calycin, epanorin, pinastric acid, rhizocarpic acid, leprapinic acid, leprapinic acid methyl ether, demethylepropinic acid, pulvinic acid, pulvinamide, pulvinic acid lactone, 2-hydroxypulvinic acid lactone, vulpinic acid and 4-hydroxyvulpinic acid were quoted from the book written by Huneck and Yoshimura (1996) and drawn by using Chem3D 17.0 [18]. Vitamin E (α-tocopherol) was used as positive control and the chemical structure was obtained from the PubChem open chemistry database [19,3]. To identify the optimized geometric and electronic structure of pulvinic acid derivatives, Gaussian 03 software with Hartree-Fock (HF) theory at the B3LYP/3-21G level was employed by ab-*initio* quantum mechanical calculations based on Density Functional Theory (DFT). Although the experimental ultraviolet/visible spectrum species in methanol were quoted from the book published by Huneck and Yoshimura (1996), GAMESS software with HF theory at the 3-21G basis set was performed to determine the predicted ultraviolet/visible spectrum species by using the geometrically optimized 3D chemical structures of compounds. Then, PASS (Prediction of Activity Spectra for Substances) online provided by Way2Drug Team was performed to determine photosensitizer, cytotoxic, cytoprotectant, free radical scavenger, antioxidant, irradiation, skin irritation, ocular toxicity, antipruritic and anti-allergic, antibacterial and anti-inflammatory potentials of pulvinic acid derivatives [20]. To determine reactive metabolites forms adduct, skin irritation, serious eye damage, serious eye irritation and eye/skin corrosion potentials of compounds, PaDEL-DDPredictor software were employed [21]. Additionally, skin sensitization model (CAESAR) 2.1.6 in VEGA QSAR platform was used to identify skin sensitization potentials of pulvinic acid derivatives [22]. After the first *p*-values ($\boldsymbol{x}$) of the compounds were determined for the activities that were mentioned above, the activities were divided into two groups as positive factors and negative factors to be a photoprotective compound and a relative weight as

percentage (w) was given for each activity by using previous experimental experience [23,3]. Then, a positive weighted average value ($W_{avg_{I(+)}}$) for the positive factors and a negative weighted average value ($W_{avg_{I(-)}}$) for the negative factors were calculated by using the formula given below. Consequently, a final p-value was calculated by addition of the previously obtained two values.

$$W_{avg_I} = w_1x_1 + w_2x_2 + \cdots + w_nx_n \quad (1)$$

$$p = W_{avg_{I(+)}} + W_{avg_{I(-)}} \quad (2)$$

3. RESULTS

The two-dimensional (2D) chemical structures and three-dimensional (3D) conformations of pulvinic acid derivatives and α-tocopherol were given in Table 1. To predict ultraviolet/visible spectrum species of the compounds, GAMESS software was employed and the obtained spectra were given in Table 1. As can be seen in Fig. 1 and Fig. 2, the predicted UV/Vis values were generally compatible with the experimentally obtained ultraviolet/visible spectrum species of the compounds in methanol. Although the absorption spectra of pulvinic acid derivatives are generally observed in ultraviolet A and ultraviolet C, the experimental spectra in methanol (MeOH) have displayed that vulpinic acid and pinastric acid could absorb the light within the ultraviolet B wavelength (Fig. 1 and Fig. 2).

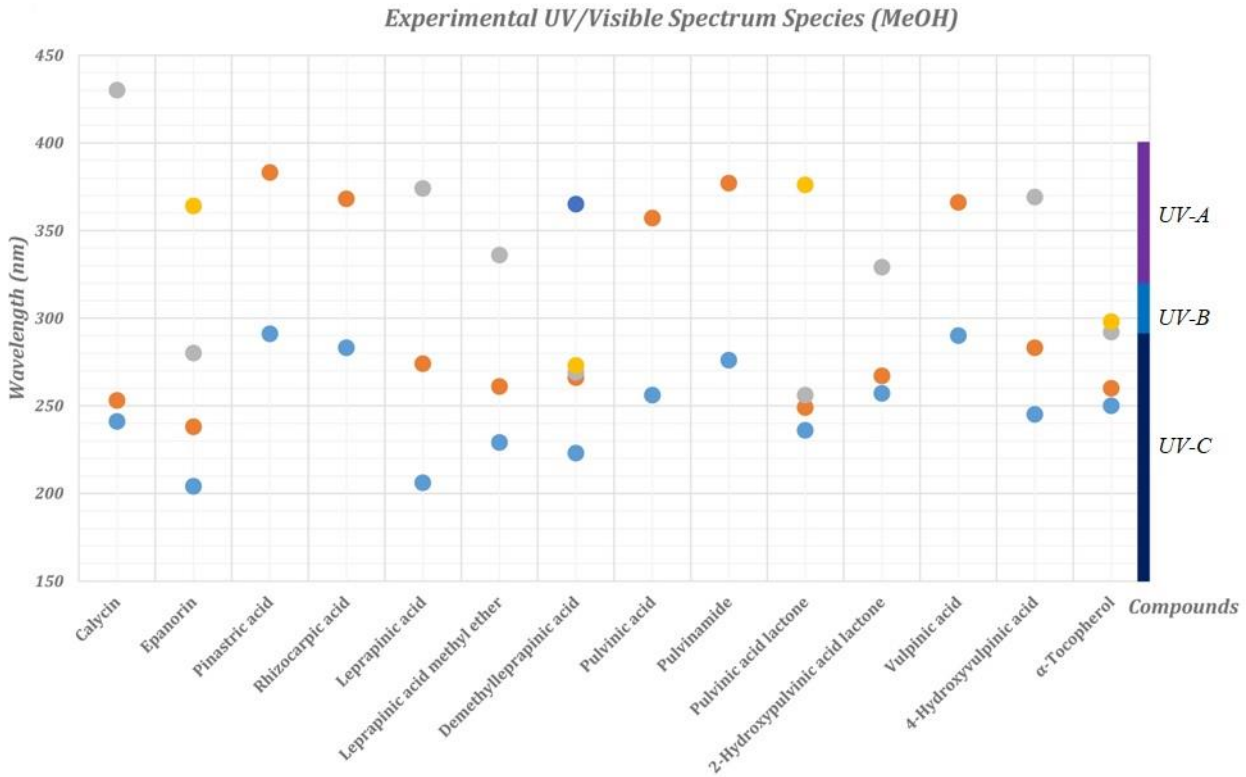


Fig. 1. Experimentally obtained UV/Visible spectrum species of the pulvinic acid derivatives and α-tocopherol in methanol.

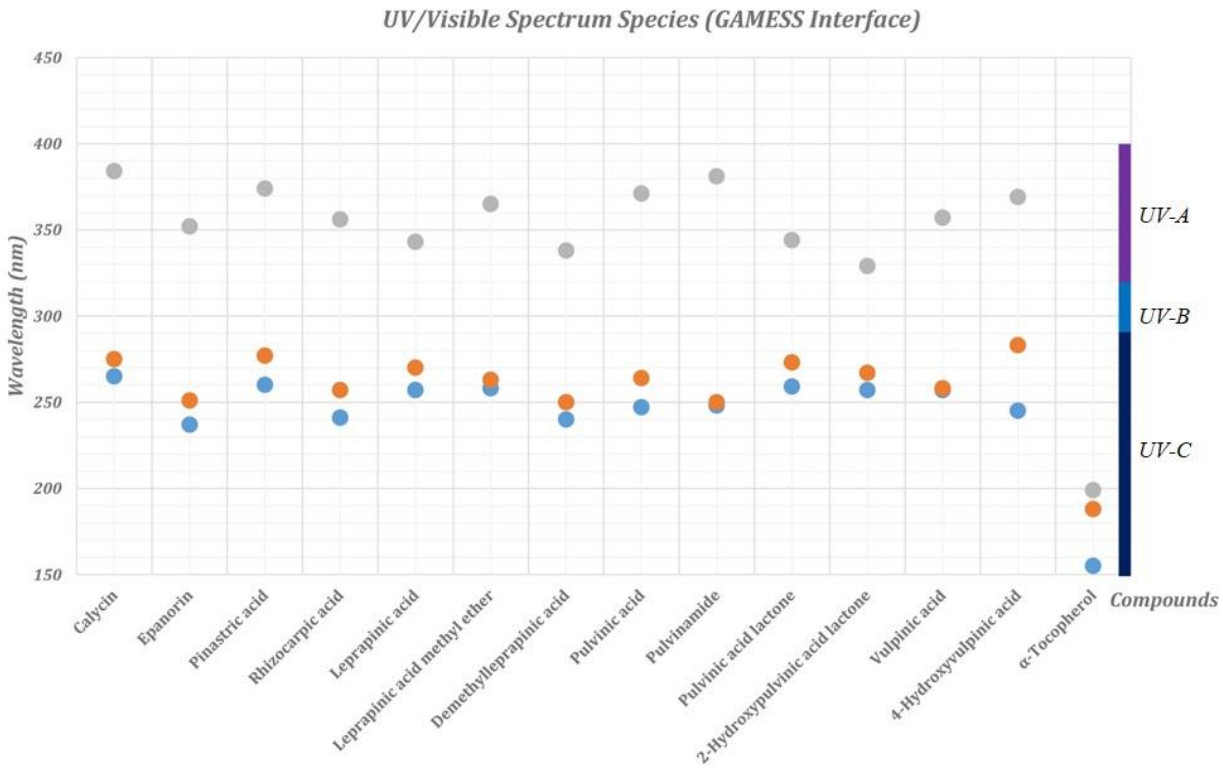


Fig. 2. Computationally obtained UV/Visible spectrum species of the pulvinic acid derivatives and α-tocopherol by GAMESS interface in Chem3D 17.0 software.

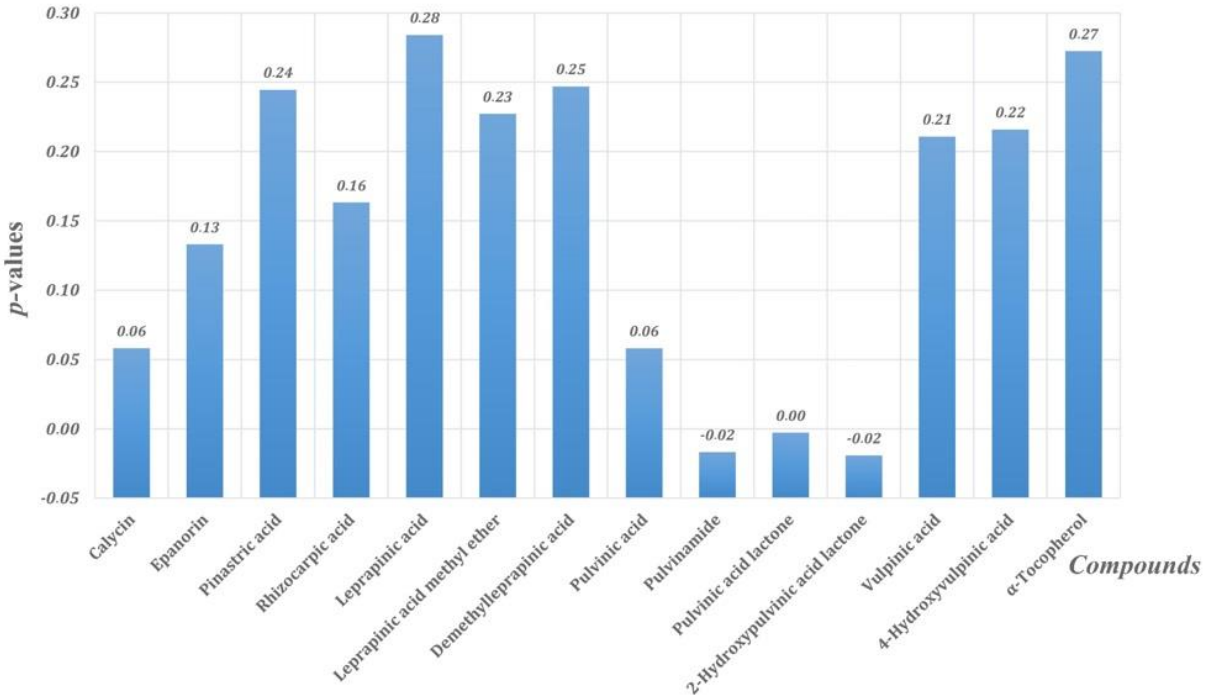


Fig. 3. The comparison of photoprotective capabilities of pulvinic acid derivative toward ultraviolet light-induced skin damages according to the statistically obtained *p*-values.

After the UV/Vis spectrum species were evaluated, some protective and harmful activities that are related to skin protection towards ultraviolet rays were computationally evaluated by using PASS, PaDEL-DDPredictor and VEGA QSAR platforms as can be seen in Table 2. After

the data were obtained from *in silico* platforms, a *p*-value was calculated for each compound and *p*-values were framed as a graph for the comparison of photoprotective capabilities of pulvinic acid derivative toward ultraviolet light-induced skin damages (Fig. 3).

4. DISCUSSION and CONCLUSION

Nature has been of great importance as a generous source of nutrition, poison and remedy throughout the history of humankind, and the ancient relationship between humankind and nature affects the people's preferences in favor of natural products for drugs, cosmetics and care products [24]. Pharmaceutical industry and cosmetic companies have therefore given more importance and allocated a significant amount of R&D budget to discover active natural products or functional ingredients [25,26].

Although the rational and best way to search for potentially active ingredients of cosmetics, drugs and care products seems to be the testing of substances obtained from the natural sources such as plants, animals or fungus, there are some limitations such as costly and/or time-consuming detection processes of the cosmetically and pharmacologically active compounds that are located within these highly structured-organisms due to the complex compound contents of them [27]. Nonetheless, lichens that are defined as a complex symbiotic organisms composed by a photobiont and a mycobiont, which have a tight metabolic relationship with each other, emerge to provide the researchers many advantages by their simple but very active secondary metabolite contents that can be easily isolated by the basic isolation method such as thin layer chromatography [28-30]. On the other hand, it should be noted that the R&D departments of companies and governments, and researchers would like to prefer R&D budget- and labor-saving alternatives such as *in silico* biological activity prediction platforms depending on the mathematical relationships of chemical structures and biological activities [13,14]. In the present study, the photoprotective and destructive activities of lichen-originated pulvinic acid derivatives were investigated by using different *in silico* platforms and the authenticity of the obtained data was evaluated, to the best of our knowledge for the first time, by comparing the experimentally obtained data in the literature. Due to the skin protection capability against ultraviolet rays, α-tocopherol was applied as positive control *in silico* models [19,3,31]. The calculated *p*-values and obtained data from the *in silico* models showed that pulvinic acid derivatives could be divided into three main groups that composed by unpromising, promising and significantly promising as the photoprotective substances toward ultraviolet-induced skin damage (Table 2 and Fig. 3).

Table 1. The two- and three- dimensional chemical structures and UV/Vis spectrum data of pulvinic acid derivatives and α-tocopherol.

*** indicates that there is no information in literature.

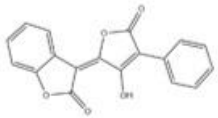
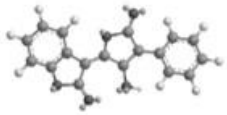
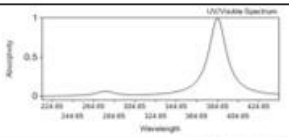
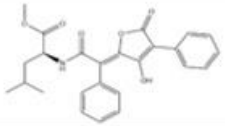
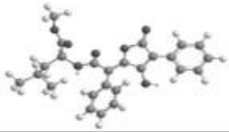
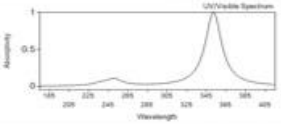
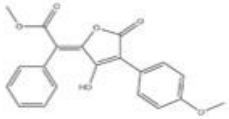
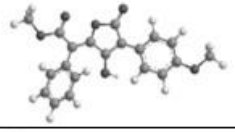
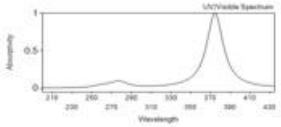
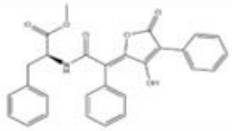
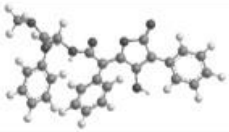
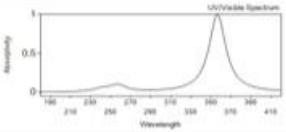
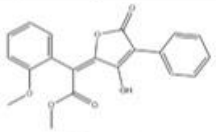
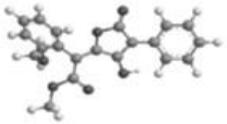
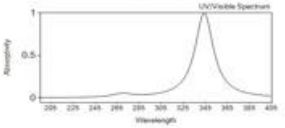
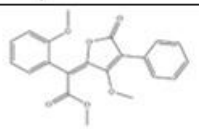
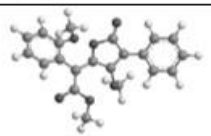
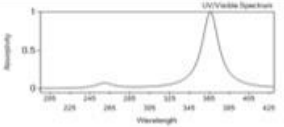
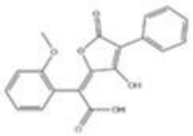
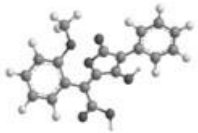
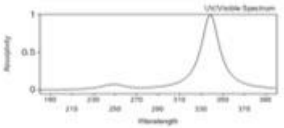
Compound	2D Structure	3D Structure	Predicted UV/Vis Spectrum Species		Spectrum	UV (MeOH)
			Oscillator Strength	Wavelength (nm)		
Calycin $C_{18}H_{10}O_5$ (306.26 g/mol)			0.0020 0.0630 0.1020	265.6615 275.8884 384.5665		241 nm 253 nm 430 nm
Epanorin $C_{25}H_{25}NO_6$ (435.45 g/mol)			0.0320 0.0930 1.0520	237.5632 251.0819 352.2280		204 nm 238 nm 280 nm 364 nm
Pinastric acid $C_{20}H_{16}O_6$ (352.33 g/mol)			0.0290 0.0880 1.0300	260.5806 277.2456 374.3486		291 nm 383 nm
Rhizocarpic acid $C_{28}H_{23}NO_6$ (469.46 g/mol)			0.0420 0.0910 1.0780	241.4493 257.2821 356.4814		283 nm 368 nm
Leprapinic acid $C_{20}H_{16}O_6$ (352.33 g/mol)			0.0020 0.0420 1.0010	257.1220 270.1182 343.8276		206 nm 274 nm 374 nm
Leprapinic acid methyl ether $C_{21}H_{18}O_6$ (366.35 g/mol)			0.0590 0.0100 1.0010	258.8398 263.5159 365.8431		229 nm 261 nm 336 nm
Demethylleprapinic acid $C_{19}H_{14}O_6$ (338.30 g/mol)			0.0220 0.0590 1.0570	240.5593 250.0186 338.2926		223 nm 266.5 nm 269 nm 273 nm 365 nm

Table 1. **(***Continued***)**

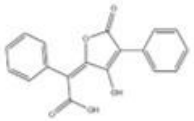
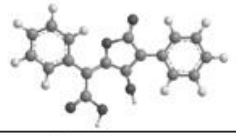
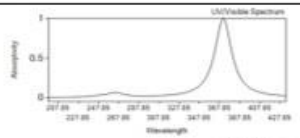
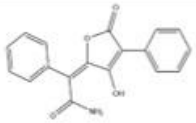
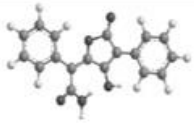
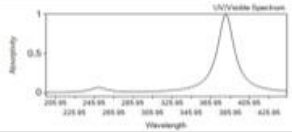
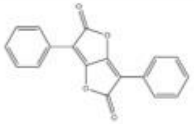
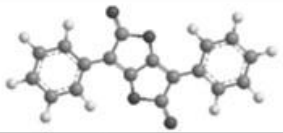
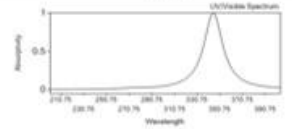
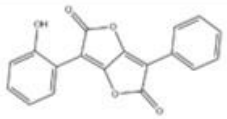
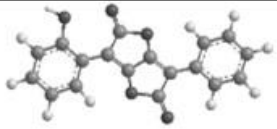
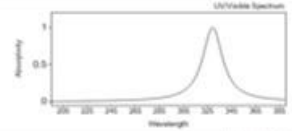
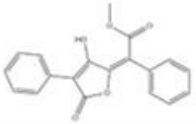
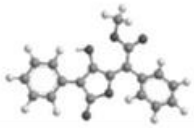
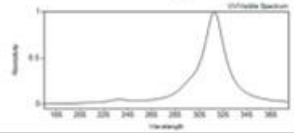
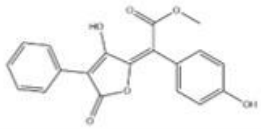
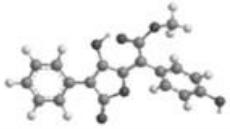
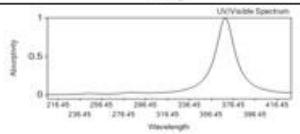
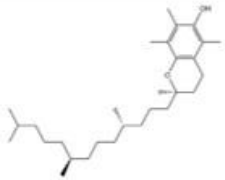
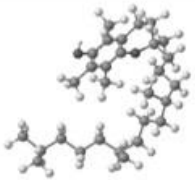
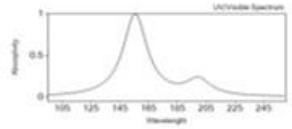
Pulvinic acid $C_{18}H_{12}O_5$ (308.28 g/mol)			0.0140 0.0640 1.1440	247.8198 264.5279 371.5440		256 nm 357 nm
Pulvinamide $C_{18}H_{13}NO_4$ (307.28 g/mol)			0.0070 0.0690 1.1990	248.6647 250.1195 381.2554		276 nm 377 nm
Pulvinic acid lactone $C_{18}H_{10}O_4$ (290.26 g/mol)			0.0010 0.0120 1.1780	259.5985 273.8773 344.8797		236 nm 249 nm 256 nm 376 nm
2-Hydroxypulvinic acid lactone $C_{18}H_{10}O_5$ (306.26 g/mol)			0.0010 0.0010 1.1700	257.2821 267.1498 329.0452		***
Vulpinic acid $C_{19}H_{14}O_5$ (322.30 g/mol)			0.0190 0.0460 1.0690	257.1220 258.4620 357.5093		290 nm 366 nm
4-Hydroxyvulpinic acid $C_{19}H_{14}O_6$ (338.30 g/mol)			0.0150 0.0230 1.0680	245.2220 283.3278 369.0007		***
α-Tocopherol $C_{29}H_{50}O_2$ (430.71 g/mol)			0.7440 0.0020 0.1450	155.5832 188.1400 199.6204		250 nm 260 nm 292 nm 298 nm

Table 2. The computationally evaluated activities on PASS, PaDEL-DDPredictor and VEGA QSAR platforms, the given relative weights as percentages (*w*) and calculated *p*-values (weighted averages).

Compound	***Photosensitizer (PASS)***	***Cytotoxic (PASS)***	***Cytoprotectant (PASS)***	***Reactive Metabolites forms adduct (PADEL)***	***Free radical scavenger (PASS)***	***Antioxidant (PASS)***	***Irritation (PASS)***	***Skin irritation (PASS)***	***Skin Irritation (PADEL)***
Calycin	0,523	0,240	0,379	Positive	0,438	0,307	0,241	0,192	Negative
Epanorin	0,307	0,296	0,284	Negative	0,275	0,221	0,285	0,186	Negative
Pinastric acid	0,351	0,223	0,497	Negative	0,407	0,288	-	0,095	Negative
Rhizocarpic acid	0,351	0,221	0,338	Negative	0,287	0,234	0,199	0,186	Negative
Leprapinic acid	0,351	0,169	0,537	Negative	0,386	0,276	-	0,170	Negative
Leprapinic acid methyl ether	0,345	-	0,476	Negative	0,235	0,131	-	0,158	Negative
Demethylleprapinic acid	0,359	0,212	0,585	Negative	0,467	0,298	0,187	0,178	Negative
Pulvinic acid	0,378	0,273	0,554	Positive	0,403	0,339	0,250	0,288	Negative
Pulvinamide	0,344	0,258	0,505	Positive	0,339	0,288	0,207	0,166	Negative
Pulvinic acid lactone	0,468	0,240	0,504	Positive	0,315	0,220	0,307	0,290	Negative
2-Hydroxypulvinic acid lactone	0,444	0,345	0,463	Positive	0,455	0,325	0,312	0,236	Negative
Vulpinic acid	0,381	0,229	0,491	Negative	0,347	0,295	0,177	0,105	Negative
4-Hydroxyvulpinic acid	0,341	0,243	0,502	Negative	0,403	0,315	0,184	0,108	Negative
α-Tocopherol	0,252	0,490	-	Negative	0,783	0,968	0,223	0,178	Positive
Relative weight (%)	*+15*	*-15*	*+15*	*-15*	*+20*	*+20*	*-15*	*-15*	*-5*

Table 2. (*Continued*)

Compound	***Skin Sensitization (VEGA TOX)***	***Ocular toxicity (PASS)***	***Serious Eye Damage (PADEL)***	***Serious Eye Irritation (PADEL)***	***Eye/Skin Corrosion (PADEL)***	***Antipruritic, anti-allergic (PASS)***	***Antibacterial (PASS)***	***Anti-inflammatory (PASS)***	***Weighted Average***
Calycin	Sensitizer Reliability *	0,371	Negative	Negative	Negative	0,471	0,384	0,413	*0,058125*
Epanorin	NON-Sensitizer Reliability *	0,408	Negative	Negative	Negative	0,390	0,174	0,666	*0,133100*
Pinastric acid	Sensitizer Reliability *	0,331	Negative	Negative	Negative	0,293	0,164	0,926	*0,244550*
Rhizocarpic acid	NON-Sensitizer Reliability *	0,273	Negative	Negative	Negative	0,244	-	0,680	*0,163300*
Leprapinic acid	Sensitizer Reliability *	-	Negative	Negative	Negative	0,253	0,164	0,921	*0,284075*
Leprapinic acid methyl ether	Sensitizer Reliability *	-	Negative	Negative	Negative	0,232	-	0,931	*0,227275*
Demethylleprapinic acid	Sensitizer Reliability *	0,285	Negative	Negative	Negative	0,310	0,229	0,906	*0,246925*
Pulvinic acid	Sensitizer Reliability *	0,385	Negative	Negative	Negative	0,391	0,270	0,938	*0,058050*
Pulvinamide	Sensitizer Reliability *	0,391	Positive	Negative	Negative	0,306	0,330	0,841	*-0,016825*
Pulvinic acid lactone	Sensitizer Reliability *	0,393	Negative	Negative	Negative	0,463	0,317	0,476	*-0,002775*
2-Hydroxypulvinic acid lactone	Sensitizer Reliability *	0,415	Positive	Negative	Negative	0,462	0,375	0,500	*-0,019225*
Vulpinic acid	Sensitizer Reliability *	0,350	Negative	Negative	Negative	0,301	0,199	0,942	*0,210775*
4-Hydroxyvulpinic acid	Sensitizer Reliability *	0,344	Negative	Negative	Negative	0,290	0,189	0,933	*0,215850*
α-Tocopherol	Sensitizer Reliability *	0,284	Negative	Negative	Negative	0,217	0,213	0,830	*0,272525*
Relative weight (%)	*-5*	*-15*	*-5*	*-5*	*-5*	*+15*	*+7,5*	*+7,5*	

Although pulvinic acid derivatives display ultraviolet profiles qualified by two absorption bands with a high absorption coefficient, some pulvinic acid derivatives such as calycin, pulvinic acid, pulvinamide, pulvinic acid lactone and 2-hydroxypulvinic acid lactone were identified as unpromising photoprotective substances due to their low *p*-values that were statistically obtained by the negative and positive factors (Table 2 and Fig. 3). Calycin, pulvinic acid lactone and 2-hydroxypulvinic acid lactone, for example, seem to be the most active photosensitizer substance by the predicted values in PASS platform but they have also serious negative effects such as the adduct formation by reactive metabolites, skin sensitization, irradiation, etc., as can be seen in Table 2. Thence, the photoprotective and other beneficial effects of these substances are suppressed by the negative activities. In the literature, there are some studies about rhizocarpic acid that is determined in the present study as promising photoprotective substance depending on the calculated *p*-value (Table 2) [32,9]. Compatible with our predicted results, Rubio and co-workers (2002) have suggested that the accumulation of rhizocarpic acid in lichens is increased depending on UV-B radiation levels [9]. On the other hand, pinastric acid, leprapinic acid, demethylleprapinic acid, leprapinic acid methyl ether, 4-hydroxyvulpinic acid and vulpinic acid were determined as the most promising photoprotective compounds toward ultraviolet-induced skin damage (Table 2). Vulpinic acid seems to be most studied one in these significantly promising compounds and previously obtained results showed that vulpinic acid is a good candidate as a photoprotective compounds against ultraviolet-induced skin damage by having no significant toxic effects and harmlessly elimination capability of the absorbed energy from the ultraviolet rays [3,5]. Similarly, the studies about pinastric acid have showed that pinastric acid have an antioxidant, photoprotective and neuroprotective acitivities in a correlation with our *in silico* data [33,34]. However, there is no reference about the protective or harmful features of other pulvinic acid derivatives, which have been identified as the significantly promising photoprotective compounds in our *in silico* data. Consequently, this paper reveals that the mathematical and computer models that have been generated by the perspective of structure–activity relationships provide significantly realistic results though these *in silico* platforms need to be improved by supporting more data about structure–activity relationships. We therefore think that researchers the computational chemistry, biology and pharmacology platforms such as Gaussian, GAMESS, PASS, PaDEL-DDPredictor and VEGA QSAR should be employed more to improve the scientific quality and discussion perspective of the produced research papers and projects, and to decrease the necessary R&D budget and workload.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Review Article**

Nutritional and Antinutritional Factors of Some Pulses Seed and Their Effects on Human Health

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Abstract: The Food and Agriculture Organization (FAO) realizes about fifteen pulses (primary and minor) such as dry peas, black beans, chickpeas, roman bean, beans, and lentils etc. cultivated worldwide over a hundred countries. Similarly pulses especially bean, chickpea and lentil are really popular in Turkish Cuisine culture and there are many meals made with the pulses in Turkey. Thus, seed quality of the pulses is too significant for producers to be sold at higher prices. Moreover, it is also important for human health with over nutrition rates and has high levels of minerals as well as folate and other B-vitamins and diminished rate of diseases particularly resulting from obesity due to the high level of fiber and protein rates. But pulses seeds have also antinutritional factors such as some enzyme inhibitors (trypsin and chymotrypsin proteinase inhibitors), phytic acid, flatulence factors, lectins and saponins, and some different allergens. The factors cause some health problem such as mineral (Fe, Zn, Mg etc.) deficiency of human body. So, we prepared the review to show situation of some pulses in Turkey and in the world and to explain some antinutritional factors (secondary metabolites) of pulses seeds besides some nutritional characteristics.

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1. INTRODUCTION

Pulses are identified as one of the earliest domesticated plants by humans and have played critical part of food, especially during the transition period from hunting-gathering times to agriculture. There are some archaeological evidences about cultivation of pea on The Fertile Crescent dating back to 11,000-10,000 years BP in Syria and Turkey [1]. Pulses are unique in comparison to other plant foods owing to higher proportions of protein contain. In fact, the protein content of pulses ranges from 17 to 30 % of dry weight which is typically twice the amount found in cereals [2], but it hasn't got some essential amino acids especially Cysteine and Methionine [3]. Despite in a relatively low number of calories, pulses are providing

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substantial amounts of vitamins and minerals. Because of diverse ecological conditions in Turkey, pulses can be grown throughout the year. They are grown on approximately 63-million-hectare area with annual production of 53 million tons in Turkey [4].

Pulses have shown numerous health benefits via the protection against cardiovascular diseases owing to having dietary fiber content [5] and combined with vitamin C improves iron absorption [6] and promotes bone health [7]. They are rich sources of complex carbohydrates, minerals as well as folate and other B-vitamins, protein [8]. Furthermore, the whole pulses are an abundant source of some key minerals include iron, zinc, selenium, phosphorous and potassium and high-quality oil and folic acid, isoflavones and tocopherol [9]. Therefore, it is said that pulses are identified as natural preservative of obesity and related disorders including coronary heart disease, diabetes, and the metabolic syndrome [10 –11]. There are recommended as part of healthy eating for lower glycemic index by health organizations globally [12].

Nowadays, there are a lot of studies about the nutritional benefits of pulses [13, 14, 80–81] and their suggestions have been that more consumption of pulses (dried seeds from the legume family such as dry peas, black beans, chickpeas, beans, and lentils) because of seeds nutritional quality [82]. Moreover, for most of them, the more legume consumption has been an elixir of the healthier life of the world [83]. But the seeds have also some compounds (antinutritional factors) such as some enzyme inhibitors (trypsin and chymotrypsin proteinase inhibitors), phytic acid, lectins and saponins, and some different allergens etc. besides protein, fiber, vitamins and minerals. The compounds are named as secondary metabolites which synthesize in plants consequence of frequent bioactivities and sometimes may cause some health problems. With this review, we tried to determine agricultural situation of some pulses in Turkey and in the world and some positive and negative effects of pulses on human health.

2. MATERIAL and METHODS

2.1. Bean

Beans usually refers to food legumes of the genus *Phaseolus*, family *Leguminosae*, subfamily *Papilionoideae*, tribe *Phaseoleae*, and subtribe *Phaseolinae*. *Phaseolus vulgaris* originated from Central and South America, where it was cultivated as early as 6000 BC in Peru and 5000 BC in Mexico. Then it was introduced to the Old World by the Spaniards and the Portuguese. It is now widespread and cultivated as a major food crop in many tropical, subtropical and temperate areas of the Americas, Europe, Africa and Asia [15]. It grows from sea level up to an altitude of 2200-3000 m where annual rainfall is between 300 and 4300 mm and average temperatures range between 15°C and 23°C. The global harvest today has been estimated approximately 18.7 million tons from nearly 150 countries (27.7 million hectares) [4]. While *P. vulgaris* is by far the most economically important domesticated species of the genus *Phaseolus*, there are four others: *P. dumosus*, *P. coccineus*, *P. acutifolis* and *P. lunatus* [16]. It has slight frost tolerance but growth stops below 10°C and frost hinders yield at various stages of growth [17–15]. Furthermore, yield of beans is also largely dependent on-air temperature of the first flowering date and then. Because the probability of the flowers that firstly opened are more likely to hold the pods than the others [18]. The common bean is a warm season legume under subtropical conditions.

Beans are an important crop in Turkey economically, since their production and export has increased significantly in recent years. Because dry weather during the maturing stage which is prevalent especially Mediterranean climate condition in a large part of Turkey, benefits seed preservation. The common bean grows well on a large variety of soils with pH ranging from 4 to 9. However, it does better on well-drained, sandy loam, silt loam or clay loam soils, rich in organic content [19, 88]. There are some bean varieties such as Akman-98, Doruk,

Karacaşehir-90, Noyanbey-98, Sarıkız, Horoz and Sarnıç suitable for climate and environmental conditions in Turkey. Today, climate change and the varieties adaptation of these is one of the most important issues in our agricultural production [89]. It generally cannot withstand waterlogging though some cultivars do well in standing water. Bean is sensitive to Al, B, Mn and high levels of Na. Deficiencies in minerals may arise in calcareous soils (Zn deficiency) and in sandy acidic soils (Mg and Mo deficiencies) [15].

2.2. Chickpea

Chickpea (*Cicer arietinum* L.) is one of the largest produced food legumes in South Asia and the third largest produced food legume globally, after common bean (*Phaseolus vulgaris* L.). Chickpea is grown in more than 50 countries (89.7% area in Asia, 4.3% in Africa, 2.6% in Oceania, 2.9% in Americas and 0.4% in Europe). India is the largest chickpea producing country accounting for 64% of the global chickpea production. The other major chickpea producing countries include Pakistan, Turkey, Iran, Myanmar, Australia, Ethiopia, Canada, Mexico and Iraq. Production area of chickpea is about 11.0 million hectares and production value is 8.8 million tons and average yield is nearly 800 $kg.ha^{-1}$ [4]. Chickpea production is almost half of bean, so it can be identified as the second most important grain legume [91]. Social factors and ecological constraints determine whether bean or chickpea are grown in a particular region [90].

There are two distinct types of chickpea cultivars named desi (microsperma) and kabuli (macrosperma) [20]. The desi type which has brown, yellow, green or black color and thick seed coat account for about 80-85% of the total chickpea area and are mostly grown in Asia and Africa [21]. While the kabuli type which has white or beige color and thin seed coat is grown small part of the world.

2.3. Lentil

Lentil (*Lens culinaris* Medik.) may have been one of the first agricultural crops grown more than 8,500 years ago. Production of the cool season annual crop spread from firstly the Near East to the Mediterranean area, then Asia and Europe and finally the Western Hemisphere. The crop has received little research attention to improve its yield and quality. It grows well in limited rainfall areas of the world [22]. The highest lentil production values of the world are made countries like Canada, Indian, Turkey, USA, Nepal and Australia. Besides most exports of lentil in the world is also done by Canada, Turkey, USA and Australia [23].

In recent years, lentil production in Turkey has increased substantially. Red lentils are grown intensely in southeastern Anatolia region [24]. Not only local varieties, but also culture varieties such as Fırat-87 (Komando), Seyran-96 and Çağıl are grown in the region. Red lentils are divided into 2 classifications as crusted lentils and red inner lentils [92]. Both groups are also divided into 2 classifications named first class and second class with some characteristics such as humidity, total foreign matter presence, % inorganic matter presence, total defective grain presence, presence of insect damaged grain, broken-shell peeled grain existence etc. [25]. Lentil growth rates are slow during early stages of vegetative growth and weeds can quickly overgrow on the crop if not adequately controlled [26]. Weeds compete with the crop for nutrients, water, and light, reducing crop yields and grain quality. Yield losses in lentil of 40-80%, as a result of weeds, have been reported [27–28].

3. DISCUSSION

3.1. Antinutritional factors of pulses and its some effects of human health

Pulses have importance both nutritional and worldwide commercial because, they are easy to store and are rich in protein and fiber [29]. However, their nutritional value is limited

by the presence of antinutritional factors such as some enzyme inhibitors (trypsin and chymotrypsin proteinase inhibitors), phytic acid, flatulence factors, lectins and saponins etc. [30]. It is noted that a significant correlation between nutritional factors (protein – starch or protein – oil) but found no correlation between antinutritional factors (phytic acid - flatulence factors or lectins – saponins) [31]. It is explained below that some of the most important antinutritional factors of pulses and their effects on the feed rate.

3.1.1. Trypsin and chymotrypsin proteinase inhibitors

Protease inhibitors are found everywhere in nature. There are a lot of studies observed various important biological functions of the inhibitors like digestion of proteins, control of blood clotting in human, signaling receptors interaction in animals and defense against insect attack in plant [31, 93 – 94]. They are present in significant amounts in plants and belong to two major groups, namely Bowman-Birk type and Kunitz type protease inhibitors. Kunitz type protease inhibitors include at least nine trypsin and chymotrypsin inhibitors [32].

Proteinase inhibitors of plants are small molecular weight proteins that are natural, defense-related proteins often present in seeds and induced in certain tissues by herbivory or wounding. Most storage organs such as seeds (*Leguminosae* and *Graminae*) contain about 1 to 10% of their total protein, which inhibit different types of enzymes [33]. Thus, their main function is thought to be in plant defense, the regulation of endogenous proteinases in addition to the prevention of unwanted proteolysis [34]. There are a number of reports in the literature related to proteinase inhibitors in various legume species. Studies discussed the role of trypsin and chymotrypsin inhibitors which decrease protein digestibility if not properly inactivated during processing [35, 36–95]. Moreover, some study showed that trypsin inhibitors play also a protective role against attack by insects [37, 99–100].

For example, 30-40% of cysteine in bean protein is found in protease inhibitor structure [38]. Protease inhibitors are resistant to small intestinal digestion. They are increase the activity of feces by binding proteases [39]. Therefore, the availability of sulfur-containing amino acids in leguminous grains is low.

3.1.2. Phytic acid

The trace elements copper, manganese, iron and zinc special attention when evaluating the nutritional adequacy of vegetarian or vegan diets. Moreover, the elements are also vital for children from the age of development and pregnant or lactating women [40]. The world population, particularly in Latin America, Sub-Saharan Africa, the Caribbean and Southeast Asia, is at risk for micronutrient intake [41]. Recent reports indicate that Fe deficiency is the most prevalent micronutrient problem in the world, affecting over 2 billion people globally [42]. An estimated 49% of the world population is at risk for low Zn intake [43]. Zn deficiency of children are common in the world [44–45]. Calcium content in rural diets in developing countries is not adequate [46] and dietary Ca deficiency has been linked to several chronic diseases, including osteoporosis [47].

Phytic acid in foods of plant origin forms a complex with dietary minerals such as calcium, zinc, iron, and magnesium and makes them biologically unavailable for absorption. Phytic acid is also widely distributed in legume seeds and it accounts for about 78% of the total phosphorus in pulses [48]. Phytic acid has also been linked to the inhibition of digestive enzymes such as protease and alpha amylases [49]. Phytic acid binds trace elements and macro-elements such as zinc, calcium, magnesium and iron, in the gastrointestinal tract are making dietary minerals unavailable for absorption and utilization by the body [50–51]. It can also form complexes with proteins, proteases and amylases of the intestinal tract, thus inhibiting proteolysis [52]. Moreover, the phosphorus in phytate has been considered to be largely

unavailable to the organism because of the limited capacity of monogastric species to hydrolyze phytate in the small intestine. Phytic acid is the major determinant of Zn absorption, especially for diets with a low animal protein content [53]. It strongly binds Zn in the gastrointestinal tract and reduces its availability for absorption and reabsorption [54]. Similarly, there is some evidence that phytic acid has also an inhibitory effect on the absorption of Fe in human [55 – 56]. Phytic acid decreases Ca absorption [57] and phytic acid breakdown improves Ca availability [79–58]. On the other hand, in spite of the presence of phytic acid, it is difficult to ascribe a negative effect to whole products on Mg absorption [101]. Effective reduction of phytic acid can be obtained via the action of exogenous phytic acid degrading enzymes [59].

3.1.3. Saponins and lectins

Saponins are secondary plant metabolites present in pulses, containing a carbohydrate moiety (mono/oligosaccharide) attached to an aglycone, which may be steroidal or triterpenoid in structure [102]. They are generally characterized by their bitter taste (those in liquorice are an exception, being sweet), their ability to foam in aqueous solutions, and their ability to hemolyse red blood cells, these latter properties being a consequence of their amphiphilic properties [85]. Ingestion of foods with saponin have been both deleterious and beneficial effects. On the one hand, saponins have unfavorable effects such as low weight in animals and hypocholesterolemia in humans [103]. On the other hand, they reduce the risk of heart diseases in humans with a diet rich in food legumes containing saponins [104]. Following oral administration, saponins are only poorly absorbed and are either excreted unchanged or metabolized in the gut. Detailed information on the fate of saponins in the animal gut is lacking, but enteric bacteria, intestinal enzymes, and gastric juices most likely cause breakdown within the gastrointestinal tract [105].

Lectins (hemagglutinins or phytohemagglutinins) are a group of proteins of nonimmune origin in nature. They are found in nature as share the property of specifically and reversibly binding to carbohydrates either in free form or as part of more complex structures. There were first described in 1888 by Stillmark working with castor bean extracts [84]. Moreover, many members of the lectinic protein family agglutinate (clump together) red blood cells. Some of the species of pulses seeds such as lentil, bean and pea also contain phytohemagglutinins (lectins) [61]. These are proteins which possess a specific affinity for certain sugar molecules. Most of the lectins contain 4 to 10% carbohydrates [62]. High levels of lectins (specialized proteins) may be found in grains (also known as cereals or pulses), legumes, dairy and plants in the nightshade family. Many other foods contain lectins but are less well studied and the amounts of lectins present are not thought to be as high or as potentially toxic [96]. The human digestive system was created to handle a variety of plant and animal proteins through the process of digestion and elimination. Some plant and animal proteins or lectins are severely toxic to humans and cannot be eaten without causing death like those in Castor beans and some mushrooms [63]. The major antinutritional toxic factor limiting the use of pulses. Although, in general, lectins are more resistant to heat-denaturation than other plant proteins, prolonged cooking can inactivate legume lectins [87]. However, as heat-processing is expensive and potentially damaging, it is usually kept to a minimum even with legumes, particularly when the product is to be used in animal nutrition [64].

Although pulses seeds also contain previously mentioned anti-nutritional factors such as enzyme inhibitors, lectins, flatulence factors, polyphenols, tannins, phytic acid and saponins. Most of them can be reduced or eliminated to some degree by different cooking techniques such as pressure cooking or any other else [65, 97–98].

3.2. Some positive effects of pulses on human health

Bean quality criteria can be listed as seed weight, wet seed weight, water uptake capacity, water uptake index, swelling capacity, cooking time etc. [66]. It can grow under higher temperatures (35°C) but this may hamper seed production. Due to their high concentrations of protein, fiber, and complex carbohydrates, beans are today one of the most important legumes in the world [67]. Moreover, bean seed is nutrient-dense, fiber-rich, and are high-quality sources of protein. The consumption of dry bean has been greatly connected with many physiological and health promoting effects such as prevention of many types of diseases [68]. Fiber has emerged as a leading dietary factor in the prevention and treatment of chronic diseases [86]. More studies show that dry bean intake has the potential to decrease serum cholesterol concentrations, improve many aspects of the diabetic state, and provide metabolic benefits that aid in weight control [70, 71–72]. Therefore, it could be said that more beans consumption is the best way for reducing the risk of some chronic diseases [69].

Chickpeas are generally considered to be a low glycemic index food [73] and it is a good source of carbohydrates and protein, and protein quality is considered to be better than the others. Furthermore, chickpea has significant amounts of all the essential amino acids except for Cysteine and Methionine which have an S-bond amino acid. In addition, it can be complemented by adding cereals to the healthy diet [74]. Starch is the major storage carbohydrate followed by dietary fiber, oligosaccharides and simple sugars such as glucose and sucrose. Although lipids are present in low amounts, chickpea is rich in nutritionally important unsaturated fatty acids such as linoleic and oleic acids. β-Sitosterol, campesterol and stigmasterol are important sterols present in chickpea oil. Ca, Mg, P and, especially, K are also present in chickpea seeds. Chickpea is a good source of important vitamins such as riboflavin, niacin, thiamin, folate and the vitamin A precursor β-carotene [75].

Lentil is defined as relatively a cheaper source of protein, rich vitamins and minerals. Moreover, the seeds can be defined as rich fiber source [60]. High fiber intakes are associated with lower serum cholesterol concentrations, lower risk of coronary heart disease, reduced blood pressure, enhanced weight control, better glycemic control, reduced risk of certain forms of cancer, and improved gastrointestinal function [78]. So, more health organizations are recommended that people need to increase the consumption of lentil both part of healthy diets for reduce of nutritional deficiencies and against some diseases such as diabetes, heart disease, cancer and cardiovascular disease.

Pulses are annually grown leguminous crops are often promoted in diet owing to their low cost and many beneficial nutritional effects [76]. It was detected significant negative correlations between some nutrients (protein-carbohydrate and protein-fat) [77]. Furthermore, it should be noted that numerous studies are available on the interactions between environmental and genetic factors on seed quality and nutritive value of different legumes [68].

4. CONCLUSIONS

Pulses seeds contain significant amount of proteins, carbohydrates, polyphenols, phytosterols, resistant starch, oligosaccharides and dietary fiber [13, 14, 80–81]. Therefore, nowadays, pulses have been defined as a full advocate against some diseases such as diabetes, heart disease, cancer and cardiovascular disease [82, 83]. Moreover, pulses are also important part of human diet against overweight with low fat content of seeds in many countries of the world. Pulses have a low glycemic index and are rich source of antioxidants, so regular intake of pulses can improve heart health and lowers blood cholesterol [10, 11, 12–102]. In spite of providing many nutritional factors of pulses seeds, there are also providing many antinutritional factors such as phytic acid, saponins, lectins, polyphenols, lathyrogens, protease inhibitors etc.

These factors can cause many illnesses such as some trace elements deficiency, unbalanced or malnutrition [40, 41, 42–46]. So, it should be noted that intense pulses intake in the daily diet over a long time may occur some negative results for human health especially for pregnant or lactating women, children of growth age and vegetarians. Furthermore, it should be kept in mind that just some cooking techniques can reduce these negativities to some degree [65,97,98].

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The phenolic content, antioxidant and cytotoxic activities of *Origanum sipyleum* from Turkey

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Abstract: O*riganum sipyleum* is a medicinal plant species belonging to the genus *Origanum* and traditionally vegetative parts of this plant have been used as medicinal tea or food additives. The current study has been designed to examine the antioxidant, cytotoxic activities and total phenolic, flavonoid and tannin contents as well as the chemical composition of the ethanol extract of *O. sipyleum*. The antioxidant capacity of *O. sipyleum* was determined using six complementary methods, β-carotene/linoleic acid and phosphomolybdenum test systems, radical scavenging (ABTS and DPPH), metal chelating and reducing power assays. The phenolics were identified using HPLC. A brine shrimp *(Artemia salina L.)* lethality test was used for determining cytotoxic activity. The ethanol extract exhibited high DPPH free radical scavenging (DPPH, IC_{50}:102.75 µg/mL), ABTS radical scavenging (ABTS, IC_{50}:88.64 µg/mL), metal chelating (20.68 %) and reducing power capacity (0.51 mg/mL). The antioxidant activities of the *O. sipyleum* with β-carotene/Linoleic acid and phosphomolybdenum were calculated as 85.59 % and 62.95 µg/mg respectively. The phenolic contents of the ethanol extract were evaluated using HPLC and determined major phenolics: caffeic acid, epicatechin and 2,5 dihydroxybenzoic. Furthermore, to gether with cytotoxic activity (LC_{50}, 327.414 µg/mL) *O. sipyleum* is also rich in total phenolic, flavonoid and tannin contents were 203.57 ± 4.62 mgGAE/g, 46.98 ± 0.34 mgQE/g and 34.55 ± 0.56 mgCE/g respectively. These results could provide addition information for the potential use of this medicinal plant as a food ingredient and as a natural antioxidant in the diet, as well as for the pharmaceutical industry.

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1. INTRODUCTION

Free radicals are unstable molecules that have been shown to react with various other materials to form new compounds that contain high levels of toxicity. Free radicals are able to induce cellular damage, oxidize protein, lipid and thereby can cause diseases [1, 2]. Antioxidants are substances that may inhibit these illnesses and due to these beneficial effects. Food and medicinal products are routinely enriched with synthetic antioxidants (BHT and

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BHA). Conversely, there is evidence of manufactured antioxidants being potentially toxic. Therefore, there is great interest in the isolation of the safer and effective antioxidants that are naturally present in plants [3-5].

Plants possess active natural compounds that differ widely in terms of biological properties and can be used in various fields, such as food ingredients, as well as medicinal and pharmacological applications. For this reason, there has been increased interest in research on the isolation and identification of these compounds [6,7]. Many plants belonging to the Lamiaceae family contain chemical compounds with antioxidant activity [6]. Among the Lamiaceae members, *Origanum* species are among the important and popular plants. They are used as an anti-diabetic, digestive, diuretic, and for respiratory problems [8]. *Origanum sipyleum* is one of the endemic species of the *Origanum* genus and used as a medicinal tea or as a food additive [9].

Previous studies on antioxidant capacities of *Origanum* species were conducted [10-13]. There were also few studies dealing with the biological activities of *O. sipyleum* [8, 9, 14], but there were no comprehensive report that has studied the phenolic composition, antioxidant and cytotoxic activities of ethanol extract of *O. sipyleum*. Therefore, more research is required on the biological activities of this aromatic and medicinal plant. With these points in mind, the objectives of the present study are to evaluate the antioxidant capacities, the cytotoxic activity, the total phenolic, flavonoid and tannin contents as well as the chemical composition of the ethanol extracts of *O. sipyleum*.

2. MATERIALS and METHODS

2.1. Plant materials and preparation of plant extracts

O. sipyleum. was collected in June 2017 from above the Çamlık forest, on the old Denizli-Kızılcabölük road, in mixed *'Pinus brutia-Quercus coccifera'* forest clearings, Denizli, Turkey The plant materials were identified and stored with voucher specimens (*O. sipyleum*; Herbarium No: No: 2017-4-92) at the private herbarium of Dr. Mehmet Cicek, a plant taxonomist from the Pamukkale University, Denizli, Turkey. The stem, flowers and leaves of *O. sipyleum* were air-dried and milled. The extractions were performed by mixing the sample (20 g) with 200 mL of ethanol and shaking at 50 ^{o}C for 6 h in a temperature controlled shaker. The extracts were filtered twice with filter paper and evaporated using a rotary evaporator under vacuum at 40-50^{o} C. The samples were lyophilized and kept at -20 ^{o}C until tested. The assays were carried out in three sample replications and values were represented as the average of three replicates.

2.2. Evaluation of DPPH free radical and ABTS radical cation scavenging activity

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was used for determining radical cation scavenging activity according to the method described by Shalaby and Shanab [15]. ABTS radical cation was prepared by reacting 7mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was held for ~16 h in a dark room. Prior to the assay, the ABTS solution was diluted with ethanol to an absorbance of 0.700±0.05 at 734 nm. A total of 900 µL ABTS solution was mixed with 100µL of the extracts. The mixture was incubated at room temperature for 15 min. and then the absorbance of this mixture was measured. The results were assessed as IC_{50} values (concentration in µg/mL that cause 50% inhibition of the ABTS radicals).

The DPPH (2,2-Diphenyl-1-picryl hydrazyl radical) free radical was used for determining the free radical scavenging activity according to the procedure of Meriga et al. [16]. The different concentrations of the extracts (1 mL) were mixed with methanolic DPPH solution (4 mL). After 30 minutes, the absorbance of the extracts was recorded at 517 nm. These outcomes

were calculated as IC_{50} values, that is the strength of the sample required to scavenge 50% of DPPH radicals.

2.3. Determination of total antioxidant capacity by β-carotene/linoleic acid and Phosphomolybdenum assay

The antioxidant capacity was evaluated using the method of Amin and Tan [17]. β-carotene (2 mg) was dissolved in chloroform (10 mL) to prepare the stock solution and Linoleic acid (20 µL) and 200 µL of 100% Tween 20 was added for one milliliter of the solution. The chloroform was removed using a rotary evaporator and then the remaining residue was added to 100 mL of dH_2O. This emulsion (24 mL) was mixed with the extracts (1 mg/mL). A spectrophotometer was immediately used to measure the initial absorbances at 470 nm. The reaction mixture was incubated for 2 hours at 50° C. Following this the measurement of the absorbance of this mixture was repeated, and a synthetic antioxidant (BHT, Butylated hydroxytoluene) was applied as the positive control.

The antioxidant capacity of *O. sipyleum* extract was also determined using the phosphomolybdenum method according to that of Prieto et al. [18]. The sample preparation and procedure followed that given by Kaska et al. [19]. The antioxidant activity of the extracts was calculated using the ascorbic acid graph.

2.4. Measurements of Ferric ion reducing power activity

The reducing power of the *O. sipyleum* was estimated using the method described by Oyaizu [20] with slight modifications. Different concentrations of the samples (1 mL) were combined with 0.2-M phosphate buffer (1 mL) and 1% potassium ferricyanide (1 mL). The mixture was kept at 50°C for 20 min. Trichloroacetic acid (10%) was added to reaction mixture. The aliquot of the upper layer (1.5 mL) was combined with the same volume of the ddH_2O and 0.1% ferric chloride. After 10 min the absorbance was read, at 700 nm.

2.5. Measurements of Metal chelating capacity

The metal chelating assay was conducted using the method of Karpagasundari and Kulothungan [21] with slight modifications. The plant sample and 3.2 mL of ddH_2O was mixed with 2 mM $FeCl_2$ (0.1 mL) solution. After 30 s, ferrozine (5 mM) was added. By adding ferrozine, the reaction was activated. After approximately 10 min at room temperature, the absorbance of the solutions was read at 562 nm. The synthetic metal chelator (EDTA) was applied as the positive control. The metal chelating activity was calculated in the following way:

$$\text{Chelating ability (\%)} = [(A_{co} - A_{samp}) / A_{co}] \times 100,$$

(A_{co} : absorbance of the control and A_{samp} : absorbance of the extract or positive control, EDTA.)

2.6. Determination of total phenolic, flavonoid and tannin contents

The total phenolic content was ascertained according to the Folin-Ciocalteu method [22], the total flavonoid content of the extracts was determined using the method of Arvouet-Grand et al. [23] and the Tannin content was investigated using the vanillin-HCL method [24]. The method of Kaska et al. [19] provided the sample preparation and procedure for determining the total phenolic, flavonoid and tannin content. The outcomes were shown as the equivalents of Gallic acid (mgGAE/g), quercetin (mgQEs/g) and catechin (mgCEs/g) for phenolic, flavonoid and tannin content respectively.

2.7. Quantification of phenolic compounds by HPLC

For the determination of the phenolic compound reversed-phase high performance liquid chromatography (RP-HPLC, Shimadzu Scientific Instruments) was used. The phenolic composition of the ethanol extract of *O. sipyleum* was determined according to the method of Caponio et al. [25] with slight modifications. The procedure followed was given by Kaska et al. [19]. Gallic, 3,4 dihydroxybenzoic, 4-hydroxybenzoic, 2,5 dihydroxybenzoic, chlorogenic, vanillic, caffeic, *p*-coumaric, ferulic, cinnamic acid and quercetin, epicatechin, rutin were used as standards and quantitative analysis was made by comparing these standards. The results were expressed as µg/g of each compound from the total phenolic compounds.

2.8. Cytotoxic bioassay

The possible cytotoxic activity of *O. sipyleum*, was evaluated using the Brine shrimp lethality bioassay [26]. The brine shrimps (*Artemia salina*) were hatched using *A. salina* eggs in a beher-beaker (1 L), filled with air-bubbled sterile artificial seawater (prepared using sea salt 38 g/L) and left to incubate under artificial light for 24-48 h at 28 ºC. The tubes containing ten nauplii, different concentration of extracts (1000, 500, 100, 50 and 10ppm) and brine solution as well as the control tubes were maintained under artificial light for 24 h at 28 ºC. The experiments were conducted in a set of three tubes per concentration and the controls. For each concentration of the extracts and the controls, the number of dead shrimps were counted and recorded using an overhead projector. The larvae were regarded as dead if no activity of the appendage was seen within 10 sec. The EPA Probit Analysis Program was used for data analysis.

2.9. Statistical analysis

The standard and the different groups were compared via t-test by using MINITAB.

3. RESULTS and DISCUSSION

An evaluation of the antioxidant capacity of plants cannot be conducted by a single standard method due to the complex structure of the compound to be analyzed. Consequently, a single assay could not accurately reflect the antioxidant capacities of the plants [27]. For this reason, several antioxidant methods (DPPH, ABTS, phosphomolybdenum, metal chelating activity etc.) were applied to evaluate the true antioxidant potential of the *O. sipyleum*.

3.1. Radical scavenging capacity (DPPH and ABTS)

A well-known mechanism for ascertaining the antioxidant activity of plants is through ABTS radical scavenging capability hydrogen donating [28]. The results of the radical scavenging capability were calculated to be a concentration, 50% of which was scavenged by ABTS (IC_{50}). The low IC_{50} value shows the high radical scavenging property. The ABTS radical scavenging capacity of extracts from *O. sipyleum* is presented in Table 1 and there were statistically differences among the radical scavenging activity of the ethanol extract of *O. sipyleum* and BHT ($t=31.47$, $df=11$, $p<0.001$).

The results of the DPPH free radical scavenging activity presented in Table 1 and the findings indicate that the ethanol extract of *O. sipyleum* exhibits radical scavenging activity. The DPPH radical scavenging activities for the ethanol extract determined in this study were higher than in methanol, water and acetone extracts and lower than in the ethanol extract from *O. sipyleum* as reported by Nakipoglu et al. [8]. The phenolic content may be attributed to the DPPH radical scavenging activity of *O. sipyleum*. Oxidative damage caused to cells by free-radicals may potentially be mitigated by these phenolic compounds [29]. As shown in the present study, the ethanol extract of *O. sipyleum* has potent radical scavenging activities and findings reveal that this extract could serve as a strong radical scavenger, and due to this

property it could be possible to use *O. sipyleum* plants in pharmacological applications as radical inhibitors or scavengers.

Table 1. Antioxidant properties of O.*sipyleum*

Sample	DPPH (IC_{50}, µg/mL)	ABTS (IC_{50}, µg/mL)	β-carotene/linoleic acid (%)	Phosphomolybdenum (µg/mg)	Power reducing activity (mg/mL)	Metal chelating activity (%)
Ethanol	102.75 ± 1.6 a	88.64 ± 2.1 a	85.59 ± 1.2 b	62.95 ± 1.36	0.51 ± 0.08	20.68 ± 2.6 b
BHT	31.76 ± 1.7 b	12.89 ± 1.2 b	93.46 ± 0.3 a	nt	nt	nt
EDTA	nt	nt	nt	nt	nt	76.41 ± 0.2 a

BHT: Standard antioxidant, nt: not tested

*Values are mean of three replicate determinations (n=3) ± standard error. Mean values followed by different letters in a column are significantly different ($p<0.05$).

3.2. Antioxidant activity (β-carotene/linoleic acid and Phosphomolybdenum method)

The one of the main causes of food quality deterioration is lipid peroxidation. A antioxidants have the capability to delay the development of toxic oxidation, prolong the storage stability of foods and maintain nutritional quality and for this reason they are used in lipid containing foods [30, 31]. Nowadays, because of their antioxidant content numerous plants are regularly used as sources of nutritional additives to inhibit lipid peroxidation. Medicinal Lamiaceae plants with high antioxidant capacities are known to be efficient in delaying the process of lipid peroxidation in fatty foods [32]. In the present study, β-Carotene assay was used to evaluate the capability of plants regarding the inhibition of linoleic acid oxidation. The results demonstrate that the ethanolic extract of *O. sipyleum* shows strong antioxidant activity (Table 1). The level of antioxidant properties could be sufficient for this plant to be a natural source of antioxidant substances for use in the food industry as a natural additive.

The results of the phosphomolybdenum assay presented in Table 1 indicate that ethanolic extract from *O. sipyleum* possesses antioxidant capacities. The antioxidant activity of extracts depended on the presence of polyphenols [33] and the powerful antioxidant activity of the ethanolic extract of *O. sipyleum* may be attributed to the presence of polyphenols.

3.3. Metal chelating and Reducing power activity

The metal chelating and reducing abilities of the ethanol extract from *O. sipyleum* were measured in this study and the results shown in Table 1. The results indicate that the ethanol extract demonstrated potential metal chelating and power reducing antioxidant capacity.

Lipid peroxidation can be activated by metal ions starting a chain reaction bringing about the deterioration of food. For this reason, metal chelating capacity is critical as it decreases the volume of catalyzing transition metal in lipid peroxidation [34, 35]. The chelating abilities of the plants contribute directly to their antioxidant properties and can be considered as important mechanisms in the oxidation process. The metal chelating ability of *O. sipyleum* and EDTA were found to be statistically significant ($t=21.03$, $df=7$, $p<0.001$) (Table 1) and the ethanol extract of *O. sipyleum* is capable of chelating Fe^{+2} ions and the chelating agents existing in plant extracts have the ability to reduce the radical formation that can cause damage to living cells [36].

The reducing ability of extracts depends on the presence of polyphenols, which may act as reductones that exert antioxidant action by breaking the free radical chains by donating a hydrogen atom [37]. It has been reported that there is a correlation between total phenolic content and reducing power and metal chelating activity [38, 39].

In the present study, the reducing ability of ethanol extract from *O. sipyleum* was measured and the result of this activity demonstrated that the ethanol extract exhibited a high reduction ability (Table 1). The reducing power and metal chelating activities of *O. sipyleum* may be dependent on total phenolic content.

3.4. Total phenolic, flavonoid and tannin contents

In the present study, the total phenolic, flavonoid and tannin content in the ethanol extract from *O. sipyleum* was determined. The total phenolic, flavonoid and tannin content of ethanol extract were 203.57 ± 4.62 mgGAE/g, 46.98 ± 0.34 mgQE/g and 34.55 ± 0.56 mgCE/g respectively. The phenolic content of the ethanol extract determined in this study were lower than in the methanol extract from *O. sipyleum* as reported by Ozkan et al. [9].

Phenolic compounds (phenolic acid, flavonoid, tannin etc.) in plants possess antioxidant effects and the antioxidative properties of polyphenols based on their various abilities, as in their high reactivity as hydrogen or electron donating agents and, their metal chelating and radical scavenging properties [40].

Although, many reports indicate that besides polyphenols, nonphenolic components present in plants, such as ascorbates, carotenoids, and pigments contribute to the total antioxidative activities [41], phenolic compounds are responsible for a major part of the antioxidant activity of many plants [28]. Establishing the phenolic compounds of plants therefore plays a significant role in the identification of their medicinal properties. In the present study it was observed that there was high phenolic content and high antioxidant activity in the ethanol extract of *O. sipyleum* indicating that the phenolic compounds present in the extracts are largely responsible for antioxidant activity.

3.5. Phenolic composition

Lamiaceae species comprise chemical compounds with biological activities. In this work, phenolic compositions of ethanol extract of *O. sipyleum* were identified using the HPLC method. The phenolic compounds determined in the ethanol extract are listed in Table 2 and the main phenolics were identified as caffeic acid, epicatechin and 2,5 dihydroxybenzoic.

Table 2. Phenolic components of *O. sipyleum*

No	Phenolic component	Retention time (min)	µg/g*
1	2,5 dihydroxybenzoic acid	17.2	4435.68
2	Chlorogenic acid	18.2	75.86
3	3,4 dihydroxybenzoic acid	10.7	72.04
4	4-hydroxybenzoic acid	15.7	147.27
5	Cinnamic acid	71.1	801.74
6	Quercetin	70.4	2591.22
7	Ferulic acid	30.1	19.77
8	*p*-Coumaric acid	26.1	68.90
9	Gallic acid	6.8	138.14
10	Caffeic acid	22.7	16787.16
11	Vanilic acid	19.2	312.51
12	Epicatechin	21.3	4653.17
13	Rutin	45.6	50.48

*based on dry weights

Caffeic acid exhibits anticarcinogenic properties that acts as a carcinogenic inhibitor [42]. The phenolic content that contribute to the antioxidant capacity of the plants [34]. These data indicated that the biological activities of *O. sipyleum* could be attributed to their polyphenol compounds.

3.6. Cytotoxic activity

The brine shrimp cytotoxic bioassay is considered to be useful tool for the preliminary assessment of general toxicity and for estimating the medium lethality concentration LC_{50} [26, 43] and universally as a test for detecting cytotoxic effects. In addition, the brine shrimp cytotoxic bioassay is highly sensitive to a variety of chemical substances [44] and only a small amount of sample is required [45].

Origanum plants from Lamiaceae family are commonly used as herbal teas, flavoring agents and medicinal plants due to their biological and pharmacological properties [8, 9]. *O. sipyleum* is one of the endemic species of the *Origanum* genus and the vegetative parts of this plant have been used as medicinal tea [9]. The lethality of the ethanol extract from *O. sipyleum* was 327.414 µg/mL (LC_{50} < 1000 µg/mL), possessed high cytotoxic activities against brine shrimp and accepted as bioactive due to lower LC_{50}. To date, various studies have been reported that Lamiaceae plants provide a rich source of phytochemical components which are considered to be the basis of various biological activities, including antioxidant, antibacterial, anthelmintic and cytotoxic activity [6, 9, 19, 46, 47]. Accordingly, it could be attributed that the presence of these active components are the basis of the cytotoxic activity. The deadly effect obtained from present study indicates the presence of potent cytotoxic components in this plant and is one that requires further investigation as according to the findings this plant extract possesses a cytotoxic effect.

4. CONCLUSION

The findings in the present work show that in different assays *O. sipyleum* possess antioxidant properties and they also show that the plant possesses rich phenolic, flavonoid and tannin compounds. Furthermore, the ethanol extract show LC_{50} values of less than 1000 µg/mL and this result indicates that ethanol extract of this plant possesses strong cytotoxic activity. According to these findings, this plant could be considered as a source of natural agents in the food industry and could be used as a new cytotoxic agent for pharmacological applications. However, further research would be required before such uses could be proposed with confidence.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

DNA barcoding and phytochemical profiling of wild plant "Lal lat tan" from Imugan, Sta Fe, Nueva Vizcaya, Philippines

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Abstract: The Philippines hosts a wide-ranging diversity of plant species with pharmacological potential. Species identification on the basis of DNA sequences has been done for some time in plants and used to detect variations among the sequences specifying genetic divergence as a result of molecular evolution during the course of time. In this study, molecular approach as well as phylogentic analysis were conducted to identify the wild plant "Lal lat tan" collected from Imugan, Sta Fe, Nueva Vizcaya. In addition, thin layer chromatography was conducted to determine the bioactive compounds present in the wild plant. For molecular approach, the genomic DNA was extracted from the young leaves using CTAB and amplified using the nrDNA ITS marker. The PCR amplified product was sequenced and subjected for search query analysis using BLAST and was identified as *Dendrocnide meyeniana*. Using chloroform+methanol as solvent system, the result revealed the presence of saponins, phenols, tannins, flavonoids, anthrones, anthraquinones, terpenes and steroids which are considered as active medicinal phytochemical constituents. Lal lat tan is a wild plant and was known for having stinger leaf that lead to severe itchiness and swelling of eyes and face when touched.

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1. INTRODUCTION

Ever since, in search for rescue to treat disease, people looked for drugs in nature. The beginnings of the medicinal plants' use were instinctive, as is the case with animals. In view of the fact that during that time there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience [1].

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Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug compounds or components because of the unmatched availability of chemical diversity [2]. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs [3]. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [4]. Medicinal plants are important source of producing valuable bioactive compounds which is great importance for the health of individuals and communities.

The medicinal values of the plants are due to the chemical substances that produce a definite physiological action on human body and are called phytochemicals [5]. After various observations and experimentations, medicinal plants were identified as a source of important medicine, therefore, treatment through these medicinal plants, began in the early stages of human civilization. Several phytochemical surveys have been published, including the random sampling approach which involved some plant accessions collected from all parts of the world. Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients. They protect plants from disease and damage and contribute to the plant's color, aroma and flavor [6]. Various method of extraction for the plant is commonly used method since the extract was rich in chemical compounds and also the bioactivity of plant extracts depends on the water concentration used in the extraction process [7].

DNA barcoding technique is a new tool for taxon recognition and classification of biological organisms based on sequences of a fragment of mitochondrial gene, cytochrome c oxidase I (COI) [8]. DNA barcoding is a diagnostic technique which based on sequence variation at a small fragment of the mitochondrial c oxidase I (COI) [9] that provides an inexpensive and simple tool for identifying novel species [10] and also for describing cryptic species which are difficult to detect phenotypically [8, 11].

2. MATERIALS and METHODS

2.1 Collection and processing of sample

The plant material was collected at Imugan Falls, Santa Fe, Nueva Vizcaya, Philippines (N 16° 11" 38.76" E 121° 6" 36.72" with elevation of 1,685 meters above sea level. Matured leaves of Lal lat tan were collected using clean knife and was placed in a large plastic bag. Sample was cleaned and the external moisture was wiped out using tissue paper. The sample was dried under full sunlight condition and kept for phytochemical analysis. Some fresh leaves were kept in a ziplock and refrigerated before DNA extraction.

2.2 Genomic DNA Extraction, PCR and Sequencing

About 2-3 g of fresh plant material homogenized using mortar and pestle with liquid nitrogen. Powdered sample was transferred in new tubes and 400µl Lysis Buffer PA1 was added and mixed using vortex. Sample was added by 10µl RNase and then mixed and then incubated at 65°C for 10 min. The lysate was loaded onto a new 2ml tube with the ISOLATE II (Bioline) filter and centrifuged in 11,000xg in two minutes. After the collection of the clear flow-through, the ISOLATE II filter was decanted and discarded. The clear supernatant was transferred carefully without disturbing the pellet to a new tube. A 450µl binding buffer PB was added and mixed using vortex. The ISOLATE II Plant DNA (Bioline) spin column was placed into a new 2ml tubes and the sample and was centrifuged at 11,000xg for one minute and the flow-through was discarded. The silica membrane was washed and dried by adding 400µl wash buffer PAW1, centrifuged at 11,000xg for one minute and the flow-through was discarded. And then washed again by adding 700µl wash buffer PAW2, centrifuged at 11,000 x g for one minute and the flow-through was discarded. And then another 200µl wash buffer PAW2 was added, centrifuged at 11,000xg for two minutes to remove wash buffer and to dry silica membrane completely. The ISOLATE II Plant DNA spin column was placed into a new tube added by 50µl preheated elution buffer PG (65°) onto center of silica membrane. It was incubated at 65°C for 5 minutes and then the step was repeated with another 50µl elution buffer PG and eluted into same tube. A 2µl stock DNA mix with 1µl loading dye was loaded into 1% agarose gel containing 1µl of gel into red (GelRed™ Nucleic acid, Biotium) and run in gel electrophoresis system (Endruro Gel XL).

Electrophoresis was carried out at 100 V for 30 minutes. The gel was viewed in gel documentation system (Enduro™ GDS). The genomic DNA was diluted 1:100 using sterilized distilled water. To identify the identity of the sample, the chloroplast gene region was amplified using the *nr*DNA ITS (Table 1) using PCR machine (2720 Thermal Cycler). One (1) µl of diluted DNA was mixed with PCR components and the PCR profile was set as follows: 35 cycles with an initial denaturation at 94°C for 5 minutes, final denaturation at 94°C also for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 45 seconds, final extension for 10 minutes at 72°C and hold at 10°C. Final mixtures of PCR components and DNA were transferred to PCR tubes and were subjected to PCR reaction. The PCR product was sent to Apical Scientific Sequencing in Malaysia for PCR purification and sequencing procedure. The sequences were queried on BLAST (basic local alignment search tool) to check the percent similarity in Genbank.

Table 1. Primers use to amplify nrDNA ITS, *rbc*L and *mat* K

Primer pair	Sequence
ITS3F	5'-GTAAGTGCCGAATTCGAAATAACTGAATGGGA- 3'
ITS4R	5'-AGGGCTGTGGGATCCTCATTCAGGTCTATCACCTC-3'

The chromatogram result was evaluated using BioEdit software [12]. Sample sequence was queried using nucleotide BLAST (basic local alignment search tool) [13]. Default search parameters on the standard nucleotide BLAST (blastn) web interface were used. Other related species sequences were downloaded and were used for evolutionary relationship and phylogenetic analysis.

2.2. Phytochemical Analysis

Dried sample was homogenize using blender. 3.0 g of dried powdered plant material was defatted with 10 mL of hexane and heated over a water bath for 5 minutes. The solvent was decanted and discarded. The residue was extracted with 10 mL of a mixture of methanol and water, CH3OH:H2O (1:1) and heated over a water bath for 5 to 10 minutes. The solution was filtered and the filtrate was labeled and stored. The plant residue was then discarded. Thin layer plate (Merck Millipore) was cut into size of 1X2 inch. The plates were marked 1cm in both ends. About 0.1 mL of solution were applied as spots on the each plates respectively; the chromatogram were developed with different solvent systems suggested in Table 2.

Table 2. Solvent system used for Thin Layer Chromatography.

Absorbent	Solvent System
Silica gel G	Chloroform- methanol (5:1) n- Butanol- acetic acid- water (4:1:5) upper phase Ethyl acetate- pyridine- water (5:1:4)

The chromatogram was air dried and visualized with the following spray reagents shown in Table 3. Positive tests are likewise indicated.

Table 3. List of plant constituents, their visualizing agents and indication of a positive test

Constituents Tested	**Spray Reagents**	**Observable Result for a Positive Test**
Saponins	Antimony (III) chloride	Intense yellow to orange visible zone appear on spraying
Phenols, Tannins, Flavonoids	Potassium ferricyanide-ferric chloride	Blue spots
Alkaloids	Dragendorff's reagent	Brown- orange visible spots immediately on spraying colors aren't stable
Cardenolides	3,5 Dinitrobenzoic acid; Kedde reagent	Blue to red- violet colored zones
Coumarins, Anthraquinones, anthrones, Phenols	Methanolic potassium hydroxide (Borntrager reagent)	Anthraquinones give orange coloration Anthrones give yellow (UV365 nm) zones Coumarins react to form blue (UV365 nm) colored zone
Anthraquinones	Magnesium acetate	Orange- violet color
Indoles	Van Urk- Salkowski Test	Blue- violet spots
Terpenes, Steroids, Phenols	Vanillin- sulfuric acid	Red-violet or purple spots.

3. RESULTS and DISCUSSION

3.1 Molecular identification and phylogeny

Lal lat tan is a small tree, growing to a height of 3 to 5 meters. Leaves have numerous, conspicuous stinging hairs. The hairs have a large bulbous base from which projects a long tapering tube that ends in a curved tip that breaks off easily. Contact with the leaves causes breaking of the tips of the hairs (trichomes) is released that causes immediate and intense skin irritation. The stings are painful and may cause the formation of blisters, immediate and possibly progressing to confluence. DNA from the plant sample was successfully extracted and was subjected in agarose gel to check for DNA quality. The gene markers were used in amplification of the genomic DNA. About 450 base pair fragments of ITS was amplified based on its expected size after PCR amplification. After sequencing the representative sequences of the plant sample were queried against GenBank nucleotide database using BLAST to determine the sequence similarity to the known plant sequence with maximum percent identity. BLAST analysis showed that the plant species collected was identified as *Dendrocnide meyeniana* (KM58432) with 99% identity.

Table 4. Identities of the specimens after BLAST analysis.

Primer used	GenBank Accession Number	Match Accession Name	Maximum % Identity
ITS	*Dendrocnide meyeniana*	KM58432	99%

The sequences were used to construct phylogenetic tree using Mega6 [14] and Clustal W [15], with a bootstrap value of 1000 replicates, respectively. The evolutionary history was inferred based on Neighbour-Joining method [16] and the evolutionary distances were computed using the Maximum Composite Likelihood Method (Figure 1).

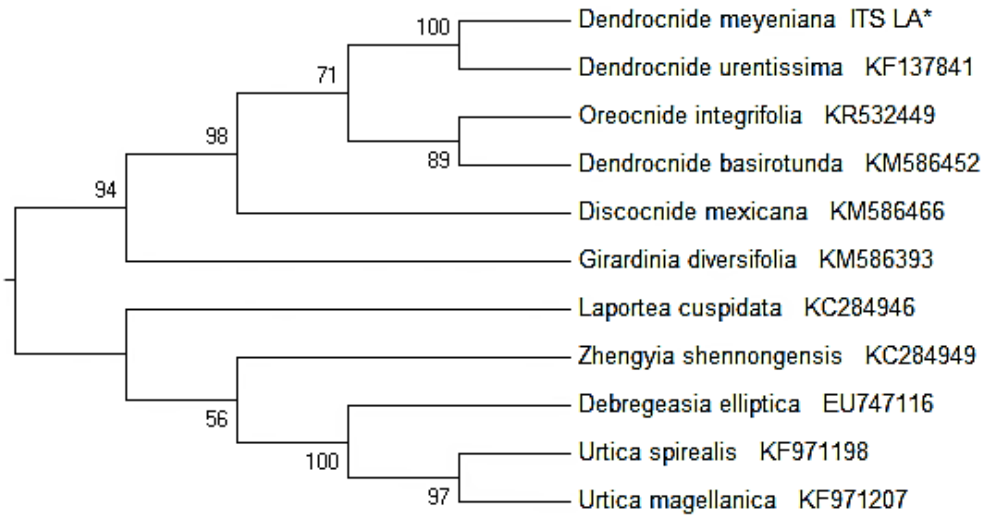

Figure 1. The molecular phylogenetic tree of the Lal lat tan inferred from nrDNA ITS gene marker

The molecular phylogeny tree involved 11 nucleotide sequences that was divided into two major branches and all are sister related taxa up to their genera. The plant sample are in the first branch. All the eleven species are angiosperms, under the order *Rosales* and a family *Urticaceae* and have the characteristics of being armed with stinging hairs.

The nettle family (Urticaceae) is a family of flowering plants. This includes about 2600 species, grouped into 54 to 79 genera according to the database of Royal Botanic Gardens, Kew [17]. The largest genera are *Pilea, Elatostema, Urtica* and *Cecropia.* Urticaceae can be herbs, subshrubs, or shrubs, rarely trees, very rarely climbing, stems often fibrous, sometimes succulent and armed with stinging hairs [18]. The stinging trichomes of *Urtica* consist of a stinging cell with surrounding pedestal cells. When a touch with the human skins, the toxin in the stinging trichomes is released to human and gives pain, wheal, or stinging sensation, and the sensation can be lasted for several hours [19][20]. It was also noted that other than used for animal defence, the function of the stinging trichomes is also regarded as secretion of metabolites [21].

Three genera of *Dendrocnide* including the *Oreocnide integrifolia* in the first branch formed into clade that are all known to be trees. *D. Mexicana* and *G. diversifolia* are herbs so that they separated from the four tree species which formed another subclade. The second group are shrubs and were clustered closely to the first branch. The longer branches in the horizontal dimensions represents the larger amount of evolutionary lineages changed.

In comparison, the plant sample *Dendrocnide meyeniana* are trees, its bark are smooth and glabrous almost throughout to the end of branchlets [22] and grows to a height of 3 to 5 meters.

3.2 Phytochemical Analysis

Chloroform and methanol extract was used as the extracting solvent. The result of the phytochemical analysis from various fraction and the RF value of the different constituents were shown in Table 5. The phytochemical screening with the extract of Lal lat tan leaves revealed the presence of secondary metabolites such as saponins, phenols, tannins, flavonoids, anthrones, anthraquinones, terpenes and steroids which are considered as active medicinal phytochemical constituents.

The intense yellow zone visible upon submerging of thin-layer plates to the Antimony (III) Chloride showed the existence of saponins in the plant material. Using Potassium ferricyanide- ferric chloride spray reagents, a blue spot were seen in the thin layer plates which indicated the presence of phenols, tannins and flavonoids. The yellow and orange zone observed as the positive result in methanolic Potassium hydroxide (Borntrager reagent) inditates the presence of anthrones, anthraquinones and phenols while negative for the presence of coumarins. And lastly, the blue-violet spots appeared in Vanillin-sulfuric acid spray reagents specifies the presence of terpenes and steroids.

Table 5. Phytochemical profiling of various fractions of Lal lat tan

Constituents Tested	Extracting Solvent (Chloroform+ Methanol)	RF Value
Saponins	+	1.402cm
Phenols	+	1.572cm
Tannins	+	1.374cm
Flavonoids	+	1.264cm
Alkaloids	-	1.582cm
Cardenolides	-	1.364cm
Coumarins	+	1.641cm
Anthraquinones	+	1.469cm
Anthrones	+	1.145cm
Phenols	+	1.000cm
Terpenes	+	1.815cm
Steroids	+	1.575cm
Anthraquinones	-	1.263cm
Indoles	-	1.544cm

+: present, - : absent

4. DISCUSSION

The typical bioactive compounds in plants are produced as secondary metabolites. Thus, a definition of bioactive compounds in plants is: secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals [23]. The phytochemical constituents detected are known to have medical importance and to be biologically active compounds that are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer [24, 25]. For example, several reports are available on flavonoid groups which exhibited high potential biological activities such as antioxidant, anti-inflammatory, antimicrobial, anti-angionic, anticancer and anti-allergic reactions [26, 27, 28, 29]. Tannins and their derivatives are phenolic compounds considered to be primary antioxidants or free radical scavengers [30] and according to research, are known to have antibacterial, antitumor and antiviral activities.

Phenolic compounds could be a natural source of antioxidants because phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables [31]. According to Enrico *et al.* [32] the essential oils, unlike antibiotics, are composed of many molecules so that bacteria cannot resist in mutant. Preventively and curatively, they are especially known for their potent antibacterial, antiviral, anti-inflammatory, anti-fungal, anti-parasitic, antipyretic, expectorant, and mucolytic effects. A large body of literature has demonstrated that the naturally occurring anthraquinones possess a broad spectrum of bioactivities, such as cathartic, anticancer, anti-inflammatory, antimicrobial, diuretic, vasorelaxing, and phytoestrogen activities, suggesting their possible clinical application in many diseases [33]. According to Mazza *et al.* [34], Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains and also has a biological activity such as anticancer, and anticholesterol activity.

Steroids has therapeutic value as anti-inflammatory agents, anabolic (growth-stimulating) agents, and oral contraceptives. Renewed interest in plant antioxidants has emerged during the recent years, probably due to the appearance of undesirable side effects of certain commercial antioxidant. In medicinal plants world, there are a huge number of different types of bioactive compounds with antioxidant activity that play a significant role in terminating the generation of free radical chain reactions [35]. This research work that focused on phytochemical analysis of the phytochemical constituents of Lal lat tan. The phytochemical analysis of the plants are important and have commercial interest in both research institutes and pharmaceuticals.

5. CONCLUSION

The molecular approached revealed that ITS gene marker possess the high discriminatory power and best among the gene markers used in obtaining the the resolution of sequence of Lal lat tan. After obtaining the sequences on GenBank using nucleotide BLAST, it was identified that the plant sample is *Dendrocnide meyeniana* with 99% identity. The phylogenetic tree showed that the branches are composed of sequences that are sister related taxa and possess matching characteristics. Different secondary metabolites that are essential and known to have medical importance and to be biologically active compounds that are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer were determined to be present in plant material as well as the absences of other phytochemicals in the plant.

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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Enhancement of stevioside production by using biotechnological approach in *in vitro* culture of *Stevia rebaudiana*

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Abstract: *Stevia rebaudiana* Bertoni, which is an important plant for the food and health sector, contains calorie-free natural sweet-tasting steviol glycosides (SGs). In the present study, the effects of different elicitors [methyl jasmonate (MeJA), salicylic acid (SA), or chitosan (CHI)] on the *in vitro* production of stevioside and rebaudioside A were carried out. For this purpose, 3-week-old *in vitro* plantlets were transferred into 250 mL flasks containing liquid woody plant medium (WPM) supplemented with MeJA, SA, or CHI at different concentrations (0, 50, 100, or 200 µM), and were exposed to these elicitors for 2 weeks. A HPLC method was developed to quantify the aforementioned SGs in the cultivated plantlets and all of the elicitor types and concentrations resulted in an increase in stevioside production ranged between 2.87 mg/g dry weight (DW) (Control) and 50.07 mg/g DW (100 µM MeJA). The highest number of shoot, node, leaf, leaf length, and biomass accumulation and shoot length were observed with application of 100 µM CHI and control, respectively. The present findings open new perspectives for increasing the stevioside production using a plant tissue culture system.

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1. INTRODUCTION

Stevia rebaudiana (Bertoni), native to the eastern Paraguayan rainforests, is a member of the family Asteraceae. Since being introduced to Europeans in 1899, steviol glycosides (SGs) have been of significant interest both commercially and scientifically, due to their intense sweetness [1]. SGs are tetracyclic diterpenes sharing the same kaurenoid precursor as

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gibberellic acid and are synthesized mainly in *Stevia* leaf tissue [1, 2, 3]. With an approximate sweetness of 300 times that of sucrose, a 4% concentration (w/v) [4], SGs are relied on as a non-caloric sweetener in many countries worldwide, including China, Japan, Korea, Australia, New Zealand, and many countries in the European Union [5, 6]. The 2 major SGs are stevioside and rebaudioside A, while other lower-concentration SGs include steviolbioside, rebaudioside B, C, D, E, F, and dulcoside A [5, 6]. These SGs, due to their intense sweetness, have become an attractive sugar alternative in the food industry [7] as well as in the health sector. Due to these benefits, increasing the production of SGs and understanding their mechanism are of great interest.

The production of secondary metabolites via plant tissue cultures, such as callus culture, cell culture, and organ culture, have some advantages over conventional agricultural production: 1) Only a small portion of tissue is necessary to establish the *in vitro* culture [8]. 2) Fluctuations in secondary metabolite concentrations due to geographical, seasonal, and environmental variations do not occur [9, 10]. 3) They offer a defined production system, which ensures a continuous supply of products, uniform quality, and yield [10, 11]. 4) It is possible to produce novel compounds that are not normally found in the parent plant. 5) These methods also provide opportunities for the production of plant products on a large scale and the improvement of secondary metabolite production [11]. It is well known that the biosynthesis of secondary metabolites in plants generally occurs in response to biotic and abiotic stresses [10, 12]. Therefore, one of the most common applications aimed at increasing the secondary metabolite production in plant tissue cultures is the use of elicitors [13, 14]. Elicitors are the signals that trigger the production of secondary metabolites [15]. Methyl jasmonate (MeJA), salicylic acid (SA) and chitosan (CHI) have been used as effective elicitors for secondary metabolite production *in vitro* for many years [12, 14, 15]. It was reported that both SA and MeJA are stress-signaling molecules that influence plant resistance to biotic and abiotic stress factors [14, 16]. Chitin, a structurally important component of many fungi, increases a plant's accumulation of soluble pathogenesis-related proteins [17]. Therefore, CHI has often been used as an elicitor in plant cell cultures to induce or stimulate the production of many secondary metabolites [18].

The biosynthesis of SGs begins in the chloroplasts [1, 19] and there is a positive correlation between the development of the chloroplast membrane system and the biosynthesis of the SGs [19]. Therefore, it was thought that the synthesis of SGs takes place especially in specialized cells or structures, and plants with well-developed leaves are able to synthesize SGs in desired quantities and in the present study, *in vitro*-grown whole plantlets were used. In our previous research, we found that because of long exposure time (four weeks), SA and MeJA effected the shoot growth negatively; however, with the concentrations of 50 µM MeJA and 100 µM SA, we obtained approximately an 8-fold increase in the stevioside content when compared to the control. Although CHI applications resulted in better shoot growth, the stevioside content remained low from SA and MeJA applications [20]. Thus, we aimed to establish a suitable method by minimizing the exposure time and chancing the culture type. Therefore, we applied elicitors to 3-week-old *in vitro* plantlets transferred to liquid culture for two weeks. Herein, the results of this elicitation study are reported.

2. MATERIAL AND METHODS

2.1. Plant material

In vitro clonally propagated plantlets obtained from single seed descent seedlings of *S. rebaudiana* in the Bioengineering Department of Ege University were used as the plant material. To obtain a sufficient number of plants, nodal segments of 4-week-old *in vitro* grown plantlets, with one axillary bud and a length of approximately 1.0 cm to 1.5 cm, were cultured

into glass tubes (23/24 × 140 mm, Lab Associates b.v., Oudenbosch, Netherlands) with plastic caps, containing 10 mL of woody plant medium (WPM) [21] supplemented with 3% (w/v) sucrose, and solidified with 0.65% (w/v) plant agar (Duchefa Biochemie B.V., The Netherlands). For plant multiplication, they were transferred every 4 weeks to fresh solidified medium.

2.2. Initiation culture

For the elicitor applications, 3-week-old *in vitro* grown plantlets were used. Development of the shoots and roots from the nodal explants of *S. rebaudiana* cultured on the basal WPM occurred simultaneously. Root formation began after one week of culturing. For elicitor applications in agitated liquid culture, 3-week-old plantlets should be removed from the culture vessels without damaging their roots. To facilitate this operation, different substrates were tested. Nodal explants were transferred to the liquid culture in tubes with the filter paper bridge (LCFPB), loofa (Luffa cylindrica) sponge (LCLS), or perlite (LCPE) and in 250-mL flasks containing sterilized liquid WPM [liquid culture (LC)]. They were also cultured in glass tubes containing semi-solidified WPM in a reduced amount of agar (0.58% (w/v)) [solid culture in reduced agar (SCRA)] (Table 1; Figure 1). Per culture vessel, 10 explants for flask applications and one explant for other applications were used. The flasks were maintained on an orbital shaker at 100 rpm under daylight conditions. The experiments were performed in triplicate. Ten explants were used for each replication and 30 explants were tested in total per treatment. The data were recorded 3 weeks after starting the culture.

2.3. Preparation of the elicitors

MeJA, SA and CHI were prepared according to procedures described in our previous paper [20]. They were added individually in liquid medium as elicitors. For the stock solutions, MeJA and SA (Sigma-Aldrich) were dissolved in 96% ethanol. Next, the solutions were diluted with distilled water in concentrations as required. Finally, the pH of both solutions was adjusted to 5.8. In the case of CHI from crab shells (Sigma-Aldrich), it was dissolved in 2.0 mL of 1% (v/v) acetic acid (Sigma-Aldrich) and then diluted with distilled water. Finally, the pH of the solution was adjusted to 5.8.

2.4. Elicitor application

Nodal explants were cultured on WPM supplemented with 3% (w/v) sucrose and solidified with 0.58% (w/v) agar. After 3 weeks of culture, rooted shoots, approximately 6–8 cm long, with 14 leaves were carefully removed from the glass tubes and transferred into 250-mL flasks containing 25 mL of liquid WPM supplemented with different types (MeJA, SA, or CHI) and concentrations (0, 50, 100, or 200 µM) of elicitors and 3% (w/v) sucrose. The stock solutions of MeJA, SA, and CHI were sterilized by filtration through a 0.22-µm syringe Millipore filter (Minisart®, Sartorius, Germany), and then added to the autoclaved and cooled liquid WPM aseptically at the desired concentrations. The cultures were continuously agitated on a rotary shaker at 100 rpm. The plantlets were exposed to elicitors for 2 weeks. All of the applications were done in triplicate, and 10 explants were used for each replication. Thirty explants were tested in total per application. After 2 weeks of elicitor applications, either elicited or control plantlets were harvested, and the data regarding the stevioside and rebaudioside A contents were investigated using HPLC.

2.5. Media and culture conditions

The pH of all of the media was adjusted to 5.8. The filter paper bridge, loofa sponge and perlite were placed in the glass tubes before autoclaving. Media were autoclaved at 121 °C with a pressure of 1.04 kg/cm^2 for 15 min. All of the cultures were maintained at 25 ± 1 °C and a 16 h photoperiod under a cool white fluorescent light (approximately 50 µmol/m^{2}s).

2.6. Stevioside and rebaudioside A analysis

The stevioside and rebaudioside A contents in the leaves of 5-week-old *S. rebaudiana* plantlets exposed to the elicitors for 2 weeks were analyzed using the HPLC method from Bayraktar et al. [20].

2.6.1. Chemicals

HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). The ultrapure water was supplied from an in-house ultrapure water system (Sartorius Arium 611, Sartorius Stedim Biotech, Göttingen, Germany).

2.6.2. HPLC sample preparation

Approximately 50 mg of leaf sample from each application was added to 5 mL of 70% (v/v) methanol and sonicated 3 times with this solvent for 20 min. The clear extracts were combined and diluted with methanol to 20 mL. Prior to the HPLC analysis, the samples were filtered with a 0.45 µm PTFE filter (Sartorius AG, Göttingen, Germany) to remove any non-dissolved particles.

2.6.3. LC-DAD Analysis

LC-DAD analyses were carried out with a Thermo Surveyor Plus HPLC instrument (Thermo Scientific, Bremen, Germany), equipped with a quaternary pump, an autosampler, a column oven and a diode array detector. For all separations, a Teknokroma RP C18 analytical column measuring 250 × 4.6 mm i.d., particle size being 5 µm (Teknochroma, Barcelona, Spain) was used. LC separations were conducted using following solvents: ultrapure water (A) and acetonitrile (B) and elution by gradient was according to the following scheme: 0-1 min 65A/35B, in 4 min to 63A/27B, in 2.5 min to 60A/40B, in 0.5 min to 5A/95B and kept at that composition for 3 min and changed to initial ratios (65A/35B) of method in 1 min. Prior to the next injection, the column was equilibrated for 3 min with the beginning conditions (65A/35B). The flow rate was 1 mL/min column temperature was 40 ºC. Detection was performed at 210 nm and UV spectra of all samples were scanned between 200-360 nm.

Quantifications of stevioside and rebaudioside A were performed using calibration curves generated by *Stevia* glycosides mixture which contains 50% rebaudioside A and 36% stevioside by mass. These percentages were determined according to relative peak areas of *Stevia* glycosides based on LC-DAD signals. Retention times of rebaudioside A was 5.75 and stevioside 6.15 minutes. Calibration curve for rebaudioside A were constituted with standard solutions between 1000.000 - 15.625 µg/mL and 720.00 – 11.250 µg/mL for stevioside. Regression coefficient of calibration curve for rebaudioside A was 0.998 and formula was peak area of rebaudioside A=8000.76 x rebaudioside A concentration (µg/mL). Similar to rebaudioside A, regression coefficient of stevioside was 0.998 and formula was peak area of stevioside=7850.76 x stevioside concentration (µg/mL).

2.7. Statistical analysis

The experiments were set up in a completely randomized design, and all of the applications were replicated in triplicate. Each replicate comprised of 10 explants. The statistical analysis of the data on various parameters was subjected to SPSS Version 16.0 (SPSS Inc., Chicago, USA). The significance of the differences among the means was carried out using the Student-Newman-Keuls (SNK) test at P = 0.05.

3. RESULTS AND DISCUSSION

3.1. Initiation culture

In the beginning, the influence of the different substrates on *in vitro* plantlet growth was determined (Table 1). The LC and LCLS showed 100% and 34.3% vitrification rate, respectively, and the results implied their unsuitability for the culture initiation (Figure 1a, b). The culture of the explants on LCPE resulted in the development of very thin shoots and small leaves, and the rooted shoots showed a 49% vitrification rate. Therefore, LCPE was not suitable for the initiation of the culture (Figure 1c). Although, in the LCFPB (Figure 1d), the development of the shoots was as good as that in the SCRA, the rooting rate was less than that in the SCRA (Figure 1e) (Table 1, 2). Consequently, the SCRA was found to be a more suitable system for culture initiation and the removal of rooted shoots with no damage.

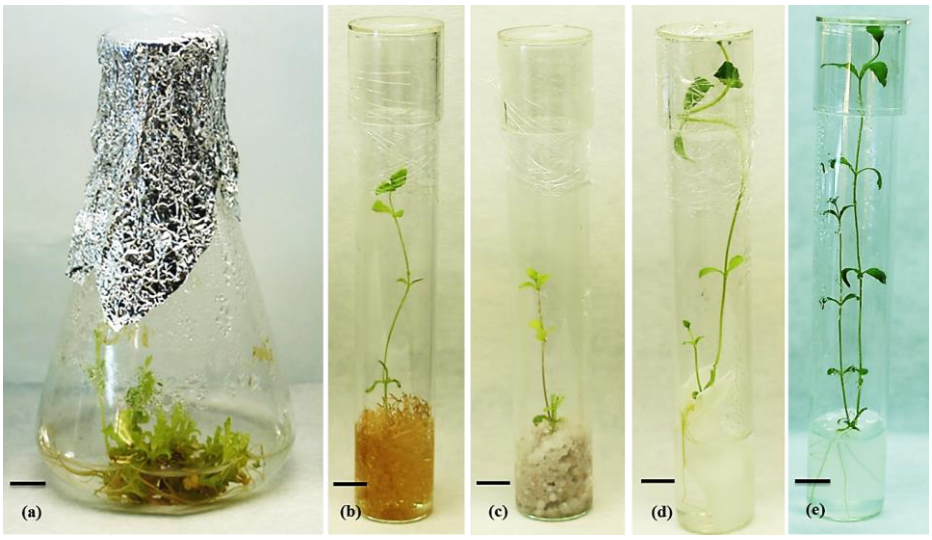

Figure 1. The growth of the plantlets after 3 weeks of the initiation culture: **(a)** Liquid culture in 250 mL flasks (LC); **(b)** Liquid culture in tubes with loofa sponge (LCLS); **(c)** Liquid culture in tubes with perlite (LCPE); **(d)** Liquid culture in tubes with filter paper bridge (LCFPB); **(e)** Solid culture in tubes with reduced agar (SCRA) (bars = 1.0 cm).

Table 1. Effect of different type of substrates on the *in vitro* shoot growth in *Stevia rebaudiana*

Substrate code	Number of shoots/explant ± SE	Shoot length (cm)/explant ± SE	Number of nodes/explant ± SE	Number of leaves/explant ± SE	Leaf length (cm)/explant ± SE	Stem diameter (mm)/explant ± SE	Vitrification (%) ± SE
LCSC	2.00 ± 0.08 b	9.89 ± 0.45 a	13.63 ± 0.88 a	29.13 ± 1.83 a	0.85 ± 0.03 a	0.62 ± 0.04 a	0.00 ± 0.0 d
LC	4.97 ± 0.54 a	1.99 ± 0.36 c	4.33 ± 0.63 c	10.67 ± 1.27 c	0.30 ± 0.02 c	0.67 ± 0.05 a	100.0 ± 0.00 a
LCFPB	1.60 ± 0.11 bc	8.43 ± 0.67 b	7.00 ± 0.39 b	16.00 ± 0.79 b	0.68 ± 0.06 b	0.56 ± 0.04 a	6.33 ± 0.17 c
LCPE	0.90 ± 0.13 c	3.10 ± 0.57 c	1.70 ± 0.25 d	4.80 ± 0.64 d	0.34 ± 0.05 c	0.25 ± 0.03 c	49.00 ± 4.13 b
LCLS	1.27 ± 0.08 bc	7.03 ± 0.50 b	2.87 ± 0.14 cd	7.73 ± 0.28 cd	0.61 ± 0.04 b	0.43 ± 0.02 b	34.33 ± 1.81 b

In each column, mean ± SE followed by the same letter was not significantly different (p=0.05) according to Student-Newman-Keuls test

LCSC: Solid culture in tubes with reduced agar; **LC:** Liquid culture in 250 ml flasks; **LCFPB:** Liquid culture in tubes with filter paper bridge; **LCPE:** Liquid culture in tubes with perlite; **LCLS:** Liquid culture in tubes with loofa sponge

Table 2. Effect of different type of substrates on the root formation in *Stevia rebaudiana*

Substrate code	Percent of explants forming roots ± SE	Number of roots/ rooted explant ± SE	Root length (cm)/ rooted shoot ± SE
LCSC	100.00 ± 0.00 a	4.30 ± 0.23 a	3.54 ± 0.14 b
LC	70.00 ± 5.77 b	2.97 ± 0.26 b	5.73 ± 0.25 a
LCFPB	80.00 ± 5.77 b	4.27 ± 0.21 a	3.41 ± 0.14 b
LCPE	56.67 ± 3.33 c	2.87 ± 0.25 b	0.98 ± 0.08 c
LCLS	96.67 ± 3.33 a	3.10 ± 0.21 b	2.99 ± 0.22 b

In each column, mean ± SE followed by the same letter was not significantly different (p=0.05) according to Student-Newman-Keuls test

LCSC: Solid culture (reduced agar) in tubes; **LC:** Liquid culture in 250 ml flasks; **LCFPB:** Liquid culture in tubes with filter paper bridge; **LCPE:** Liquid culture in tubes with perlite; **LCLS:** Liquid culture in tubes with loofa sponge

3.2. *In vitro* shoot growth and biomass accumulation

The effect of some elicitors (SA, MeJA and SA) on the *in vitro* shoot growth, biomass and SGs production of *S. rebaudiana* was investigated. For this purpose, 3-week old *in vitro* plantlets of *S. rebaudiana* were exposed to elicitors for 2 weeks in the liquid culture.

Information on the growth parameters of shoot cultures exposed to elicitation for 2 weeks was presented in Figure 2. Plantlets exposed to CHI at different concentrations showed the best response regarding shoot number among the applications (Figure 3a, b). The highest number of shoots per explant was obtained in the application with 100 µM CHI, which led to a shoot number of 3.60, followed by application with 200 µM CHI, with 3.10 shoot number (Figure 2a). Especially in plantlets with shoot tip necrosis, the effect of apical dominance on lateral buds was removed and shoots were induced from lateral buds. This resulted in more than two shoots formation (Figure 3c).

No significant differences were obtained in the shoot length per explant between applications (Figure 2b). Two-week elicitor treatment did not suppress shoot elongation. The maximum shoot length (8.67 cm/explant) was observed in the control medium (liquid WPM) followed by all of the elicitor applications except with 200 µM MeJA, and they were statistically placed in the same group. The shortest shoot length was obtained as 6.97 cm from 200 µM MeJA application.

Plantlets exposed to CHI at the concentration of 100 µM had the highest number of nodes (6.93 nodes per explant), number of leaves (23.67 leaves per explant), and leaf length (0.68 cm) (Figure 2c, d, e). Elicitor applications induced more leaf necrosis than non-treated plantlets (control). The necrosis rate was predominantly observed in the SA and MeJA treatments at the concentrations of 100 and 200 µM and CHI treatment at the concentration of 200 µM. Percentage of leaves showing necrosis ranged between 8.35% (control) and 24.07% (200 µM MeJA) (Figure 2f). As regards biomass production, CHI induced the highest plantlet fresh (153.7 mg per explant) (FW) and dry (35.7 mg per explant) weight (DW) at the 100 µM concentration (Figure 2g, h). According to our previous study, 4-week elicitation with SA and MeJA, especially at the 100 and 200 µM concentrations, resulted in high level necrosis formation in MeJA applications and the development of very thin and short shoots with short internodes and small leaves in SA applications. CHI applications gave generally best response regarding plant growth and biomass production. In the present study, the negative effect of MeJA and SA on plant growth was minimized by reducing exposure times of these elicitors. CHI also showed the same responses with our previous study.

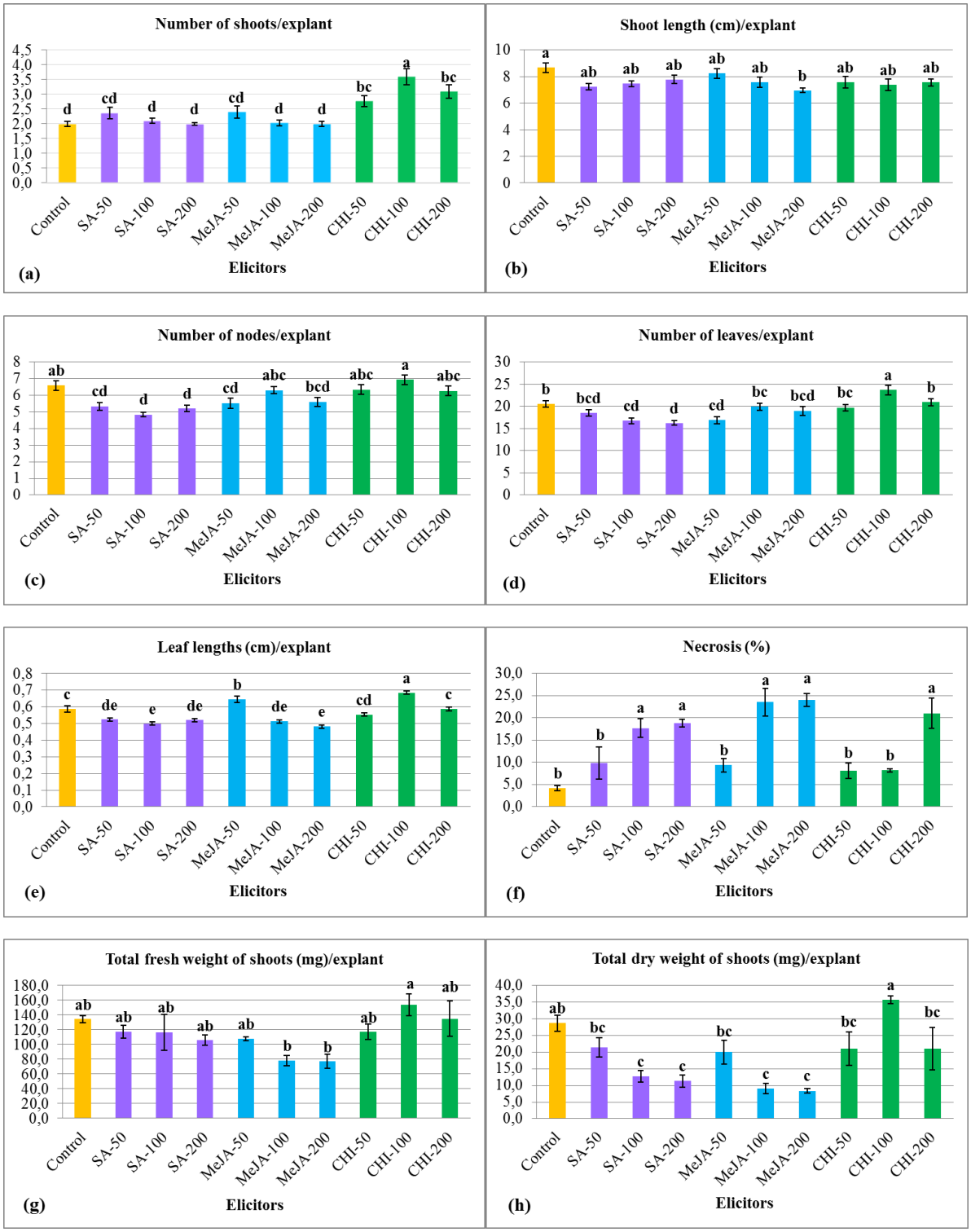

Figure 2. Effect of different type and concentrations of elicitors on *in vitro* shoot growth (a-f) and biomass production of *Stevia rebaudiana* after 2 weeks of culture. In each column, mean ± SE followed by the same letter was not significantly different (p=0.05) according to Student-Newman-Keuls test.

Figure 3. The growth of the plantlets after 2-week CHI elicitation **(a, b)** and plantlet showed shoot tip necrosis **(c)**.

3.3. Steviol glycosides production

There are several methods to increase the production of secondary metabolites in *in vitro* plant culture systems, such as elicitation, two-phase culture, hairy root culture, and immobilization, but elicitation is the most widely used method to enhance the secondary metabolite production. The elicitors mimic the effects of stresses, and so stimulate the plant defense system, which results in increased biosynthesis of secondary metabolites in plant tissues [22]. To date, a lot of plant tissue culture studies have proven that elicitors increased secondary metabolite production of medicinal plants [14]. In the present study, similar stimulatory effects were also observed for the *in vitro* plantlets of *S. rebaudiana*, resulting in increased yields of stevioside production.

All of the MeJA doses provided higher metabolite content than the control plants, and the 100 µM dose was more efficient than the other concentrations with regards to the metabolite yield. The highest quantity of stevioside (50.07 mg/g DW) was 17.4-fold higher than the controls (2.87 mg/g DW) (Figure 4). SGs in *S. rebaudiana* share a common pathway with gibberellic acid and both are synthesized via the 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway that occurs in plastids and mainly in leaves [1, 3, 23]. It has been proven extensively that Jasmonates, a phytohormone, induce plant secondary metabolites with incredible structural variety [23]. It has been reported that MeJA alters gene expression in the MEP pathway as well as the concentrations of products derived from these genes [23]. The pot plants of *S. rebaudiana* were inoculated with *Rhizophagus fasciculatus* (Thaxt.) C. Walker & A. Schüßler, an arbuscular mycorrhizal fungus, to improve the yield of SGs. It was observed that arbuscular mycorrhizal fungi colonized *Stevia* plants showed a significant increase (more than three-fold) in the concentration of JA when compared with the controls [23]. *Stevia* plants originated from *in vitro* culture and growing in a hydroponic system were exposed to MeJA, spermidine (SPD), SA, and paclobutrazol (PBZ) at the concentration of 100 µM for 24, 48, 72, and 86 h to examine the effect of these elicitors on SGs' contents and transcriptional levels of fifteen genes responsible for biosynthesis of SGs. It was found that MeJA and SPD showed positive effect on the transcription of SGs biosynthetic genes at 48 h of exposure [3]. In the present study, MeJA applications to *in vitro* plantlets of *S. rebaudiana* at different concentrations showed a similar effect and increased the stevioside production when compared to the control plants (Figure 4).

When the data were evaluated in the SA-treated plants, stevioside production was stimulated by SA treatments. At the 3 different concentrations (50, 100, and 200 µM), 100 µM dose of SA was more effective than the 50 and 200 µM concentrations with regards to the stevioside yield and the stevioside content reached up to 26.66 mg/g DW (9.3-fold higher) with the 100 µM dose (Figure 4). Belonging to the glycosyltransferase (GT) family, UDP-glycosyltransferases (UGTs) are a very diverse group of plant enzymes able to transfer a sugar residue from an activated donor to an acceptor molecule. UGTs play a role in the production of SGs in *S. rebaudiana* [24]. It has been reported in many studies that some elements, such as SA, JA, or some foreign compounds, can regulate GTs [25]. This may be because the stevioside biosynthesis increased in the SA-treated *in vitro* grown plantlets.

In the case of the CHI, compared to the controls, the 50 µM and 200 µM CHI applications affected the production same and all the SA applications raised apparently stevioside production. The stevioside content increased from 2.87 mg/g DW to 25.13, 31.24 and 25.50 mg/g DW at the 50, 100 and 200 µM concentrations, respectively (Figure 4). CHI is a linear polysaccharide that originates from the deacetylation of chitin, which is the main structural component of the cell wall of plant pathogen fungi. It is known that CHI mimics the effects of several pathogenic fungi and so activates the biosynthesis of defense-related secondary metabolites in plants [18]. Similar to other secondary plant metabolites, SGs may also behave in a defensive manner as feeding deterrents or antimicrobial agents against specific herbivores, pests, or pathogens [2]. Therefore, application with CHI increased the level of stevioside production when compared with the controls. In our previous work, the long exposure time (four weeks) of CHI resulted in better shoot growth than the MeJA and SA-treated explants and the stevioside content was lower than that of these applications [20]. In the present study, we improved the stevioside contents by reduced the exposure time of CHI.

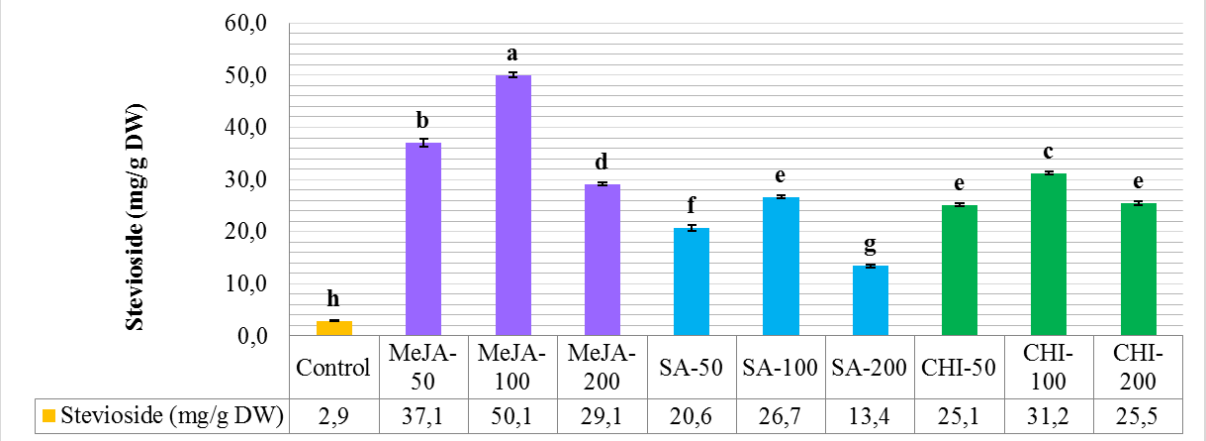

Figure 4. Effect of different type and concentrations of elicitors on stevioside production of *Stevia rebaudiana* after 2 weeks of elicitation culture. In each column, mean ± SE followed by the same letter was not significantly different (p=0.05) according to Student-Newman-Keuls test.

In the previous studies aimed at the increasing the SGs yield with elicitors in *in vitro*, Mejía-Espejel et al. [26] applied the SA (10 and 100 mM) and MeJA (10 and 100 mM) to the calli obtained from leaf segments of *S. rebaudiana* and increased the stevioside content 9.8 times compared to that of the leaves of plants grown under greenhouse conditions. They obtained the highest rebaudioside A contents (34.6 times higher than in leaves) by applying 10 mM of SA. Álvarez-Robles et al. [27] elicited *in vitro* shoot cultures of *S. rebaudiana* with methanol at different concentrations. They determined the stevioside and rebaudioside A production as 42 mg/g DW and 10.50 mg/g DW, respectively. Golkar et al. [28] enhanced the

stevioside and rebaudioside A concentrations up to 32.34 mg/g DW and 3.40 mg/g DW in callus culture exposed to 45 mg/L silver nanoparticles (Ag NPs) and 0.25 mg/L SA, respectively. Javed et al. [29] cultured the nodal explants of *S. rebaudiana* on the media containing different concentrations of Copper oxide (CuO) nanoparticles (NPs) and determined the highest stevioside and rebaudioside A production as 2.06% and 4.17% in the shoots exposed to 10 mg/L CuO NPs, respectively. In the present study, much higher contents of stevioside could be achieved and the stevioside content reached its maximum value with the 100 µM MeJA-treated *in vitro* plantlets (50.07 mg/g DW). In our previous work, with 50, 100 and 200 µM concentrations of MeJA and SA, we obtained tiny shoots with necrotic leaves from 4 weeks MeJA and SA applications in agar culture. The stevioside contents of the 100 and 200 µM MeJA- and 200 µM SA-treated plantlets were not determined due to the week growth and the necrotic formation in the leaves. The stevioside content increased 8.05-fold (12.53 mg/g DW) and 8.89-fold (13.84 mg/g DW) with the 50 µM applications of both MeJA and SA, respectively [20]. In the present study, the exposure time was reduced up to two weeks and the stevioside content was improved without inhibiting plant growth.

Among the three elicitors, the stevioside content reached its maximum value (50.07 mg/g DW), which was 17.4-fold higher than that of the control plants, in the 100 µM MeJA-treated *in vitro* plantlets. When the concentrations of all off the elicitors were compared, the 100 µM dose was found to be more effective than the other concentrations in stevioside production. After 2 weeks of elicitation, no rebaudioside A production was observed in all the applications and control.

4. CONCLUSION

In the present study, all of the elicitors (MeJA, SA, and CHI) tested resulted in an increase in the stevioside synthesis when compared to the untreated *in vitro* plantlets. *In vitro* plantlets synthesized the highest stevioside content (50.07 mg/g DW) when they were exposed to 100 µM MeJA. This result is 17.4-fold higher than in the control plants. Elicitor application can be made to plants grown in bioreactors and field or hydroponic system for increasing SGs contents.

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