International Journal of Secondary Metabolite

e-ISSN: 2148-6905

IJSM
International Journal of Secondary Metabolite

http://www.ijate.net/index.php/ijsm
http://dergipark.gov.tr/ijsm

IJSM

Volume: 5
Number: 3
2018

International Journal of Secondary Metabolite (IJSM)
is a peer-reviewed online journal

e-ISSN: 2148-6905

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Volume 5, Issue 3, September, 2018

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Publisher	:	İzzet KARA
Frequency	:	4 issues per year (March, June, September, December)
Online ISSN	:	2148-6905
Website	:	http://www.ijate.net/index.php/ijate http://dergipark.gov.tr/ijsm
Design&Graphic	:	IJSM

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hosted by Turkish JournalPark ACADEMIC

International Journal of Secondary Metabolite

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- JournalTOCs
- Akademik Türk Dergileri İndeksi
- International Innovative Journal Impact Factor (IIJIF)

ISSN:2148-6905

ISSN:2148-6905

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Research Article

International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 171-185

DOI: 10.21448/ijsm.430703

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

Plant Taxa Used in the Treatment of Diabetes in Van Province, Turkey

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Abstract: Despite the richness of local flora and medicinal plant utilization, there is no any report on documentation of antidiabetic botanicals used in Van province. Therefore, the present study aimed to record accumulation of the traditional antidiabetic medicinal plants of Van province in order to preserve the valuable local medicines knowledge, which has been threatened by urbanization. Antidiabetic folk medicines were determined via field surveys conducted in 1065 settlements during the period of 2014-2017 through medicinal plants questionnaire and structured face-to-face interviews (600 informants) with local people who are well known in the province for their long practice in traditional medicine. 69 plant taxa (35 species with undocumented antidiabetic medicinal use in scientific literature for Turkey) including five endemic and one rare to Turkey belong to 16 families (principally Asteraceae and Lamiaceae) were recorded for their traditional antidiabetic use. 52 different vernacular names were detected which were mainly indicate morphological characteristics. Infusion prepared from leaf and flower organs were found as the most common preparation method of local medicines in the province. Use value analysis showed that *Rheum ribes*, *Urtica dioica*, *Scutelleria orientalis* subsp. *pichleri*, *Diplotenia cachrydifolia*, *Teucrium polium*, *Rosa canina*, *Campanula glomerata* subsp. *hispida*, *Rumex scutatus*, *Helichrysum plicatum* subsp. *plicatum* and *Tanacetum balsamita* subsp. *balsamita* might serve promising pharmaceutical agents for diabetes treatment.

ARTICLE HISTORY

Received: 10 April 2018
Revised: 26 April 2018
Accepted: 30 May 2018

KEYWORDS

Diabetes,
Medicinal Plants,
Van,
Asteraceae

1. INTRODUCTION

Diabetes is among metabolic disorders that characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both [1]. The total number of diabetic patients estimated to rise from 171 million in 2000 to 366 million in 2030 across the world and through its long-term effects; it is a cause of highest morbidity rate around the globe [2, 3]. Acarbose, metformin, miglitol and voglibose are among commercially available synthetic antidiabetic drugs commonly used in the management of diabetes, which have potentially hazardous side effects such as liver problems and diarrhoea. Phytotherapeutics and/or phytopharmaceuticals are accepted as complementary medicines or an alternative to conventional medicines with fewer side effects. Phytochemicals identified from traditional medicinal plants present an exciting opportunity for the development of newer antidiabetic

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ISSN-e: 2148-6905 /© IJSM 2018

agents. Some natural herbal sources that have extraordinary antidiabetic potential are tea polyphenols, pine bar extract, ginsenosides, condensed tannins, epigenine, charantin, and kotalanol [3].

Local people of Van province have been used various medicinal plants in the treatment of diabetes for a long time. Antidiabetic preparations have been used as valuable alternative and/or complementary agents to conventional medicines in the province. Though the richness of local flora and common medicinal plant utilization, there is no any report on documentation of antidiabetic botanicals used in Van province in the scientific literature. Hence, the present study was aimed to (i) document plant taxa used in the treatment of diabetes by local people of Van province for centuries which has been threatened by urbanization, (ii) analyse data via determining the most efficient plant taxa for diabetes in order to make contribution to antidiabetic drug discovery, (iii) analyse the local names, plant part(s) used, preparation and utilization methods of these folk medicines since local names, formulation of traditional remedies and methods of their preparation can assist to pharmaceutical studies such as the proper extraction method, therapeutic effect, pharmacological dose and body intake form [4].

2. MATERIAL AND METHODS

2.1. The study area

The study area is located in the Eastern Anatolia Region of Turkey at an approximate altitude of 1.800 m above sea level and with an area of 19,069 km^2. It is surrounded by Iran to the east, Ağrı to the north, Van Lake, Bitlis and Ağrı to the west, Siirt, Şırnak and Hakkari to the south (Figure 1). The study area consisted of 1065 settlements (counties, villages and hamlets), belong to the Iran-Turan Plant Geography Region, and situated in B9 and C9 grid square, which is one of the main endemism centres in Turkey [5]. The dominated climate is continental characterized by cold, long and snowy winters, short and rainy springs, and hot and dry summers.

Figure 1. The study area

The study area is surrounded by chains of high mountains from north, south and east which represents mountainous fields with numerous highlands (Nordiz, Sündüs, Abaza, Tirşîn, Nebirnav etc.) that contribute to a rich biodiversity. There are several mountains (Başet, Artos, Kavuşşahap, Karadağ), stream and rivers (Karasu, Zilan, Güzelsu, Özalp, Müküs, Bendimahi,

Bahçesaray, Çatak), valleys (Bendimahi, Zilan, Hoşap, Memedik, Çatak and Havasor), lake (Van gölü, Erçek gölü, Keşiş gölü, Akgöl, Zernek), lowland (Gürpınar, Erciş, Muradiye and Hoşap), gateways (Güzeldere, Kerapet, Kurubaş) and wetlands (Bendimahi, Çelebibağ, Çaldıran, Edremit) in the province. The geomorphological differences in the field contribute to the natural vegetation. Van province has been an important settlement from ancient times. Several instances of historical and cultural settlements are located in the province because of being homeland of various civilizations including Urartians, Persians and Ottomans, which contributed the province in terms of culture, civilization and agriculture. The economy of the province largely depends on stockbreeding and agriculture.

2.2. Antidiabetic herbal medicine data collection

This study conducted in 1065 settlements bounded to Van city between the periods of 2014-2017. In order to obtain comprehensive antidiabetic folk medicine data of Van province, the extensive ethnobotanical surveys (Appendix) and face-to-face interviews were carried out with 600 different local people who are well known in the province for their long practice in traditional medicine. The interviews were conducted in accordance to the requirements of the International Society of Ethnobiology Code of Ethics. Interviews were generally conducted in the fields, gardens, teahouses, highlands and village houses. During data collection surveys, demographic characteristics of the local people, vernacular/local names of plant species, preparation and utilization methods in traditional medicine were recorded. Moreover, live plant samples were collected from wild by help of those local people in order to identify their scientific names.

2.3. Plant materials

The field studies were carried out over a period of 4 year (2014-2017). Collector and herbarium numbers, family names, scientific plant names, endemism and risk categories, local names, plant part(s) used, preparation and utilization methods of antidiabetic plant materials collected were recorded properly and presented in Table 2. During this period, 69 taxa belong to 16 family (Spermatophyta (Angiospermae)) were collected from wild areas with no apparent physical damage at vegetation time (flowering, fruit and seed periods) for proper botanical nomenclature analysis (Table 2).

Herbarium samples were prepared from fresh plant materials through standard herbarium techniques. Scientific identities (family and species names) were determined according to the plant identification literatures: Flora of Iran [6], Flora of Iraq [7], Flora of Turkey [5, 8-9] and comparison with the specimens of Van Pharmaceutical Herbarium (VPH). Scientific names of plant samples were confirmed by using The Plant List (www.theplantlist.org) and International Plant Name Index (IPNI: http://www.ipni.org) and alphabetically ordered (Table 2). Herbarium samples prepared from plant materials have been stored at Van Pharmaceutical Herbarium, Faculty of Pharmacy, Van Yuzuncu Yil University, Turkey and collector and herbarium numbers were given properly (Table 2). The endemism and risk categories were specified properly [10-11] as presented in Table 2.

2.4. Statistical analysis

Statistical analysis of this study was performed by using Use Value (UV) as described previously [12]. Use value is a quantitative method that demonstrates the relative importance of species utilization locally, was calculated according to the following formula: UV = U/N, where UV refers to the use value of a species; U to the number of citations per species; and N to the number of informants. The UV values of the antidiabetic plant taxa were presented in Table 2.

3. RESULTS

3.1. Demographic characteristics of informants

As presented in Table 1, 600 local people were interviewed during field surveys. The majority of the respondents were male, low educated (literate and / or primary school) and ≥ 31 aged group. The local knowledge of herbal medicines had the lowest level in female, university educated and ≤ 30 aged groups. The number of male respondents were approximately 1.75 fold that of the female respondents (Table 1).

Table 1. Demographic characteristics of the informants (n=600)

	Number	%
Age		
20-30	110	18
31-49	200	33
50 and above	290	48
Sex		
Male	380	63
Female	220	36
Educational level		
Literate	210	35
Primary school	140	23
Secondary school	110	18
High school	80	13
University	60	10

3.2. Demographic characteristics of informants

Table 2. Database of plant taxa used in the treatment of diabetes in Van province

Plant species, endemism-rare IUCN [a]	Voucher specimen/ Herbarium Numbers [b]	Vernacular or Local Name(s)	Plant part(s) used	Preparation Method	Utilization Method [c]	NI [d]	UV [e]	Recorded Ethnobotanical Antidiabetic Use	
								Eastern Anatolia	Turkey (except E.Anatolia)
APIACEAE									
1. *Diplotaenia cachrydifolia* Boiss. (R-VU)	AD671 /VPH260	Sîyabo	Leaf, Root	Raw eaten Decoction	RAW, DOGBM	218	0.36	[12, 13]	-
2. *Eryngium bornmuelleri* Nab. (END-NT)	AD672/ VPH261	Tusî	Leaf	Infusion	DOGE	32	0.05	[13]	-
3. *Ferula orientalis* L.	AD673/ VPH262	Heliz	Leaf	Infusion	DOGAM	78	0.13	[13, 14]	-
4. *Ferula rigidula* DC.	AD674/ VPH263	Heliz	Leaf	Infusion	DOGAM	49	0.08	[15]	-
5. *Heracleum persicum* Desf.	AD675/ VPH264	So(y)	Leaf	Infusion	DOGESM	37	0.06	[13]	-

ASTERACEAE										
6.	*Achillea arabica* Kotschy	AD720/ VPH265	Bovîjan	Flower	Infusion	DOGAM	53	0.09	-	-
7.	*Achillea millefolium* L. subsp. *millefolium*	AD721/ VPH266	Bovîjan	Aerial parts	Decoction	DOGD	47	0.07	-	-
8.	*Anthemis cretica* L. subsp. *anatolica* (Boiss.) Grierson	AD72/ VPH267	Kulîlîk	Leaf , Flower	Infusion	DOGAM	26	0.04	-	-
9.	*Artemisia absinthium* L.	AD723, VPH268	Granguruh, Tahlişk, Bevüjana kuvî	Leaf Flower	Infusion	DOGD	82	0.14	[12-14]	[16-18]
10.	*Centaurea iberica* Trev. ex Spreng.	AD72 VPH269	Tahliş	Leaf	Infusion	DOGD	21	0.03	-	[19]
11.	*Centaurea glastifolia* L.	AD725/ VPH270	Tahliş	Leaf	Infusion	DOGD	13	0.02	-	-
12.	*Centaurea pterocaula* Trautv.	AD726/ VPH271	Tahliş	Leaf	Infusion	DOGD	19	0.03	-	-
13.	*Centaurea saligna* (K.Koch.) Wagenitz (END- LC)	AD727/ VPH272	Tahlişa spi	Leaf, Flower	Infusion	DOGD	27	0.05	-	-
14.	*Crepis hakkarica* Lamond (END- EN)	AD728/ VPH273	Tahliş	Leaf, Flower	Decoction	DOGD	7	0.01	-	-
15.	*Helianthus tuberosus* L.	AD729/ VPH274	Sevik	Tuber	Raw eaten	FC			[12,13, 15]	[20, 21]
16.	*Helichrysum arenarium* (L.) Moench subsp. *aucheri* (Boiss.) P.H.Davis & Kupicha (END--LC)	AD730/ VPH275	Herdemcan	Aerial parts	Decoction	DOGTD	62	0.1	[13]	-
17.	*Helichrysum armenium* DC. subsp. *armenium*	AD731/ VPH276	Herdemcan	Aerial parts	Decoction	DOGTD	74	0.12	[13]	-
18.	*Helichrysum pallasii* (Sprengel) Ledeb.	AD732/ VPH277	Herdemcan	Leaf, Flower	Infusion	DOGTT	41	0.07	[13]	-
19.	*Helichrysum plicatum* DC. subsp. *plicatum*	AD733/ VPH278	Herdemcan	Aerial parts	Decoction	DTGAM	157	0.26	[13,15, 22, 23]	-
20.	*Gundelia colemerikensis* Fırat (END-VU)	AD734/ VPH279	Kengerzer	Whole parts	Raw eaten	DOGD	52	0.09	-	-

21.	*Onopordum acanthium* L.	AD735/ VPH280	Kivar	Seed	Decoction	DTGD	58	0.1	-	-
22.	*Psephellus karduchorum* (Boiss.) Wagenitz (END- VU)	AD736/ VPH281	Giya brînok	Leaf, Flower	Infusion	DOGAM	15	0.02	-	-
23.	*Scorzonera latifolia* (Fisch. & C.A.Mey.) DC. var. *latifolia*	AD737/ VPH282	Nermend	Leaf	Raw eaten	EFLDEW	109	0.18	[24]	
24.	*Tanacetum balsamita* L. subsp. *balsamita*	AD738/ VPH283	Papatya	Leaf, Flower	Infusion	DTCTDTW	134	0.22	-	-
ARACEAE										
25.	*Arum rupicola* Boiss. var. *virescens* (Stapf) P.C	AD681/ VPH284	Kahrî	Tuber	Decoction	DOCDOW	8	0.01	[15]	
BERBERIDACEAE										
26.	*Berberis vulgaris* L.	AD682/ VPH285	Êmîş	Fruit	Raw eaten	CPDTM	37	0.06	[15]	-
CAMPANULACEAE										
27.	*Campanula glomerata* L. subsp. *hispida* (Witasek) Hayek	AD683/ VPH286	Nojda	Aerial parts	Decoction	DOCBS	176	0.29	-	-
CUCURBITACEAE										
28.	*Bryonia multiflora* Boiss. & Heldr.	AD684/ VPH287	Jurî ruvî	Fruit	Raw eaten	CFDFW	11	0.02	-	-
FABACEAE										
29.	*Astragalus gummifer* Lab.	AD714/ VPH288	Gunîzer	Root	Decoction	DOCDTW	69	0.12	[13, 15]	-
30.	*Astragalus longifolius* Lam. (END)	AD715/ VPH289	Girgunî	Root	Decoction	DOCDTW	31	0.05	[13]	-
31.	*Astragalus amblolepis* Fischer	AD716/ VPH290	Girgunî	Root	Decoction	DOCDTW	6	0.01	-	-
32.	*Astragalus halicacabus* Lam.	AD717/ VPH291	Çekçekok	Root	Decoction	DOCDTW	17	0.03	-	-
33.	*Astragalus pycnocephalus* Fischer	AD718/ VPH292	Gunî	Root	Decoction	DOCDTW	5	0.01	-	-
34.	*Lathyrus tuberosus* L.	AD719/ VPH293	Xenc	Tuber	Raw eaten	CFTDTW	95	0.16	[13, 14]	

HYPERICACEAE										
35.	*Hypericum perforatum* L.	AD685/ VPH294	Sîc	Flower	Infusion	DOGTHD	40	0.07	-	[17, 25]
36.	*Hypericum scabrum* L.	AD686/ VPH295	Sîc	Flower	Infusion	DOGTHD	83	0.14	-	-
LAMIACEAE										
37.	*Nepeta betonicifolia* C.A. Mey. subsp. *betonicifolia*	AD692/ VPH296	Nojda	Leaf, Flower	Infusion	DOCD	19	0.03	-	-
38.	*Nepeta lamiifolia* Willd.	AD693/ VPH297	Nojda	Leaf, Flower	Infusion	DOCD	7	0.01	-	-
39.	*Salvia limbata* C.A.Mey.	AD694/ VPH298	Bareşa spî	Leaf, Flower	Infusion	DOCD	56	0.09	-	-
40.	*Salvia macrohlamys* Boiss. & Kotsch	AD695/ VPH299	Çirçirk	flower	Infusion	DOCDTW	48	0.08	-	-
41.	*Salvia poculata* Nab.	AD696/ VPH300	Bareş	flower	Infusion	DOCDTW	71	0.12	-	-
42.	*Salvia trichoclada* Bentham	AD697/ VPH301	Bareş	Leaf, Flower	Infusion	DOCDTW	13	0.02	-	-
43.	*Salvia verticillata L.* subsp. *verticillata*	AD698/ VPH302	Bareş	Leaf, Flower	Infusion	DTCBS	104	0.17	-	-
44.	*Scutelleria orientalis* L. subsp. *pichleri* (Stapf.) Edmondson	AD699/ VPH303	Qêsêlmehmud	Leaf, Flower	Infusion Raw eaten	DTCBS	253	0.42	-	-
45.	*Stachys lavandulifolia* Vahl var. *lavandulifolia*	AD700/ VPH304	Bareş	Leaf, Flower	Infusion	DTCBS	20	0.03	-	-
46.	*Teucrium chamaedrys* L. subsp. *syspirense* (C.Koch) Rechf	AD701/ VPH305	Neman	flower	Infusion	DTCD	9	0.02	-	[19, 24]
47.	*Teucrium orientale* L. var. *puberulens* Ekim	AD702/ VPH306	Neman	Leaf, Flower	Infusion	DTCD	5	0.01	-	-
48.	*Teucrium polium* L.	AD703/ VPH307	Qeselmehmuda şin	Leaf, Flower	Infusion	DOGTES	202	0.34	[13, 15, 22, 23, 26, 27]	[24, 25, 28-30]
49.	*Thymus kotschyanus* Boiss. & Hohen. subsp. *kotschyanus*	AD704/ VPH308	Catir	Leaf, Flower	Infusion	DOCAMTM	51	0.09	-	-

50.	*Thymus kotschyanus* subsp. *kotschyanus*	AD705/ VPH309	Catir	Leaf, Flower	Infusion	DOCAMTM	23	0.04	[15]	-
51.	*Ziziphora tenuior* L.	AD706/ VPH310	Catira kuvî	Leaf, Flower	Infusion	DOCD	39	0.07	-	-
ORCHIDACEAE										
52.	*Dactylorhiza umbrosa* (Kar. & Kir.) Nevski	AD676/ VPH311	Sahlep	Tuber	Decoction	DOCDTW	51	0.09	-	-
PAPAVERACEAE										
53.	*Fumaria schleicheri* Soy. Will. subsp. *microcarpa* (Hausskn) Liden	AD677/ VPH312	Nişatir	Aerial parts	Decoction	DOCD	12	0.02	-	-
54.	*Papaver bracteatum* Lindl.	AD678/ VPH313	Xişxaş	Seed	Raw eaten	DC	44	0.07	-	-
PLANTAGINACEAE										
55.	*Plantago lanceolata* L.	AD679/ VPH314	Giyamembel	Leaf	Raw eaten	DC	71	0.12	[12]	-
56.	*Plantago major* L. subsp. *major*	AD680/ VPH315	Belghevîzar	Leaf	Infusion	DOGBM	30	0.05	-	[31]
POLYGONACEAE										
57.	*Rheum ribes* L.	AD687/ VPH316	Rêvas	Root	Decoction	DOGTDES	322	0.54	[12-15, 23, 27]	[32]
58.	*Rumex acetosella* L.	AD688/ VPH317	Tirşok	Sap	Decoction	DOGD	124	0.21	[26]	[18, 33]
59.	*Rumex scutatus* L.	AD689/ VPH318	Tirşka tirş	Leaf	Raw eaten	DC	159	0.27	[34]	[32, 35]
60.	*Rumex tuberosus* L. subsp. *horizontalis* (Koch) Rech.	AD690/ VPH319	Tirşok	Leaf	Infusion	DTGD	76	0.13	[13, 27]	[32]
PRIMULACEAE										
61.	*Primula auriculata* Lam.	AD691/ VPH320	Belg sîsîn	Leaf	Infusion	DOGD	13	0.02	-	-
ROSACEAE										
62.	*Cerasus avium* (L.) Moench	AD707/ VPH321	Helhelok	Dry fruit	Decoction	DOGESM	24	0.04	-	-
63.	*Crataegus monogyna* Jacq. subsp. *monogyna*	AD708/ VPH322	Guhîş	Fruit	Decoction	DOGTD; DC	67	0.11	-	[16, 17]
64.	*Malus sylvestris* Mill. subsp. *orientalis* (A.Uglitzkich) Browicz var. *orientalis*	AD709/ VPH323	Sevtirşk	Dry fruit	Decoction	DOGAM	108	0.18	[15]	-
65.	*Rosa canina* L.	AD710/ VPH324	Şîlank	Fruit	Decoction Raw eaten	DOGBM; DC	161	0.29	[22, 26]	[24, 29, 33, 36]

66.	*Rosa heckeliana* Tratt. subsp. *vanheurckiana* (Crépin) Ö.Nilsson	AD711/ VPH325	Şîlank	Fruit	Decoction Raw eaten	DOGBM; DC	18	0.03	-	-
67.	*Rosa pisiformis* (Christ) D. Sosn. (END-NT)	AD712/ VPH326	Şîlank	Fruit	Decoction Raw eaten	DOGBM; DC	42	0.07	-	-
68.	*Rubus sanctus* Schreber	AD713/ VPH327	Tutirik	Fruit	Raw eaten	DC	9	0.02	-	[24]
URTICACEAE										
69.	*Urtica dioica* L.	AD670/ VPH328	Gezînk	Aerial parts	Decoction	DOGTHD	289	0.48	[13-15, 22, 26, 34]	[24, 36, 37]

[a] **END:** Endemic**, R:** Rare**; EN**: Endangered, **LC**: Least concern, **NT**: Near Threatened, **VU**: Vulnerable; [b] **AD:** Abdullah Dalar**; VPH:** Van Pharmaceutical Herbarium [c] CFDFW: Consuming 4-5 fruits daily during 4-5 weeks; CFTDTW: Consuming 4-5 tubers daily during 2-3 weeks; CPDTM: Consuming 5-6 pieces daily during 2 months; DC: Directly consuming; DOCDOW: Drinking one tea cup daily during one week; DOCDTW: Drinking one tea cup daily during 2-3 weeks; DOCBS: Drinking one tea cup before sleeping; DOCD: Drinking one tea cup daily; DOGAM: Drinking one tea glass after meal; DOCAMTM: Drinking one tea cup after meals during two months; DOGBM: Drinking one tea glass before meal; DOGE: Drinking one glass in the evening; DOGESM: Drinking one glass on empty stomach in the morning; DOGD: Drinking one tea glass daily; DOGTD: Drinking one tea glass twice daily; DOGTES: Drinking one tea glass twice daily on empty stomach; DOGTHD: Drinking one tea glass thrice daily; DOGTT: Drinking one tea glass thrice daily; DTCBS: Drinking two tea cups before sleeping; DTCD: Drinking two tea cups daily; DTCTDTW: Drinking two tea cups 3 times a daily during 3 weeks; DTGAM: Drinking three tea glasses after meal; DTGD: Drinking two tea glasses daily; EFLDEW: Eating four leaves daily during eight weeks; FC: Freshly consuming; RAW: The plant is eaten raw on an empty stomach in the morning.[d] **NI:** Number of informants, [e] **UV**: Use value.

Table 2 presents plant species, endemism and rare status, risk categories, voucher numbers, vernacular name(s), plant part(s) used, preparation and utilization methods, number of informants, use values and recorded ethnobotanical antidiabetic use of these species according to scientific literature. A total of 69 antidiabetic plant taxa belong to 16 family (Spermatophyta (Angiospermae)) were determined in the study area. All antidiabetic botanicals used in the province were detected as wild, which were gathered during the vegetation time by local people. No any complication occurred based on the utilization of plant species used as traditional antidiabetic medicine in Van province was recorded during ethobotanical field surveys.

Among these antidiabetic botanicals, 35 of them including *Achillea arabica*, *Achillea millefolium* subsp. *millefolium*, *Anthemis cretica* subsp. *anatolica*, *Centaurea glastifolia*, *Centaurea pterocaula*, *Centaurea saligna*, *Crepis hakkarica*, *Gundelia colemerikensis*, *Onopordum acanthium*, *Psephellus karduchorum*, *Tanacetum balsamita*, subsp. *balsamita*, *Campanula glomerata* subsp. *hispida*, *Bryonia multiflora*, *Astragalus amblolepis*, *Astragalus halicacabus*, *Astragalus pycnocephalus*, *Hypericum scabrum*, *Nepeta betonicifolia* subsp. *betonicifolia*, *Nepeta lamiifolia*, *Salvia limbata*, *Salvia macrohlamys*, *Salvia poculata*, *Salvia trichoclada*, *Salvia verticillata* subsp. *verticillata*, *Scutelleria orientalis* subsp. *pichleri*, *Stachys lavandulifolia* var. *lavandulifolia*, *Teucrium orientale* var. *puberulens*, *Thymus kotschyanus* subsp. *kotschyanus*, *Ziziphora tenuior*, *Dactylorhiza umbrosa*, *Fumaria schleicheri* subsp. *microcarpa*, *Papaver bracteatum*, *Primula auriculata*, *Cerasus avium*, *Rosa heckeliana* subsp. *vanheurckiana* and *Rosa pisiformis* were reported for the first time for their traditional antidiabetic use for Turkey according to recorded scientific literature presented in Table 2.

Among antidiabetic folk medicines, 5 of them were identified as endemic (*Eryngium bornmuelleri*, *Centaurea saligna*, *Crepis hakkarica*, *Psephellus karduchorum* and *Rosa pisiformis*) and one as rare (*Diplotenia cachrydifolia*) (Table 2). According to risk categories, there are one taxa (*Crepis hakkarica*) as endangered, two taxa (*Diplotenia cachrydifolia* and *Psephellus carduchorum*) in vulnerable, two taxa (*Eryngium bornmuelleri* and *Rosa pisiformis*) in near threatened and two taxa (*Centaurea saligna* and *Helicrysum arenarium*) in least concern (Table 2).

With regards to the local names, 52 local plant names corresponded to 69 plant species used by local population detected in the study area. The local names were used generally for expressing morphological characteristics (herdemcan, kengerzer, gunizer, belghevizar) or taste (tahliş, tirşok, tirşkatirş) (Table 2).

Some local names assigned by the locals to the medicinal plants refer to more than one taxa such as tahliş that is used for naming four different taxa, herdemcan (4), bares (4) and şilank (3) (Table 2). The genus taxa distribution showed that *Astragalus* and *Salvia* were the most represented taxa (5 taxa each) in the province in terms of diabetes treatment followed by *Centaurea* (4 taxa), *Helichrysum* (4 taxa), *Teucrium* (3 taxa), *Rumex* (3 taxa), *Rosa*, (3 taxa), *Ferula*, *Achillea*, *Hypericum*, *Nepeta*, *Thymus* and *Plantago* (2 taxa each) (Table 2).

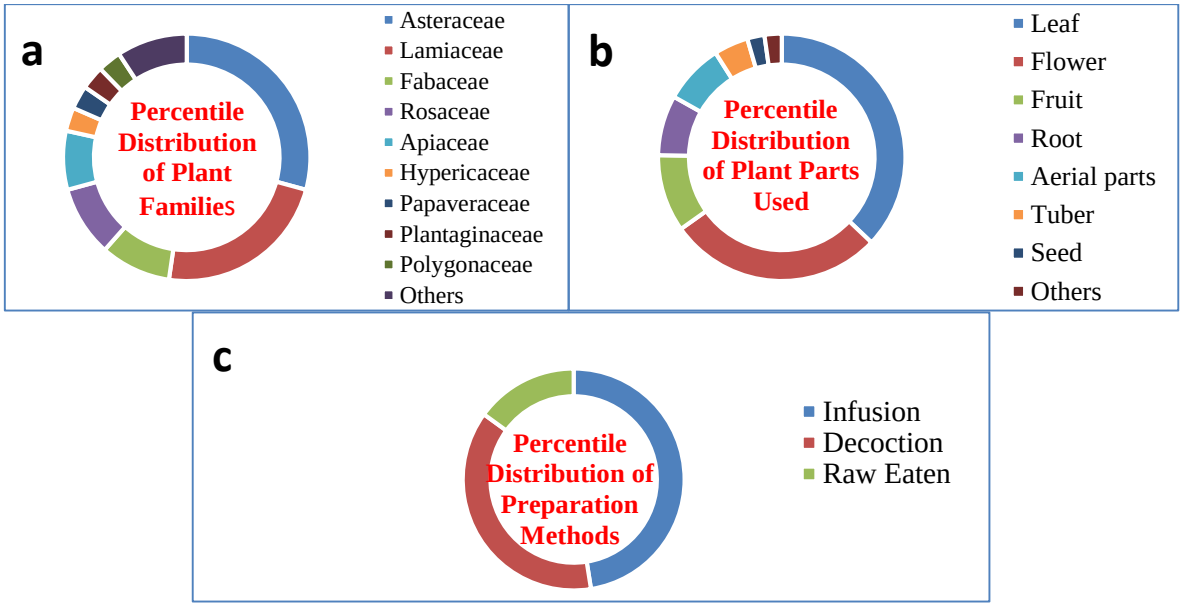

Figure 2. Percentile distribution of a) Plant families, b) Plant parts used and c) preparation methods.

The percentile distribution of plant families was presented in Figure 2a. Asteraceae was found as the most utilized plant family represented by 19 taxa (30%) and followed by Lamiaceae (15 taxa; 23%) (Figure 2a). The other most utilized plant families were Fabaceae (6 taxa; 9%), Rosaceae (6 taxa; 9%), Apiaceae (5 taxa, 8%). Leaf (37%) and flower (28%) parts were found as the major plant parts utilized in antidiabetic folk remedies followed by fruit (10%) and root (8%) (Figure 2b). With regards to preparation methods (Figure 2c), only 3 methods including infusion, decoction and raw eaten were detected with the superiority of infusion (47%).

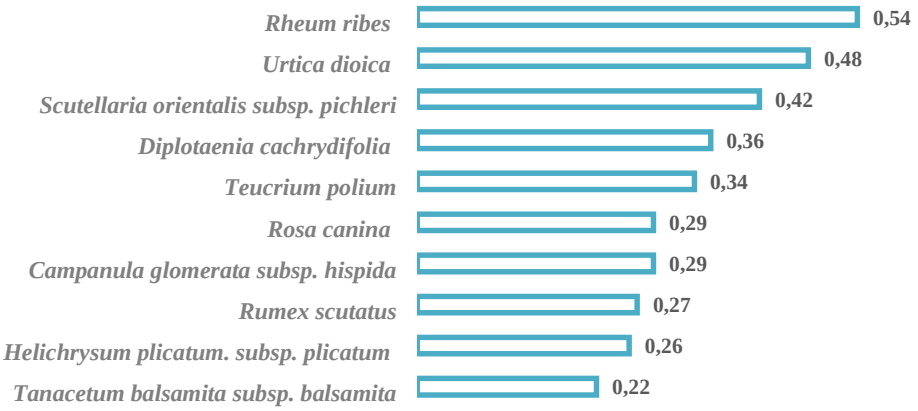

Figure 3. Plant taxa had the highest UV

Figure 3 presents the plant taxa with high use values of antidiabetic botanicals of Van province. The highest use value was detected in *Rheum ribes*, followed by *Urtica dioica*, *Scutelleria orientalis* subsp. *pichleri*, *Diplotenia cachrydifolia*, *Teucrium polium*, *Rosa canina*, *Campanula glomerata* subsp. *hispida*, *Rumex scutatus*, *Helichrysum plicatum* subsp. *plicatum* and *Tanacetum balsamita* subsp. *balsamita* (Figure 2). The lowest use value was found in *Crepis hakkarica*, *Arum rupicola*, *Astragalus amblolepis*, *Nepeta lamiifolia*, *Teucrium orientale* var. *puberulens* (UV: 0.01) (Table 2).

4. DICCUSSION AND CONCLUSION

Demographic characteristics of the informants findings indicate that familiarity of the local population with antidiabetic botanicals and their uses were inversely proportional with their level of education. Moreover, the number of male respondents were approximately 1.75 fold that of the female respondents (Table 1). The reason behind of this matter is probably due to the higher interaction rate of the male respondents with nature who were mostly shepherds and/or farmers.

Among all plant families, Asteraceae was found as the most utilized family for therapeutic properties, which was also reported in various ethnobotanical surveys conducted in Eastern Anatolia by several researchers [12, 15, 27]. It was reported that Asteraceae was the richest family across the world (among 452 vascular plant families) and Turkey (167 plant families) [38, 39] which have been identified by botanists until now. The prevalence of the Asteraceae family across the world including various ecological zones and different climatic conditions including our study area can be related to their wide tolerance broadness against several stress factors including Ultraviolet rays and cold, which are among the main stress factors in Van province.

The selection of proper plant part(s) or their products for targeted ailment treatment in folk medicine varies due to cultural context and as well as the ecological conditions which affect the production of bioactive compounds. Within this study, leaf and flower had superiority use compare to seed, tuber, stem, fruit and root. UV-B damage protection is one of the most significant roles of chemical compounds synthesized in plants [40]. Leaf and flower organs had the largest area in flowering plants and are the most exposed plant parts to the UV-B radiation and hence production of chemical compounds was relatively higher than those of the other plant organs such as stem, root and fruit, which can be one of the main reason of the superior usage of leaf and flower parts in Van province by local people in traditional medicine.

The vegetative plant parts of endemic plant taxa are the most utilized plant organs in Van province for antidiabetic purposes and unconscious and excessive collection of these plant

species can cause serious problems in the context of continuation of their generation. Endemic plant taxa (particularly *Psephellus karduchorum*) detected within this study are in risk categories and therefore some practises must be adopted for their conservation. For instance, these endemic plant taxa can be cultivated via plant tissue culture techniques or their populations can be scrutinized. Also local people, scientific researchers or local medicinal plant suppliers must be informed for avoiding excessive collection of these plant materials.

The main preparation methods detected in this study were infusion and decoction, which indicate that local remedies used in the treatment of diabetes in Van province mainly focused on hydrophilic compounds that might be among the major contributors of the antidiabetic effects.

A high number of plant taxa (69 species) used in the treatment of diabetes were detected within this study, which was remarkably higher than those studies conducted in Turkey. For instance, eight antidiabetic plant taxa were reported for Hakkari [12], 32 for Alaşehir (Manisa) [41], 24 for Hatay [19], 15 for Malatya [27], 9 for Espiye (Giresun) [33] and 15 for Esenli (Giresun) [42]. The higher number of antidiabetic plant taxa detected within this study can be explained by the limitation of modern medicine facilities and carbohydrate-based food habits. Pastoral farming and semi-nomadic life style, the rich local flora and abundance of mountainous fields, multiple numerous highlands, lowlands and valleys in the province let local people of Van province to utilize extensive medicinal practices on wild plants.

Use value (UV) was applied in order to detect the most used plant taxa and their effective healing potential(s) in certain disorders for therapeutic features. Namely, plant taxa with high value might have therapeutic potential since local people have used them for a long time with minimum side effects. Our findings showed that *Rheum ribes*, *Urtica dioica, Scutelleria orientalis* subsp. *pichleri, Diplotenia cachrydifolia, Teucrium polium, Rosa canina, Campanula glomerata* subsp. *hispida, Rumex scutatus, Helichrysum plicatum* subsp. *plicatum* and *Tanacetum balsamita* subsp. *balsamita* might serve promising pharmaceutical agents for diabetes treatment.

Plant taxa used in traditional medicine are based on observations of their effectiveness derived from their utilization on humans over centuries. This study presents a vast number of plant sources, selected by traditional wisdom, to cure diabetes in Van province for the first time. Until now, our knowledge regards the chemical composition and mechanism of actions of these antidiabetic folk medicines is limited. Traditional medicinal plants presented in this study may provide valuable leads for the identification of natural compounds for pharmaceutical uses. These plants, selected by generations of local people based on trial and error method, represent a vast source for the identification of novel and efficient antidiabetic agents

Acknowledgements

The author would like to thank to all indigenous people of Van province for their hospitality, interest and sharing their valuable traditional medicinal plant knowledge regards to diabetes. The author thanks to Mr. Muzaffer Mükemre of the Biology Department in Van Yuzuncu Yil University for help in plants collection from wild and botanical nomenclature.

Conflict of Interests

Authors declare that there is no conflict of interests.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 186–199

DOI: 10.21448/ijsm.416258

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

Cold Resistance of Plant Species of the Genus Vitex l. Introduced in M.M. GRYSHKO National Botanic Garden of NAS of Ukraine

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Abstract: Results of current studies of flavonoids content in vegetative and reproductive organs and their dynamics under cold stress in plants of the genus *Vitex* L. introduced in the M.M. Gryshko National Botanic Garden (NBG) of NAS of Ukraine are summarized. The varying effects of flavonoids on different plant organs during adaptation to different climatic and weather conditions of introduction are analyzed. The relation of dynamics of these compounds and the pigment level, photosynthetic rate, breath, transpiration, morphological and anatomical specifics of stomata is observed.

ARTICLE HISTORY

Received: 15 January 2018
Revised: 20 February 2018
Accepted: 28 April 2018

KEYWORDS

Vitex species, flavonoids, cold stress, vegetative and reproductive organs, physiological processes, pigments.

1. INTRODUCTION

Since ancient times, people were surrounded by plants known for their nutritional, medical and decorative properties. If such plants were absent in the local flora, the seeds, planting materials or plants were taken far away and introduced locally. At all times, the botanical gardens were engaged in enriching regional floras with new and exotic species.

Conserving and enhancing plant species diversity and varieties with introduced species are still relevant; the introduced plants have complex valuable economic and biological properties, which define their diverse use. Of many medical and odoriferous plants, species of the genus *Vitex* L. are prospective for introduction under the conditions of the forest-steppe zone of Right-Bank Ukraine. These plants are characterized by valuable medicinal, nutritional, aromatic, melliferous, technical and decorative properties, and can be used for different purposes in pharmaceutical industry, cosmetology, food and landscape construction, particularly.

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ISSN-e: 2148-6905 /© IJSM 2018

Taxonomically, the genus *Vitex* L. belongs to Magnoliophyta or Angiospermae, class Rosopsida, order Lamiales, suborder Lamiineae, family Verbenaceae, and subfamily Viticoideae [1].

More than 250 species (or more than 380, according to various sources [2]) are included in the genus. They are common in the Caucasus, Central Asia and Asia Minor, Mediterranean, North America, Northwest Africa, and New Zealand. In Ukraine, these plants grow in Crimea and in 1960s they were cultivated in the south of Ukraine and in Transcarpathia [3-6].

Vitex plants were brought to the American continent from Europe and the East. Today they are widely grown and naturalized there, from Maryland, the District of Columbia and New York to the states of Florida, Missouri, Arkansas, Texas and New Mexico [6].

Plants of the genus *Vitex* are widespread in tropical and subtropical regions in nature. They prefer wet areas near the banks of streams and rivers (especially near river mouths, in the ditches and valleys), coastal and seaside areas, sandy and dried-up alluvial soils, rocky areas near the sea, as well as limestone slopes, sunny and hot areas and grooves [7].

Plants of three *Vitex* species, which are part of collection of aromatic plants of the M.M. Gryshko National Botanic Garden of NAS of Ukraine, belong to the group of scientific objects which represent national treasure of our country. Species and varieties of plants of the genus *Vitex* are the important source for creating varieties with high promise for selection, which will enrich the biodiversity of the cultivated flora of Ukraine.

Plants of the studied *Vitex* species are bushes up to 2.9 m high with the upright stems covered with silky glandulosous in the conditions of introduction. Plant stems are usually perennial, but in adverse hibernation conditions they can freeze to the root cervix or to the level of snow cover. In spring the shoot system is restored by the sleeping buds (Fig. 1).

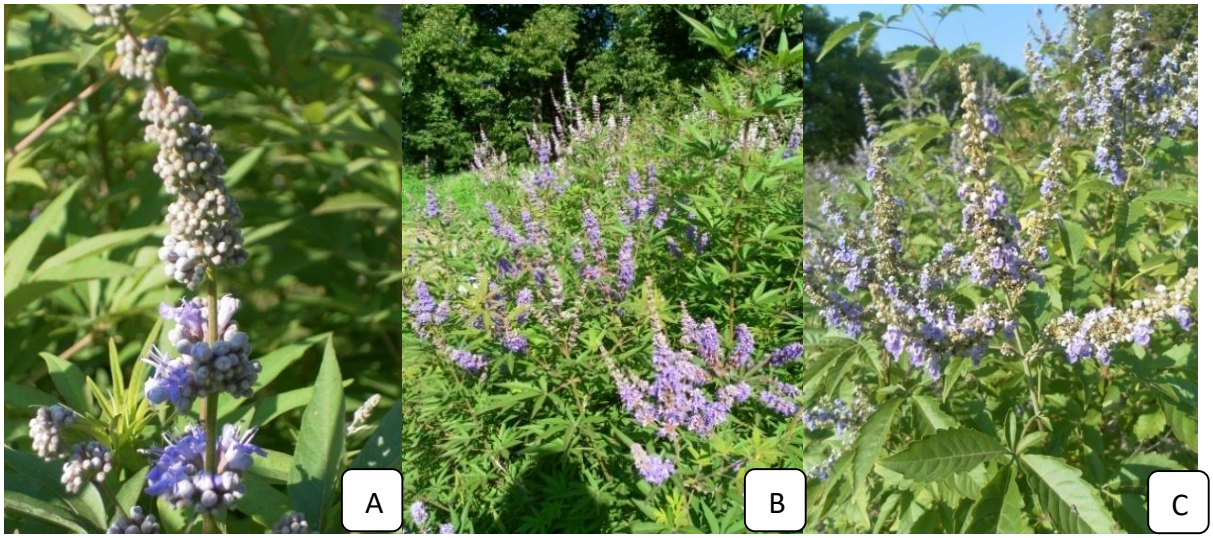

Fig. 1. Plants of species of the genus *Vitex* L. from the M.M. Gryshko NBG collection: A – *V. agnus-castus*; B – *V. cannabifolia*; C – *V. negundo*.

It has been established that species of the genus *Vitex* are plants with the late start and late end of vegetation period in the conditions of introduction. The beginning of plant growth is observed in the III decade of April – the II decade of May with the sum of active air temperatures above +10°C – 120-190°C and the average air temperature +16–22.5°C daily. The

duration of vegetation for the forest-steppe of Right-Bank Ukraine is 167±15.10 days for *V. agnus-castus*, 169.3±18.88 for *V. cannabifolia* and 170.7±18.56 days for *V. negundo* [8].

The flower cluster of *Vitex* plants is thyrsus. The plants have floral reversion and start blooming in III decade of June to the II decade of July when the sums of active temperatures are 525-900°C. Blooms last for 67-77 days in *V. agnus-castus*, 110-115 days in *V. cannabifoliata* and 91-115 days in *V. negundo* [8].

The flower of *Vitex* plants is complete, hermaphrodite, xenogamous, symmetrical, irregular, tetracyclic with a double perianth formed with calyx and sertulum different in appearance and colors, and dichlamydeous. Flower formula for the studied *Vitex* species is $\uparrow Ca_{(5)}Co_{(2+3)}\ A2_{,2}\ G_{(2)}$. The flower cumulosol is 5-bladed or 5-dentate, and remains on fruit. The surface is ribbed with a webbing of veins, densely covered with glandular and non-glandular hairs. The sertulum is symmetrical, zygomorphic, sympetalous, separated into the sympetalous cylindrical tube, the 5-bladed slanting curvature (the immature part) and the point of transition from tube to bend, the throat. The lower dorsal sepal is the three-bladed, large (the typical landing place for pollinators in all Lamiales) with wavy edge and a ring of hairs; the upper dorsal sepal raised over it, formed by accreted curves of two petals.

By morphology of pollen grains, the species can be divided into plants with pollen with erratic, netted surface and numerical perforation (*V. agnus-castus* and *V. cannabifolia*) and plants with smooth and netted surface of pollen with a large number of small openings (*V. negundo*). The largest pollen grains (35.6×19.5 microns) are found in *V. agnus-castus*, the smallest (30.3×16.1 microns) has *V. negundo* [8].

The fruit is small stinging, spherical, dry four-nested stone fruit with the lignified endocarp [9], from 3 to 4 mm in diameter [3-5, 10] breaking into four monospermous nut-like mericarps. The fruits ripen in late summer and fall [11]. There are good harvests of seeds almost annually. Typically, there are four nut-like mericarps per fruit in *V. agnus-castus*, and fewer (two or three) in *V. cannabifolia* and *V. negundo* due to the reduction of dissepiments and seed embryos.

The blooming phase and end of vegetation are the optimum periods for accumulation of secondary metabolites in plants [12].

The largest quantities of essential oil (0.24-0.65%) are found in the *V. agnus-castus* plants, *V. cannabifolia* produces almost as much (0.06-0.31%), the smallest amount is typical for *V. negundo* (0.04-0.13%). The maximum content of essential oils is typical for blossoming, fructification and end of vegetation periods. The blend composition of essential oil is one of species-specific characters of plants of the genus *Vitex*. 1.8-cineole, sabinene, citrene and α-pinene are typical for *V. agnus-castus, β*-caryophyllene, sabinene, caryophyllene oxide are typical for *V. cannabifolia* and *V. negundo* [8].

The plants of the studied species during the adaptation and acclimatization developed certain frost resistance, sufficient to survive the frosts of –25 to –30°C. The shoot tissues are damaged significantly by the frosts over –30°C, causing freezing to the root crown or to the level of a snow cover. The plants *of V. cannabifolia* have the best frost resistance abilities, *V. negundo* have the worst, and the plants *of V. agnus-castus* are in between [13].

Aim: climate changes, extreme weather conditions and environmental disasters affect the plants, changing their resistance and species ranges. Besides, introduction has an environmental impact on plant organisms, which remains a relevant and unresolved problem. The aim of our research was to elucidate the mechanism of formation of resistance to cold, low positive temperatures and weak frosts, to identify an adaptive component formed under cold stress, to discover the level of resistance to stress of the studied *Vitex* plants for preservation of the diversity, successful cultivation and optimum usage of plant raw materials.

2. MATERIALS AND METHODS

Plants of the introduced species of the genus *Vitex* from the collection of aromatic plants of department of cultural flora of M.M. Gryshko NBS of NAS of Ukraine (*Vitex agnus-castus, V. cannabifolia, V. negundo)* were studied. The content of flavonoids expressed as rutine was defined according to V.Yu. Andreyeva and G.I. Kalinina [14] in modification of V.F. Levon [12]. Samples of two grams of dry leaves were taken for laboratory analyses. The photosynthetic rate, the intensity of photorespiration, transpiration and dark respiration were studied according to A.T. Mokronosov [15]. The content of chlorophylls and carotenoids pigments was measured according to M.M. Musiyenko [16]. The measurement of stomata cells of species of the genus *Vitex* took place in the AxioVision program.

3. RESULTS AND DISCUSSION

The mechanism of formation of plant resistance to new climate conditions is necessarily connected with morphological and biochemical, physiological and molecular changes. The ability to resist both abiotic and biotic factors of the environment is the general prerequisite for the survival of a plant. Low temperatures and temporary freezing are some of the important abiotic stresses that cause damage to plants, reduce their immunity and cause yield loss in important crops.

When the plant prepares to and withstands stress, its levels of expression of different cold-responsive genes oscillate, specific protective compounds (proteins, amino acids, sugars and polyamines) are accumulated, the composition of the membrane changes, the activity of antioxidant systems increases, metabolic and physiological processes are reorganized, and it synthesizes a number of biologically active compounds to overcome cold [17].

Flavonoids are a group of biologically active compounds of polyphenolic origin with the general formula C6-C3-C6, accumulated in all plant organs as glycosides. Flavonoids are some of the main nutrients responsible for resistance to adverse conditions and protection of plants from various harmful environmental influences. Biotic and abiotic stress can lead to the intensification of biosynthesis of flavonoids in different anatomical parts of a plant [18].

Over 300 of flavons, flavonols and isoflavonoids from different plants are known today. Flavonoids are divided into catechins, anthocyans, chalcones, flavanones, flavones, flavanols according to the oxidation level of the pyran fragment [19]. Flavonoids are found in all plant parts, but are distributed unevenly. They are active metabolites of a plant cell, accordingly to localization in a plant. There are more flavonoids in actively functioning organs like flowers, fruits (color and aroma), leaves, sprouts, and in surface tissues with protective functions. The flavonoids content is lower in shoots, and the least is in the root system [20].

Flavonoids are the main pigments in flower. In leaves they block ultra-violet radiation which is destructive for nucleic acids and proteins, and usually selectively absorb blue-green and red light, important for photosynthesis [21]. Flavonoids are also in buds, pollen of flowers, trunk cortex, in wood and in plant secretions [22]. There is insufficient data on flavonoids in seeds [20]. Different organs and tissues of plants can be different not only by quantitative, but also by qualitative content of flavonoids [18].

There are plenty of data on biosynthesis of flavonoids. The biosynthesis of flavonoids in plants happens in three steps: formation of the main part (polyketide and shikimate pathways), further development of different C6-C3-C6 flavonoids, and the final modification by hydroxylation, methylation, glycosidation causing the differentiation of flavonoids in each class. The chalcones are essential for biosynthesis of flavonoids of different classes [20, 23-25].

Numerous studies have shown that the biosynthesis of flavonoid backbones, and their modifications are influenced by a variety of internal and external factors. It is revealed, firstly, that the multi-stage process of flavonoid formation in plants is regulated by a complex enzyme system. Secondly, the genetic factor plays an important role in the regulation of biosynthesis of flavonoids. Along with the internal factors important for the biosynthesis and regulation of flavonoids, the environmental factors also have the influence. Light, higher and lower temperature, humidity, chemical composition of soil, extreme conditions of the environment have the strongest influence on the biosynthesis of flavonoids among the external factors. Consequently, the set of external factors of the environment and conditions of growth definitely affect the biosynthesis of flavonoids [20, 22, 26].

Oxidation of flavonoids occurs during the ripening of seeds. Oxidation results in polymeric compounds in the surface seed cover, which leads to darkening of the coatings and reduction of water permeability [27, 28]. During the oxidative transformation of flavonoids, intracellular oxygen and water are used to prevent germination and spoilage of seeds [27, 29]. The flavonoids of seed coating, namely proanthocyanins, are important for preserving the seeds during storage. The presence of these compounds in the seed shell ensures the resistance to mechanical damage, water stress, pathogenic microorganisms, and pests [18]. This fact is extremely important since it enables the selection and creation of new plant varieties with extended storage period. In addition, according to modern studies, flavonoids in non-transparent impenetrable seed shells can protect them against UV-light, providing the opportunity to transport seeds in space [30].

The biological function of flavonoids should be mentioned. Due to the chemical basis, flavonoids take part in the oxidoreduction processes of the plant. Many flavonoid compounds have the antioxidant properties [20, 25]. The chelating compounds can be formed with different metals and flavonoids [31, 32]. Polysaccharides binding indicate their involvement in carbohydrate metabolism, proteins binding indicate their participation in the formation of some enzymes that take an active part in many biochemical processes. The presence of flavonoids in chloroplasts explains their participation in photophosphorylation and other biochemical processes in chloroplasts [20].

Flavonoids are also considered as signals increasing the sensitivity of plant roots to mycorrhiza and inducing the start of symbiosis with tuberous bacteria and mycorrhizal fungi [18].

Flavonoids are important in plant immune responses. Due to phytoncide properties, they are involved in the formation of plant resistance to diseases, fungal and especially bacterial infections. Phenolic compounds are essential for the healing of mechanical damage and regeneration of plants, as well as for the protection of cells from the radiation, free radicals, mutagens, oxidants. In addition, flavonoids can absorb the shortwave light, turning it into longer wavelengths. In combination with anthocyanins they regulate the distribution of light energy absorbed by the leaf, without participation in photosynthesis on a direct basis [33].

Phenolic compounds are instrumental in utilizing excessive active oxygen formed in photosynthesis [27]. Any abiotic stress causes the overproduction of H_2O_2 in chloroplasts, mitochondria and peroxisome plant cell together with the release of peroxidase and catalase from these organs. A significant amount of H_2O_2 diffuses into the vacuole as the main site of accumulation of flavonoids able to neutralize H_2O_2 and other reactive forms of oxygen [18].

A lot is known about the impact of flavonoids as activators of IAA oxidase on the growth and development of plants, during IAA biosynthesis or as its nonspecific inhibitors. Consequently, flavonoids are not just by-products of metabolism, they are essential in many life processes of plants [33]. In addition, the content of flavonoids depends on and can indicate

the level of environmental pollution [34]. In general, the main function of flavonoids as secondary metabolites is ecological in the broad sense. These are biochemical means by which the rooted-in plant solves its ecological problems of survival throughout its existence [35].

In our previous research we have found that the quantitative content of flavonoids of the studied *Vitex* plants is influenced by a complex of factors related to the metabolism processes. It is reliant on the internal rhythm of plant development and water exchange during the vegetation period. In spring, summer and fall months, there are two periods while the flavonoid content in leaves is maximum, during budding and at the end of vegetation. It was found that plants of *V. agnus-castus* and *V. cannabifolia* species tend to accumulate more flavonoids than *V. negundo* plants. It is species-specific irrespective of plant's age [8].

Let us review the flavonoid content dynamics in the plants of the genus *Vitex* at the end of the growing season under stress caused by the first frosts. Before the frost, the highest level of flavonoids was 1.132 mg/g in *V. agnus-castus*, the medium level of 0.885 mg/g in *V. negundo*, and the lowest, 0.783 mg/g, in *V. cannabifolia*. After the first frosts, the level of flavonoids in *V. agnus-castus* decreased, in *V. negundo* it remained stable and in *V. cannabifolia* it slightly increased. After more frosts, the level of flavonoids decreased to 0.949 mg/g in *V. agnus-castus*, remained stable in *V. negundo* and slightly increased to 0.906 mg/g in *V. cannabifolia* (Fig. 2).

The freezing of water in the intercellular ducts and cells, accompanied by dehydration, osmotic shock, mechanical injury of the membranes cause the damage and death of hibernating plants [36]. Cold damage is accompanied by destructive processes, namely loss of turgor, chlorophyll decomposition, protein hydrolysis, decreased photosynthesis and respiration, which cause the damage of ultra structure of chloroplasts and mitochondria [37]. Reduced sensitivity of plants to frost indicates complex chemical processes in cells, the development of cryoprotective systems that can mitigate the effect of low temperatures on plant by minimizing the probability of ice formation inside cells, maintaining supercooled liquid water, reducing the freezing temperature for cellular solutions and ice formation in the intercellular space and lowering the cellular dehydration through loss of water and ice crystals in the intercellular ducts [37].

From the experimental data, it is evident that plants of *V. cannabifolia* had an increased content of flavonoids during the first and second frosts (Fig. 2). This can be explained by the fact that flavonoids are osmotically active low molecular weight substances that form hydrophilic colloids, retain water, protect plant proteins and act as cryoprotectants for the studied plants. The appropriate response of the plant is to accumulate flavonoids due to cold stress, resulting in greater stability and ensuring the survival of that plant and its whole species.

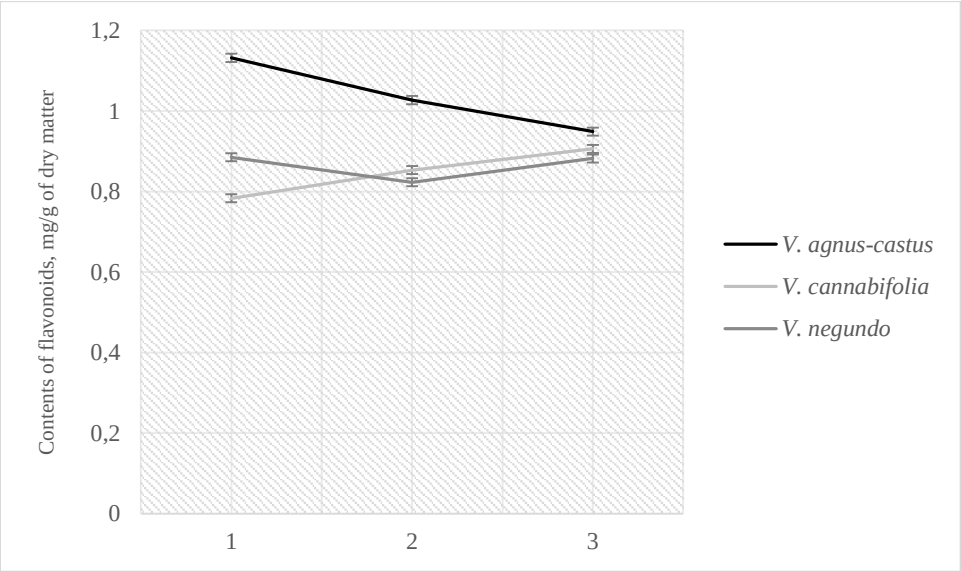

Fig. 2. Dynamics of accumulation of flavonoids during the frosts in leaves of *Vitex* plants: 1 – before the frosts, 2 – after the first frosts, 3 – after the second frosts

Plants of *V. agnus-castus* type had the highest level of flavonoids in leaves at the beginning of the experiment, but lost a lot of them during the frost period. Thus, they lost cryoprotectors and the possibility to resist cold stress. In contrast, *V. negundo* plants did not loose a significant amount of flavonoids due to the first frosts and restored their level after the second frosts.

According to the dynamics of the flavonoids content in the species *V. cannabifolia* and *V. negundo*, the plants reacted adequately to cold stress and their adaptability to the conditions of introduction is higher. The reaction of *V. agnus-castus* was opposite: its flavonoids were consumed without later restoring the level necessary for water retention and colloidal state of cells to ensure survival.

It is important to note that the plants obtained a certain level of frost resistance, sufficient to withstand the frost of -25°C during acclimatization, according to our previous studies [13]. The highest frost resistance was found in *V. cannabifolia* plants, the average in *V.agnus-castus*, and *V. negundo* plants were the most vulnerable to frost.

According to L.G. Kosulina [38], different organs of thermophilic cultures have different resistance to cold. The content of flavonoids in vegetative and reproductive organs of *Vitex* plants varies from 0.729 up to 0.949 mg/g, regardless of species and organ (Fig. 3).

It is essential that flavonoids are adaptive in fruits or seeds during the rest periods. In particular, seeds of plants of the genus *Vitex,* as is typical for plants of temperate and subtropical climate, belong to type of seeds with deep exogenous physiological rest (V_3). It is caused by a strong physiological inhibition of germination mechanism and manifests in reduced gas permeability of seed surface and special physiological condition of the embryo. Organic or physiological rest delays the germination of seeds even when all the conditions are favorable**,** and is connected with properties of seeds or fruits [39].

The oxidized flavonoids in the dry, leathery and ligneous pericarp of the studied *Vitex* species perform protective function, controlling gas and water intake of fruit, and providing physiological rest for seeds allowing embryos to ripe. The highest level of flavonoids, 0.893 mg/g are in fruits of *V. cannabifolia*, average level, 0.802 mg/g in *V. negundo*, and the lowest level, 0.729 mg/g is in *V. agnus-castus* (Fig. 3).

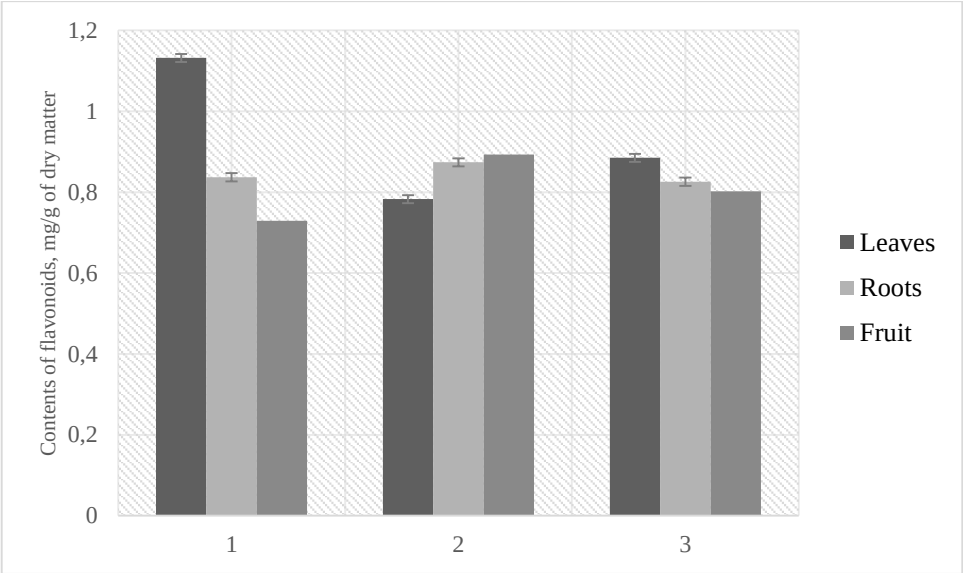

Fig. 3. Content of flavonoids in vegetative and reproductive organs in plants of the genus *Vitex* L.: 1 – *V. agnus-castus*, 2 – *V. cannabifolia*, 3 – *V. negundo.*

Besides, our conclusions are similar with the results obtained by D. Tepfer (2012) and I.D. Anikeeva (1990) in influential space research of the mechanism in seeds counteracting the effects of interplanetary space and the role of flavonoids of seed membranes in this process. They found that flavonoids protect sunflower and flax seeds from UV irradiation, temperature fluctuations, and even vacuum conditions during the transportation of seeds to Mars. The chemical samples of flavonoid UV screens were destroyed by UV light, but their overall ability to absorb UV was retained. Even if it would be impossible to store seeds, the components (e.g., DNA) could remain viable during transportation in space [30, 40].

The tendency to accumulate the highest level of flavonoids in leaves, average in roots and minimum in fruits is revealed for species of *V. agnus-castus* and *V. negundo.* The tendencies for the maximum accumulation of flavonoids in fruits, average in roots and minimum in leaves (Fig. 3) are characteristic of *V. cannabifolia*. Obviously, the importance of these compounds differs for different plant organs. They ensure reproduction success and survival in fruits and seeds, surviving as a separate individual in roots, and protective situational reaction for the fast restoration of leaves.

Along with flavonoids, the photosynthetic rate and transpiration are the defining physiological adaptations which influence stamina, general productivity and cropping capacity. Respiration is important for adaptation processes of plants as a source of power. The low temperatures cause the reduction of oxygen intake rate and inhibit respiration in heat-loving plants [38].

The highest indicators of photosynthesis with the minimum level of transpiration were obtained in *V. negundo* plants (14.3 mg of CO_2/(dm^2×h)), the average level (13.0 mg of CO_2/(dm^2×h)) was typical for *V. cannabifolia* and the lowest level of gas exchange functions (7.3 mg of CO_2/(dm^2×h)) for *V. agnus-castus* (Table 1).

Table 1. The comparative characteristic of gas exchange processes for plants of the genus *Vitex* L.

Species of the genus *Vitex*	Photosynthesis	Photorespiration	Dark respiration	Transpiration
	mg of CO_2/(dm^2×h)			mg of H_2O/(dm^2×h)
V. negundo	14.3	4.1	2.3	1.25
V. cannabifolia	13.0	2.1	1.3	2.13
V. agnus-castus	7.3	2.9	1.7	2.33

It is obvious that the plants are more thermoresistant with higher levels of photosynthesis, and their physiological processes are faster, more proficient and productive. The processes of photosynthesis, photorespiration and transpiration in *Vitex* plants are interconnected, interrelated and vary depending on the size, density and status of stomata cells (Table 2).

Table 2. Size and density of stomata of leaf areas of plants of the genus *Vitex* L. (per unit surface of leaf area)

	Vitex agnus-castus		*Vitex cannabifolia*		*Vitex negundo*	
Quantity of stomata, units/mm^2	103.6±0.50		156.1±0.33		275.2±0.45	
Size of stomata, microns	length	width	length	width	length	width
	16.24±0.45	12.40±0.31	20.19±0.75	15.18±0.51	19.57±0.78	13.50±0.96

In addition, the gas exchange parameters in *V. negundo* plants (with the average size of stomata densely located on the leaf surface) are characterized by a minimum transpiration level (1.25 g of H_2O/(dm^2×h)) with the maximum indicators of photosynthesis (14.3 mg of CO_2/(dm^2×h)), photorespiration (4.1 mg of CO_2/(dm^2×h)) and dark respiration (2.3 g of H_2O/(dm^2×h)). Thus, it can be assumed that the physiological processes in plants of this species are the most balanced for the conditions of forest-steppe of Right-Bank Ukraine.

Plants of *V. cannabifolia* (with the largest stomata and average density of stomata) have the highest indicators of transpiration (2.13 g of H_2O/(dm^2×h)), and average of photosynthesis (13.0 mg of CO_2/(dm^2×h)) and photorespiration (2.1 mg of CO_2/(dm^2×h)). Thus, the balance of processes and fitness of those plants in introduction is average.

Plants *of V. agnus-castus* (the smallest stomata and low density of stomata) lose the largest amount of water due to transpiration (2.33 g of H_2O/(dm^2×h)), need constant replenishment, have the lowest intensity of photosynthesis (7.3 g of H_2O/(dm^2×h)) and dark respiration (1.7 g of H_2O/(dm^2×h)). Thus, the plants of this species are the most sensitive and vulnerable in forest-steppe condition of the Right-Bank Ukraine.

The status of the pigment system and the plastid apparatus is a significant indicator of the plant resistance to unfavorable environmental conditions, and in particular to the cold, because the pigments are closely related to proteins and lipids [38]. Low temperatures destruct the plastid structure, which leads to the inhibition of photosynthesis. The ratio of chlorophyll *a* to chlorophyll *b* characterizes the relationships between plant and environment. It is important to note that the increase of chlorophyll content *b* is a sign of stress that requires a lot of energy and leads to a certain depletion of the plant. In addition, a high level of correlation of chlorophyll content with the content of carotenoids also indicates the presence of stress (Table 3).

Our previous studies have shown that the pigment content in plants of the genus *Vitex* (regardless of their species and age) is dynamic throughout the vegetation season (Table 3). The

maximum content of chlorophyll *a* and carotenoids was observed at the beginning of the vegetation, in the phase of regrowth and budding, the minimum content was in the flowering phase; the chlorophyll *b* content was maximum in the flowering phase. After the flowering phase until the end of the growing season, there is a tendency for all species for gradually decrease the content of all pigments.

The high amount of pigments in leaves of investigated *Vitex* plants in the first half of their vegetation directly correlates with intensive plant growth, high productivity of photosynthesis, and preparation for flowering. We agree with N.I. Yakushkina [41] and E.R. Hyubbenet [42] that the increased chlorophyll level can be used as an indicator of the plant's readiness for flowering.

During mass blossoming of plants of the genus *Vitex* the ratio of pigments changed: levels of chlorophyll *a* and carotenoids sharply decreased, chlorophyll *b* content increased twice or thrice, and was considerably prevalent over two other pigments. The increase of chlorophyll *b* content is the sign of stress conditions, and the blossoming is a stressful stage in the life of a plant. The energy expenses are greater and exhaust the plant to an extent. Plants of *V. negundo* species had the highest level of chlorophyll *b* in the flowering stage (0.98 mg/g), *V. cannabifolia* was characterized by the average level (0.83 mg/g), and the lowest level (0.78 mg/g) was found in *V. agnus-castus* plants.

The high ratio of chlorophylls to carotenoids contents also highlights the plant stress. During blossoming, such indicators of this ratio were recorded: the highest (39.25 mg/g) for the *V. negundo,* average (20.85 mg/g) for *V. agnus-castus* and the lowest (19.00 mg/g) for *V. cannabifolia*.

The carotenoids are important for the reproductive process of plants, thus their content is minimum in leaves during blossoming. At the same time, according to references, the quantity of these pigments considerably increases in anthers and petals of plants [41].

By their reaction to temperature conditions, plants of the genus *Vitex* belong into the second group of moderately thermophilic plants that can be damaged by not severe, short-term frosts (0 to –3°C) but will not perish, and without considerable damages can survive the decrease of temperature below +5°C. Such short-term decreases of temperature are entirely possible in the regions of their origin and growth.

Hence, thermophilic plants of the genus *Vitex* can withstand the specified temperature range. This possibility manifests itself in qualitative content of pigments and their ratio and in general content of chlorophyll, intensity of gas exchange processes, dynamic content of flavonoids, functional status of plants and is developed under the influence of not a single one, but a system of factors.

Table 3. The content of photosynthetic pigments in leaves of plants of the genus *Vitex* L. according to vegetative stages (dry substance, mg/g)

Species of the genus *Vitex*	Pigments	Regrowth and budding	Blossoming	Fructification	End of vegetation	Ratio	Regrowth	Blossoming	Fructification	End of vegetation
V.agnus-castus	Chlorophyll *a*	1.80±0.03	0.47±0.01	0.98±0.02	0.71±0.01	*a / b*	5.81	0.60	6.53	5.07
	Chlorophyll *b*	0.31±0.002	0.78±0.02	0.15±0.004	0.14±0.004	*a+b*	2.11	1.25	1.13	0.85
	Carotenoids	1.22±0.03	0.06±0.002	0.59±0.005	0.53±0.01	*a+b/carot.*	1.73	20.83	1.92	1.60
V.cannabifolia	Chlorophyll *a*	1.86±0.01	0.50±0.01	1.10±0.01	0.37±0.002	*a / b*	5.03	0.60	5.24	5.29
	Chlorophyll *b*	0.37±0.001	0.83±0.02	0.21±0.006	0.07±0.002	*a+b*	2.23	1.33	1.31	0.44
	Carotenoids	1.30±0.03	0.07±0.001	0.61±0.005	0.34±0.002	*a+b/carot.*	1.72	19.0	2.15	1.29
V.negundo	Chlorophyll *a*	2.10±0.07	0.59±0.02	0.91±0.06	0.55±0.01	*a / b*	3.82	0.60	5.69	4.23
	Chlorophyll *b*	0.55±0.01	0.98±0.03	0.16±0.01	0.13±0.003	*a+b*	2.65	1.57	1.07	0.68
	Carotenoids	1.25±0.09	0.04±0.006	0.52±0.03	0.40±0.01	*a+b/carot.*	2.12	39.25	2.06	1.70

4. CONCLUSION

Plants of three species of the genus *Vitex* L. were studied. The plants were acclimatized in forest-steppe conditions of Right-Bank Ukraine as perspective introduced species with valuable medicinal, nutritional, aromatic, melliferous, technical and decorative properties that can be used in many segments of the industry of Ukraine, mostly in pharmaceutics, cosmetology, food processing and landscape construction. As a result:

– in the conditions of introduction, different strategies of resisting cold stress in plants were revealed: increasing the levels of cryoprotectors (such as flavonoids) in vegetative and reproductive organs in *V. cannabifolia*, maintaining their contents at certain stable level in *V. negundo*, or losing them in *V. agnus-castus.* Therefore, the content of flavonoids and their dynamics can be considered indicative of stressful condition in plants of the genus *Vitex* and their adaptive ability during the introduction;

- the highest level of flavonoids in fruits (0.893 mg/g) was in plants of *V. cannabifolia*, the average level (0.802 mg/g) in *V. negundo* plants, and the lowest (0.729 mg/g) in *V. agnus-castus* plants. This provides deep physiological stillness for seeds and possibility of reproduction of plants in new climate conditions;

– the most tolerant to cold are the plants of *V. negundo,* followed by plants of *V. cannabifolia*, and the least tolerant to low temperatures are the plants of *V. agnus-castus* due to the intensity of photosynthetic rate, transpiration and respiration processes which are directly connected with the size and density of stomata on a leaf surface and the content of pigments.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 200-209

DOI: 10.21448/ijsm.450830

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

Antioxidative Defence Mechanisms in Tomato (*Lycopersicum esculentum* L.) Plants Sprayed with Different Pesticides and Boron compounds

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Abstract: Oxidative stress was investigated in *Lycopersicum esculentum* L. by applying various pesticides and boron compounds for two years, near Ortaca-Muğla. The field sections were treated separately by commercial pesticides and boron compounds i.e. tarimbor (TB), boric acid (BA), laser (LA), zoom (ZO) and admiral (AD). During first year, boric acid (BA-1) caused highest increase in total chlorophyll (TCh) content (158.41 µg g^{-1}) while the second dose (BA-2) decreased it (103.11 µg g^{-1}). During second year, higher doses of tarimbor (236.49 µg g^{-1}) caused increase in total chlorophyll (TCh) while ZO treatment decreased it (142.55 µg g^{-1}) (control: 149.55 µg g^{-1}). TB-1 caused the highest increase in proline content (33.52 nmol g^{-1}) while highest reduction was observed in boric acid (BA-2) (22.51 nmol g^{-1}) as compared to control group (26.77 nmol g^{-1}). During the first year, an increase of boric acid and tarimbor concentrations decreased malonaldehyde (MDA) while during the second year, both increases and decreases were observed in the MDA amount. Highest superoxide dismutase (SOD) amount was found in the first year ZO treated plants i.e. 70.35 unit SOD/mg protein while TB-1 treatment caused the highest decrease in the SOD amount i.e. 35.21 unit SOD/mg protein (control: 45.23 unit SOD/mg protein).

ARTICLE HISTORY
Received: 30 March 2018
Revised: 16 July 2018
Accepted: 20 July 2018

KEYWORDS
Lycopersicum esculentum L.,
Antioxidative enzymes,
Malonaldehyde MDA,
Boron stress,
Pesticides

1. INTRODUCTION

Oxidative stress can be defined as an imbalance between the production of free radicals (reactive oxygen species) and antioxidant defenses that can be natural or anthropogenic. The latter include pesticides, air pollutants and heavy metals [1]. Pesticides have a crucial role in the pest management and control of plant diseases. However, its excessive use particularly in terms of their application at high doses may increase especially in developing countries. Thus, it is important to optimize their use for safeguarding the consumers and producers as well as reducing environmental hazards [2,3]. Pesticide use is very common in vegetable production in glasshouse conditions to protect pest-induced damage. However, the use of pesticide at high rates may cause toxicity problem, which can deleteriously affect plant growth and development.

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ISSN-e: 2148-6905 /

Foliar applications of pesticides may negatively affect leaf photosynthesis by partially blocking stomatal pores which allow exchange of gases. Therefore, reduction in photosynthetic activity can delay time of fruit harvesting or affect adversely fruit quality, as well as visible damage due to chemical toxicity [4]. The pesticides used in the present experiment such as thiamethoxam and acetamiprid belong to the neonicotinoid insecticide group. They were introduced worldwide about a decade ago as they have lower toxicity and high activity against pests and insects [5,6]. Abamectin is a macrocyclic lactone and is a major fermentation component of avermectins, natural products produced from the soil bacterium *Streptomyces avermitilis*. It shows insecticidal, acaricidal and nematicidal activities [7]. Pyriproxyfen is known as an insect growth regulator (IGR). It is an analogue of a juvenile hormone (JHA). It is being widely used as an insecticide for the control of different pests [8,9]. Plants have a well-developed anti-oxidative machinery to prevent cellular membranes from toxic effects caused by reactive oxygen species [10,11]. It is reported that reactive-oxygen species (ROS) are responsible for various stress-induced damages to cellular structures. It is widely accepted that chemical toxicity results in oxidative stress due to the production of ROS [12-15]. Under stress conditions plants may alter the activities of ROS scavenging enzymes, such as superoxide dismutase (SOD; EC1.15.1.1) and catalase (CAT; EC1.11.1.6) [16]. To date, there seems to be no knowledge available in the literature on functioning of antioxidative defense machinery to pesticides, such as abamectin, thiamethoxam, pyriproxyfen and acetamiprid. Thus, we hypothesized that application of different pesticides at high doses could cause oxidative stress and hence regulate antioxidative defense system in tomato plants. Therefore, in the present study we examined the effects of pesticides sprayed at higher doses on key plant growth attributes, production of ROS and activities of antioxidant enzymes in the leaves of tomato plants.

Boron is an important microelement in plant nutrition. The main functions of boron relate to cell wall strength and development, cell division, fruit and seed development, sugar transport, and hormone development. Plants uptake boron in slight amount whereas its higher level is toxic to plants [17]. Boron-deficient tissues are brittle or fragile, while plants grown on high boron levels may have unusually flexible or resilient tissues [18].

Herein we report the study of various boron containing fertilizer (boric acid and tarimbor), and pesticides on *Lycopersicum esculentum*. Unlike literature reports, we did not found the high amount of boron as toxic. We used three types of pesticides i.e. Admiral, Laser and Zoom trademarks to study the changes in the physiology and biochemistry of the stated plant.

2. MATERIAL AND METHODS

The experiments were performed at the fields located in Mugla province (district Ortaca). The field was divided into various blocks that were divided into control, zoom (etoxazole, sumitomo), laser (Spinosad, Dow Agro Sciences), admiral (Pyripyroxyfen, sumitomo), boric acid and tarimbor randomly. The stated insecticides and acaricide were used according to the suggested amount of manufacturers. Tarimbor ($Na_2B_8O_{13}.4H_2O$) and boric acids were sprayed on the plants. The blocks were separated from each other's by a separating bands (SB). Total 30 blocks as a whole were studied.

Plant culture and treatments

The study was performed for two years on tomatoes (first year *Lycopersicum esculentum* var. Bestona F1, second year *L. esculentum* var. Manyla F1). First three weeks, only the development of plant was observed. After that time, boron and pesticide treatments were applied depending upon the procedure of experiment. On the 14^{th} day of the treatments, 30 leaves were collected from each block of plants for physiological studies. For biochemical studies, 20 leaves were collected from each block separately. The leaves were stored in the

refrigerator immediately after plucking, till they arrive at the lab. Leaves were stored at -20 ºC in the lab freezer till further studies. Tables 1 and 2 shows the experimental design of the field where the study was performed.

Table 1. Experimental design of the field (2010) first year

Entrance									
ZO	SB	BA (1-1)	SB	TB (2-1)	SB	AD	SB	AD	SB
SB	BA (2-1)	SB	C	SB	ZO	SB	TB (1-3)	SB	BA (1-2)
TB (3-3)	SB	LA	SB	BA (2-3)	SB	LA	SB	C	SB
SB	ZO	SB	TB (3-1)	SB	AD	SB	BA (3-3)	SB	TB (2-2)
TB (1-1)	SB	C	SB	BA (1-2)	SB	TB (3-2)	SB	BA (1-3)	SB
SB	BA (3-2)	SB	TB (2-3)	SB	LA	SB	BA (2-2)	SB	TB (1-2)
Entrance									

SB: Separation Band; C: Control irrigation water; BA: Boric acid; BA-1: 2ppm; BA-2: 5 ppm; BA-3: 10 ppm; TB-1: 20 ppm; TB: Tarimbor; TB-2: 40 ppm; TB-3: 80 ppm; ZO: Zoom 35 mL /100 L; LA: Laser, 30 mL/100 L; AD: Admiral 50 mL/100 L

Table 2. Experimental design of the field (2011) second year

BA 3-1		TB 1-1
SB		*SB*
TB 3-2		BA 2-2
SB		*SB*
TB 2-1		TB 3-3
SB		*SB*
C-2		AD
SB		*SB*
LA		ZO-1
SB		*SB*
TB 1-2		BA 1-1
SB		*SB*
BA 3-3		TB 2-2
SB		*SB*
C-1		BA 1-3
SB		*SB*
ZO		BA 2-1
SB		*SB*
BA 3-2		TB 1-3
SB		*SB*
LA		AD
SB		*SB*
TB 3-1		ZO
SB		*SB*
BA 1-2		C-3
SB		*SB*
TB 2-3		LA
SB		*SB*
BA 2-3		AD

Entrance

SB: Separation Band; C: Control irrigation water; BA: Boric acid; BA-1: 2ppm; BA-2: 5 ppm; BA-3: 10 ppm; TB-1: 20 ppm; TB: Tarimbor; TB-2: 40 ppm; TB-3: 80 ppm; ZO: Zoom 35 mL /100 L; LA: Laser, 30 mL/100 L; AD: Admiral 50 mL/100 L

Chlorophyll determination

Chlorophyll content was determined by the standard procedure of Strain and Svec [19] with slight modification.

Leaf free proline content

Leaf free proline content was determined by the procedure developed by Bates et al. [20].

Antioxidant enzymes and malondialdehyde

Fresh leaf material (500 mg) was well grounded in sodium phosphoate buffer (50 mM) that contained 1 % soluble polyvinyl pyrrolidine. Superoxide dismutase (SOD) was determined according to Giannopolitis and Reis [21], and Cakmak et al. [22]. Catalase activity was determined by the procedure of Bergmeyer [23]. Ascorbate peroxidase activity was determined by the Nakano and Asada [24] procedure. POD was performed according to Herzog and Fahimi [25]. The Bradford [26] protocol was employed to estimate total soluble proteins.

Leaf malondialdehyde (MDA) contents

MDA content was determined according to Madhava Rao and Stresty [27] procedure.

Statistical analysis

The obtained data was subjected to SPSS (v. 14) analytical software to work out variance analysis. Significant differences among mean values were assessed using LSD test at $p \leq 5$ %.

Soil characteristic analysis

The soil samples were analyzed for Zn, Fe and Cu according to DTPA [28]. K, Ca and Mg concentrations were determined according to Thomas [29] procedure. Na concentrations were analyzed by Knudsen et al. [30]. These analyses were performed using atomic absorption at 1 N concatenation with neutral ammonium acetate. Water soluble phosphorus was determined by Bingham [31] colorimetric spectroscopic procedure. Plant consumable boron was determined by 0.01 M manitol + $CaCl_2$ extraction by ICP-AES instrument [32]. pH and EC were determined by pH-EC meter.

Yield analysis

Tomato fruit production was analyzed on weekly bases from the beginning of the crops fruit giving to the end of the harvest for two years.

3. RESULTS AND DISCUSSION

As stated in the method section, the study was performed for two years. This study covers the chlorophyll, carotenoid, lipid peroxidation, proline, proteins analyses and antioxidative (superoxide dismutase: SOD, peroxidase: POD, ascorbate peroxidase: APOD, catalase: CAT) enzyme inhibition activities after the treatment of boron compounds (tarimbor and boric acid) and pesticides (Zoom, Laser and Admiral) over *L. esculentum*. The results were compared with the control group plant leaves on fresh weight basis (FW).

3.1. Total Chlorophyll (TCh) and carotenoid (Car) analysis

Pesticides and boron treated *L. esculentum* showed a decrease in the chlorophyll amount during first year, while an increase was observed during the second (Table 3). After first year treatments, total chlorophyll (TCh) amount showed a decrease except the third dose of tarimbor (TB-3). Boric acid (BA-1) caused highest increase in the TCh (158.41 μg g^{-1}, FW) while least amount was observed in the second dose (103.11 μg g^{-1}, FW) of boric acid (BA-2) as compared to the control (136.85 μg g^{-1}, FW) (Figure 1).

During the second year, an increase was observed in the total chlorophyll amount except Zoom (ZO) treatment. Highest total chlorophyll increase was observed after the treatment of tarimbor (236.49 μg g^{-1}, FW) while ZO treatment decreased it (142.55 μg g^{-1}, FW); even less than the control (149.55 μg g^{-1}, FW). Carotenoids amount observed after the pesticides and

boron treatments were statistically insignificant ($p \leq 0.05$). As a whole, they carotenoids were decreased during first year treatments while increased during second year (Figure 2).

Table 3. Effect of pesticides and different boron compounds on the total chlorophyll and carotenoid (μg g^{-1} FW) contents (year 2010&2011)

Treatments	2010		2011	
	TChl	Car	TChl	Car
C	136.85ab	15.21ab	149.55b	23.84bc
BA-1	158.41a	16.51a	177.10ab	25.77abc
BA-2	103.11c	11.61b	221.46ab	35.44a
BA-3	146.06a	15.12ab	193.65ab	32.41abc
TB-1	131.75ab	15.82a	176.33ab	26.54abc
TB-2	115.99bc	12.32ab	205.31ab	31.76abc
TB-3	139.53ab	15.07ab	236.49a	33.72ab
LA	145.01a	16.18a	205.53ab	30.97abc
ZO	152.52a	14.93ab	142.55b	21.36c
AD	153.53a	14.81ab	228.97ab	31.47abc

Note: values followed by different letters, in the same column, are significantly different at $p \leq 0.05$.

While studying pear tree, Wang et al. [33] obtained similar results to our first year treatments. They subjected the plant to boron stress that decreased the chlorophyll A, chlorophyll B, carotenoids and chlorophyll A/B amounts. On the other hand, Tort et al. [34] treated *Capsicum annuum* L. with Captan fungicide with various doses (2.5, 5, 7.5 g L^{-1}). They found an increase in the TCh and carotenoids (Car) after the prescribed dose; while increasing the concentration, decreased the TCh and Car. Öztürk and Tort [35] treatment cyprodinil fungicide at three different doses. TCh, chlorophyll A and B increased after first two doses while decreased after the third dose when compared to the control. Our results showed similarities with the reported ones. Oxidative stress can increase the chlorophyllase enzyme that will further decrease the leaves chlorophyll content [36].

3.2. Proline and Lipid peroxidation (MDA) analysis

Ten samples were analyzed for proline content where the first one was the control *L. esculentum*. During first year, boric acid treatment caused an increase in the proline amount with the increase in boron amount. On the contrast, an increase in the tarimbor amount decreased the proline amount. In pesticide treatments, ZO caused an increase while LA and AD caused a decreased in the proline amount. First treatment of tarimbor (TB-1) caused the highest increase in proline amount (33.52 nmol g^{-1}, FW) while highest decrease was observed in boric acid (BA-2) (22.51 nmol g^{-1}, FW) as compared to control (26.77 nmol g^{-1}, FW).

Tomato leaves showed 41.88 nmol g^{-1} on fresh weight basis during the second year treatments. During this stage, all treatments, except TB-1, reflected an increase in the proline amount as compared to the control. The first dose caused a decrease in the proline content in the leaves (39.84 nmol g^{-1}). The following two doses increased the proline content to 48.23 and 46.70 nmol g^{-1}, respectively. Highest amount of proline was observed in BA-1 treated plants (63.70 nmol g^{-1}, FW). The tested three commercial pesticides i.e. LA (42.09 nmol g^{-1}), ZO (47.31 nmol g^{-1}) and AD (42.19 nmol g^{-1}) caused an increase in proline content on fresh weight basis, where Zoom caused the highest increase (seen in Table 4).

By looking the whole picture of 2010, boric acid put the plant into slight oxidative stress with increase in its amount. On the other hand, proline amount in the tarimbor treated plant leaves was found less than the control in all three doses. It means that tarimbor is more suitable than boric acid in case of oxidative stress. In the second year of the study, all treatments, except

TB-1, put the plants intro oxidative stress by increasing the proline amount as compared to the control.

Ardıç [37] studied the oxidative stress in two varieties of *Cicer arietinum* L. i.e. cv. "Gökçe" and "Küsmen 99" by using boron. When the plant was treated with 400 mmol, the amount of proline in the plant was increased three times in "Gökçe". In a previous study [38] on *Solanum lycopersicum* L., we applied 4 various insecticides. Leaf free proline content increased in the leaves of tomato plants sprayed with increasing doses of pesticides. Proline content was highest in the leaves of plants sprayed with the highest dose of thiamethoxam. Karabal et al. [39] studied two types of barleys i.e. hamidiye (boron sensitive) and anadolu (boron resistant). They did not found any correlation of boron with the proline amount.

Table 4. Effect of pesticides and different boron compounds on the Proline and MDA (nmol g^{-1} FW) contents (year 2010&2011)

Treatments	2010		2011	
	Proline	MDA	Proline	MDA
C	26.77a	1.65abc	41.88c	0.87a
BA-1	25.67a	2.07a	63.70a	0.92a
BA-2	22.51a	1.95a	55.71ab	0.99a
BA-3	27.37a	1.33bc	45.26bc	0.82a
TB-1	33.52a	1.38bc	39.84c	0.88a
TB-2	30.7a	1.96a	48.23bc	0.91a
TB-3	25.92a	1.22c	46.70bc	0.98a
LA	22.85a	1.3c	42.09c	0.92a
ZO	29.68a	1.83ab	47.31bc	0.98a
AD	25.92a	1.62abc	42.19c	0.90a

Note: values followed by different letters, in the same column, are significantly different at $p \leq 0.05$.

Malondialdehyde (MDA) was also analyzed in the leaves of *L. esculentum* for two years. During the first year study, an increase of boric acid and tarimbor dose decreased MDA while during second year; both increases and decreases were observed in the MDA amount. Highest MDA was found in BA-1 (2.07 nmol g^{-1}, FW) treated plants while the least amount of MDA (1.22 nmol g^{-1}, FW) was found in TB-3 as compared to the control (1.65 nmol g^{-1}, FW). During second year analyses, lipid peroxidation test showed 0.87 nmol g^{-1} malondialdehyde (MDA) in tomato leaves. Highest MDA was found in BA-II (0.99 nmol g^{-1}, FW) (seen in Table 4).

MDA decreased during both years analyses as a whole after the boron treatment when compared to the control, but the decrease was statistically not significant ($p<0.05$). It means that the treatments did not show any damage to the cell membrane. In pesticide treatments, MDA was increased in all pesticides applied as compared to the control.

Zabalza et al. [40] applied imidazolinone herbicide over beans and observed an increase in the MDA amount in the leaves. Our results show parallel behavior with this study in case of externally applied dose. In another study, oxyfluorfen and diphenyl ether (herbicides) were used over wheat and barley leaves [41]. On comparison with the control, the workers found the decrease in the amount of MDA. Pogosyan et al. [42] used 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid over *Pisum sativum* and observed less MDA than in the leaves of control. These results are in contrast of our study.

3.3. Antixodative enzymes

There are various enzymes in plants that can increase or decrease during the stress conditions. Most important enzymes are superoxide dismutase (SOD), ascorbate peroxidase

(APOD), peroxidase (POD) [43] and catalase (CAT) that makes the antioxidative defense mechanism [44]. APOD and CAT cleans H_2O_2 from chloroplasts and mitochondria. SOD removes superoxide anions while POD catalyze the breaking hydrogen peroxide.

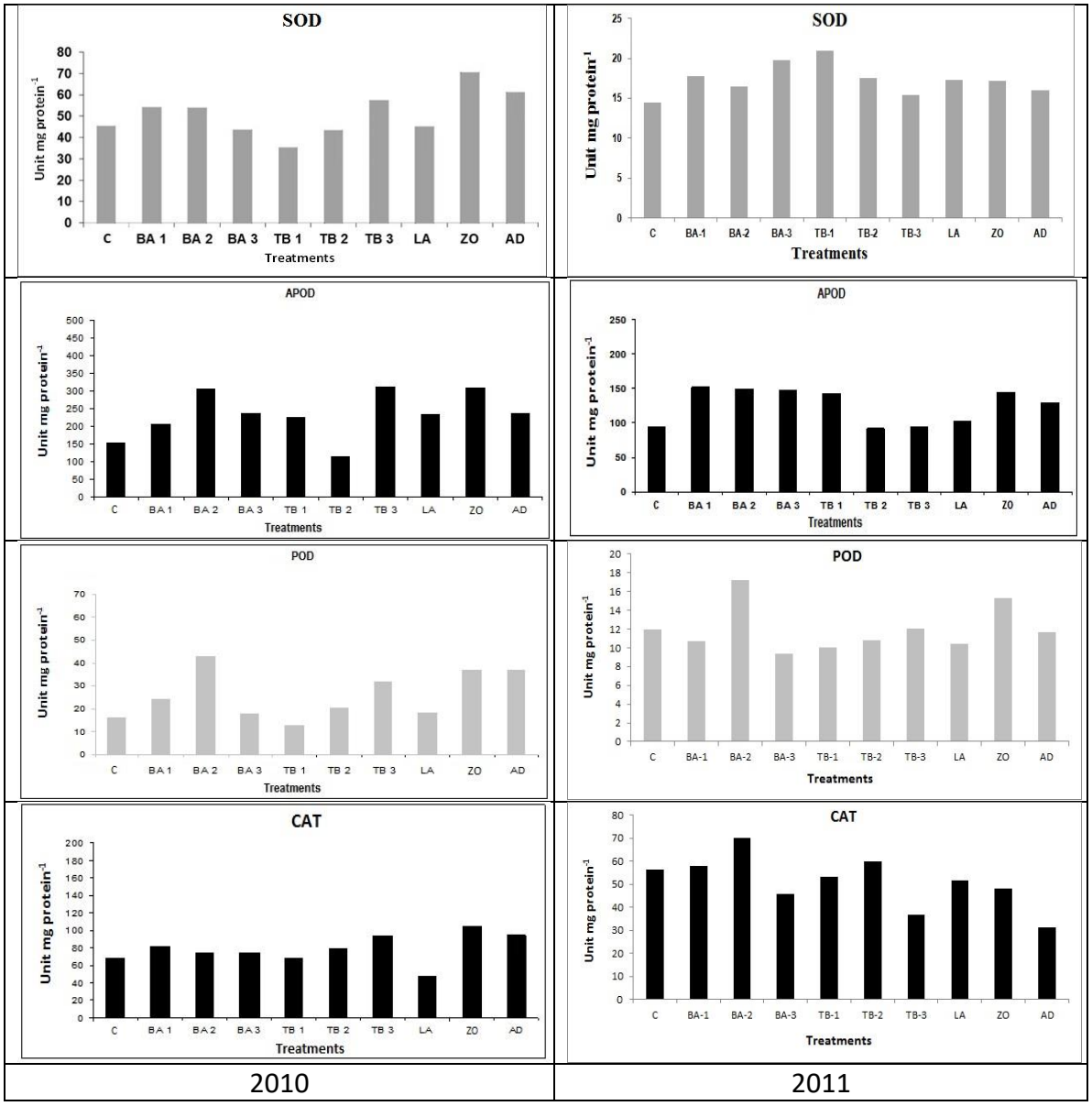

Figure 1. Superoxide dismutase (SOD), ascorbate peoxidase (APOD), peroxidase (POD) and catalase (CAT) levels of tomato plants sprayed with various levels of pesticides and boron compounds.

During 2010 and 2011, protein per gram amount of the stated enzymes were increased as compared to the control group. In 2010, highest SOD amount was found in plants treated with ZO i.e. 70.35 unit SOD/mg protein while tarimbor I treatment caused the highest decrease in the SOD amount i.e. 35.21 unit SOD/mg protein (control: 45.23 unit SOD/mg protein). Results obtained for APOD, POD and CAT were also analogues. Similar results were also obtained for SOD and POD by Kaya et al. [45] while studying tomatoes. In this study in general, pesticide treatment to *L. esculentum* caused an increase in all enzyme amounts. However, treatment of boron, if not at toxic levels, do not show notable stress. Tepe and Aydemir [46] applied boron to lentil and barley and found higher amounts of APOD and CAT. That is why the SOD amount was found less as compared to the control group. During 2011, all the pesticide treatments increased the amount of plant enzymes except CAT, which was very near to the control amount.

4. CONCLUSION

In this study, *Lycropersicum esculentum* L. was studied for its oxidative stress by using pesticides and boron compounds. Boron showed toxicity even if slight amount is higher than the required to the plant. So it is suggested to analysis boron amount in the soil before its external use as a fertilizer. We utilized boron and pesticides in the prescribed amount that is why no notable toxicity was observed. It was also found that if the boron amount is less than the required amount, the yield will be less. The use of pesticides is not too much important if the area has no disease spread in tomatoes. We observed slight decrease in the total tomatoes yield after the use of pesticides while the control group did not get any disease.

Acknowledgment

We are thankful to the National Boron Research Institute, Ankara, Turkey (BOREN) (project No: BOREN 2009/Ç0225) and BAP (Scientific Research Projects) Muğla Sıtkı Koçman University Muğla Turkey (Project No: BAP-2011/15) for their financial support.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 210-216

DOI: 10.21448/ijsm.453016

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

Effects of NaCl applications on root growth and secondary metabolite production in madder (*Rubia tinctorum* L.) root cultures

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Abstract: Madder (*Rubia tinctorum* L.) is a valuable plant rich in anthraquinones having dying properties and biological activities. This study was carried out to determine the effect of sodium chloride (NaCl) applications on the root growth and secondary metabolite accumulation in adventitious roots of madder. For this aim, adventitious roots derived from stem explants in *in vitro* conditions were cultured in MS medium containing different concentrations of NaCl (0, 1, 2, 3 and 4 g/l) for 7 days. Then roots were evaluated in terms of root growth index, total AQ, alizarin, purpurin and total phenolic contents. Based on the results, root growth decreased in line with the elevating level of NaCl while secondary metabolite accumulation significantly increased with NaCl applications compared to the controls. It was determined that NaCl at 3 g/l concentration was the most effective application in terms of total AQ, alizarin, purpurin and phenolic accumulation.

ARTICLE HISTORY

Received: 17 April 2018
Revised: 23 July 2018
Accepted: 27 July 2018

KEYWORDS

Rubia tinctorum, root culture, *in vitro*, sodium chloride, secondary metabolite

1. INTRODUCTION

Plants are major sources of valuable secondary metabolites used in pharmaceutical, cosmetic, perfumery and food industries because of both their antioxidant, antimicrobial and biologic activities and their colour, flavor and fragrance properties [1]. In recent years involving commercial importance of secondary metabolites has increased the interest in alternative techniques such as cell and tissue and organ culture in the production of bioactive substances in plants [2]. As an alternative to traditional methods, plant tissue culture techniques play vital roles in the production of desirable compounds from plants [3]. Production of secondary metabolites by cell and tissue cultures has a lot of distinct advantages including independent from seasonal and geographical constraints, reliable, simpler and more predictable production compared to *in vivo* in the whole plant [1]. Moreover, *in vitro* secondary metabolite production also provides an important strategic advantage in order to remove the danger of extinction in the plants collected from nature.

Madder (*Rubia tinctorum* L.) is a perennial plant rich in anthraquinone (AQ) derivatives including alizarin and purpurin in its roots and rhizomes. AQs with biological activities and dye

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ISSN-e: 2148-6905 /

properties are very valuable compounds in the textile, food and pharmaceutical industries [4-9]. In madder, in order to obtain the highest amount of AQs, roots and rhizomes of 3 years old plants collected from nature are used. Continuously wild-collecting of plants from nature can cause the extinction of plant in the near future [10]. For this reason, *in vitro* secondary metabolite production ensures significant advantages not only provide short-term and high-yield production of the required AQs but also prevent the risk of plant extinction. Furthermore, it is possible to enhance secondary metabolite accumulation by applying some external applications to plant cells, tissues and organs in *in vitro* conditions. Salt is one of the most important external applications that can be applied to *in vitro* cultures to increase valuable bioactive metabolite accumulation. The previous studies indicated that salinity influenced the growth, secondary metabolite production and their composition in cell, tissue and organ cultures of different plants [11-15].

Phenolic compounds defined as organic metabolites containing benzene ring are also another valuable compounds used in food, cosmetic, perfumery and pharmaceutical industries Because of having antioxidant and biological activities [16].

This study was carried out to determine the effect of different concentrations (0, 1, 2, 3 and 4 g/l) of sodium chloride (NaCl) applications on growth index, total AQs, alizarin, purpurin and total phenolic contents in adventitious roots of madder under *in vitro* conditions.

2. MATERIAL AND METHODS

2.1. Plant Materials and Induction of Adventitious Roots

In this study, internode parts of shoots of three-year-old madder were used as plant materials. After washing tap water, shoots were surface-sterilized in 20% (v/v) commercial sodium hypochlorite solution supplemented with 0.1% Tween 20 for 10 min, then rinsed three times with sterilized water. For obtaining the adventitious roots from internode parts, the method of Kubota et al. [17] was used. Briefly, internode parts of shoots (1 cm long) were planted horizontally in MS medium [18] containing 20 g/l sucrose, 2 g/l gelrite agar, 2.5 mg/l indole acetic acid (IAA) and 0.1 mg/l kinetin and cultured 4 weeks. The induced adventitious roots were cultured in the MS liquid medium of the same composition for 4 weeks, then maintained in the MS liquid medium containing 30 g/l sucrose. Adventitious roots were subcultured two times using same media after every 4 weeks. Cultures were grown at 25±1 ºC in the dark and liquid cultures were agitating on an orbital shaker at 100 rpm.

2.2. NaCl Applications

About 250 mg of adventitious roots were transferred to 30 mL of MS liquid medium, containing 30 g/l sucrose, in 100 mL flasks and maintained at 25°C on a shaker (100 rpm) in a growth chamber under dark conditions. NaCl at 1, 2, 3 and 4 g/l concentrations was added to the root cultures at 7 days after inoculation. Control was supplemented distilled water as in stock solutions of NaCl. After 7 days, adventitious roots were harvested, washed with distilled water, weighed and used in the analyses. Experiments were performed in triplicate and three flasks were used for each replication.

2.3. Growth Index

The harvested roots were washed several times with distilled water, soaked with tissue paper to remove the surface water. The growth index of the roots was calculated using the following equation:

$$\text{Growth index} = \frac{(\text{fresh weight of harvested roots} - \text{fresh weight of inoculated roots})}{\text{fresh weight of inoculated roots}}$$

2.4. Determination of AQs

AQs were extracted from the roots by the method of Schulte et al. [19]. Briefly washed roots were dried at 50 ^{o}C until a constant weight and dried root samples were ground into a fine powder using a mortar and pestle. Samples were extracted twice with boiling 80% ethanol until the tissues were colorless. The ethanol fractions were pooled and filtered by using 0.45 µm Whatman micro filters.

The quantitate analyses of total AQ, alizarin and purpurin in the roots were done by using a PG Instruments spectrophotometer (T70 Plus Dual Beam/ Arlington, USA). The content of total AQ was calculated from the absorbance values of the extract at 434 nm, using a molar extinction coefficient of alizarin (ε434=5.5) [19]. The absorbances of alizarin and purpurin at 572 and 516 nm, respectively were measured and their amounts were calculated by the calibration curves of the standard compounds Alizarin and purpurin contents were performed by the method of Shin [20] and expressed as mg/g DW. Data presented are the average of three measurements.

2.5. Determination of Total Phenolic Content

Total phenolics of the root samples were extracted twice with 70% ethanol containing 0.2% hydrochloride acid in an ultrasonic water bath. Total phenolic contents were determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method [21], calibrating against gallic acid standards and expressing the results as mg gallic acid equivalents (mg GAE/g DW). Data presented are the average of three measurements.

2.6. Statistical Analyses

Data were performed by using analysis of variance (ANOVA) using SPSS 16.0 for Windows Software Package and the means were separated by Duncan's multiple range tests.

3. RESULTS AND DISCUSSION

The results pertaining to the effect of NaCl on growth index of the adventitious roots under salt stress during the culture period of 7 days is as shown in Figure 1. The growth index significantly decreased in parallel with the increase in the salt concentrations (p<0.05). The highest growth index was found in the control roots as 0.36 while the lowest value (0.21) was obtained from the roots treated with 4 g/l of NaCl. Based on the results it can be noticed that NaCl applications had negative effect on the adventitious root growth of madder. Similarly, as reported before by Nartop et al. [15], the stress induced by salt reduced biomass of callus and suspension cultures in madder. Previously reported studies have shown that increased NaCl applications decrease the growth of *in vitro* cultures in several plants, such as Fenugreek calli, Cassia acutifolia and Panax ginseng [11, 22, 23]. The excessive intake of NaCl causes reduction of growth, even cultures can lose their survival. This may be due to the increased osmotic pressure, specific ion toxicity and ionic imbalances as mentioned previously by Munns [24]. Because NaCl stress lead to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm to vacuoles [25].

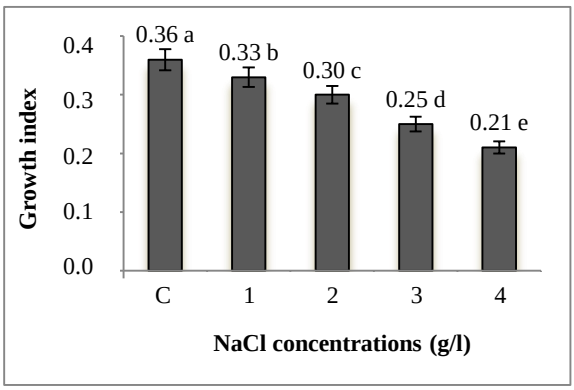

Figure 1. Effect of NaCl applications on the growth index of roots.

Amount of alizarin, an AQ derivative, also changed depending on the NaCl applications. All NaCl applications were significantly increased the alizarin accumulation in adventitious root cultures compared to control roots (p<0.05). As shown Figure 2(b), the highest alizarin content (3.79 mg/g) was found in 3 g/l of NaCl treated roots. The application of NaCl also improved the content of purpurin, another important AQ derivative, significantly (Figure 2(c)) and the highest purpurin content was determined in the roots treated with 3 g/l of NaCl as 0.69 mg/g. When NaCl concentration increased to 4 g/l, purpurin content decreased according to that of 3 g/l of NaCl. Stress applications significantly enhanced the total phenolic contents as compared to controls (Figure 2(d)). Maximum phenolic content (26.82 mg/g) was measured from the roots raised under 3 g/l of NaCl while the lowest level (13.19 mg/g) was obtained from the control roots.

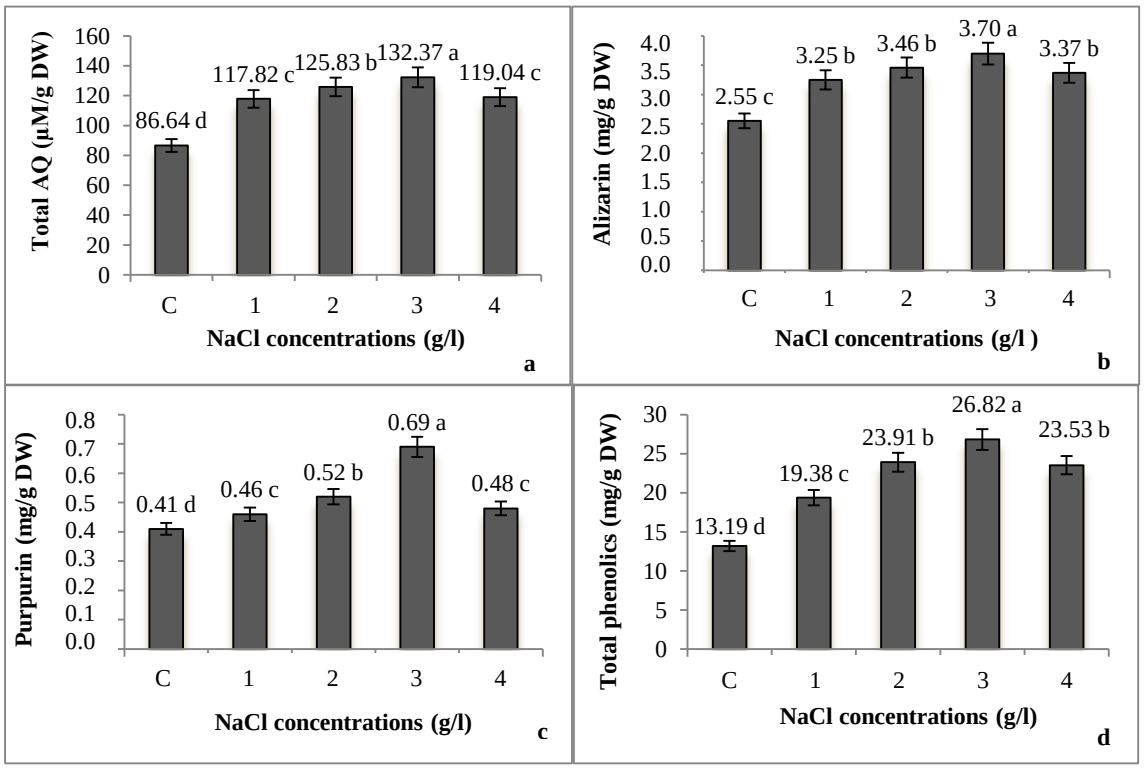

Figure 2. Effect of NaCl applications on secondary metabolites a) total AQ content b) alizarin content c) purpurin content d) total phenolic content.

According to our results, it was determined that all the applications increased the amount of the corresponding secondary metabolites significantly, and the highest values were obtained from the roots treated with 3 g/l of NaCl. Furthermore high concentrations of NaCl decreased

both growth of cultures as well as secondary metabolite biosynthesis. Similarly, Hussein and Aqlan [11] reported significant decline in the growth and secondary metabolite accumulations of fenugreek callus cultures under high concentrations of NaCl. Salt stress reduced growth of adventitious roots, whereas accumulation of AQs and phenolics were enhanced compared to controls. The reverse correlation between growth and biosynthesis of secondary metabolites was also reported by several authors [26-28]. In another study, the effect of NaCl on alizarin and purpurin contents in callus and cell suspension cultures was investigated [15], the highest amounts of alizarin and purpurin in callus cultures were obtained from 100 mM NaCl treatment while the optimal concentration for alizarin and purpurin in cell suspensions was determined as 200 mM NaCl**.** A similar study, Nazif et al. [22] reported that salt stress caused an increase of AQ in *Cassia acutifolia* cell suspension cultures. NaCl leads to cellular dehydration and as a result, the cytosolic and vacuolar volumes decrease. Thus NaCl often causes not only ionic but also osmotic stress and removal of water from the cytoplasm [29]. Plants have developed complex mechanisms such as developing enzymatic and non-enzymatic antioxidant defense mechanisms, for adaptation to the osmotic, ionic and oxidative stresses that are induced by the salt stress [30]. Secondary metabolites as non-enzymatic antioxidants accumulate under different environmental stresses and they acts as membrane stabilizer during abiotic stress [31]. It is well known that exposure to salinity induces the *in vitro* production of secondary metabolites such as phenols, terpenes and alkaloids [13, 14, 23, 32] when applied at the appropriate dose and at the correct stage of cultures.

4. CONCLUSION

As a result of this study, salt stress implementation method for higher toatal AQs, alizarin, purpurin and phenolic production in R. tinctorum L. root cultures was found to be effective. Application of NaCl to adventitious root cultures of madder decreased root growth index but induced AQ and phenolic accumulation when it was applied the appropriate concentration. In this study, the highest production of AQs and phenolic substances in the secondary metabolite production was found in the 3 g/l of NaCl concentration. To conclude, the salt induced the production of valuable phytochemical in plants and thus NaCl may be a promising compound for use in adventitious root cultures because of its positive effects on secondary metabolite production in madder.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 217-223

DOI: 10.21448/ijsm.453009

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

Molecular identification and phytochemical profiling of kamiling (wild toxic plant) using thin layer chromatography

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Abstract: Some plants may only be known by the people in their own respective place. Consequently, there is a need for the authentication and further study of wild toxic plant known by indigenous people. The genomic DNA from the leaves of the plant Kamiling collected in Imugan, Santa Fe, Nueva Vizcaya, Philippines was extracted and amplified using *mat*K and *rbc*L gene markers. Sample sequences was queried on GenBank using Basic Local Alignment Search Tool (BLAST) revealed that the sample sequences were identified as *Semecarpus cuneiformis* using both on *matK* and *rbc*L markers. The leaves of Kamiling was subjected through Thin Layer Chromatography (TLC) and it revealed important phytochemicals such as alkaloids, coumarins, anthraquinones, anthrones, tannins, flavonoids, higher alcohols, phenols, steroids and essential oils. Since there is a growing awareness in correlating the phytochemical constituents of plants with their pharmacological activity, wild toxic plant can be one of the preferences for medicinal uses.

ARTICLE HISTORY

Received: 30 March 2018
Revised: 16 July 2018
Accepted: 20 July 2018

KEYWORDS

TLC,
*mat*K,
*rbc*L,
wild toxic plant

1. INTRODUCTION

Exploring new medicine have been common to people and plays a big role with the application of plants in this field. A large number of plants with different roles on curing specific disease have been identified. Every plant has its own component that could have been use for treating disease. And so as people continue exploring to develop and determine the uses of plants which can be found in area that is conserved.

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ISSN-e: 2148-6905 /© IJSM 2018

Kamiling is wild tree that is mostly found in habitat of dry grassland, open slopes, moist and dry thickets this is a small tree growing to a height of 12 meters. Leaves are crowded at the end of the branches, lanceolate-obovate to oblong-obovate, 10 to 25 centimeters long, hairy, whitish beneath, rounded or somewhat pointed at the tip and usually pointed at the base. In the Philippines the leaves of the tree are regarded as poisonous, it can cause severe itchiness and swelling of eyes and face when touch. The tree is native usually in range of Taiwan, Indonesia, and Philippines [1]. Although many persons are probably immune to poisoning from the hairs on the leaves, in the Philippines the plant is usually regarded as poisonous, with reports of severe contact dermatitis. The sap is considered a violent contact poison which can cause painful swelling and minute blistering pustular skin eruptions. [2].

Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions and to defend against attack from predators such as insects, fungi, and herbivorous mammals. Many of these phytochemicals have beneficial effects on long-term health when consumed by humans, and can be used to effectively treat human diseases [3].

With the progress of phytochemical research more and more plant constituents have been isolated and identified. For the alkaloids alone, there are over 5,500 compounds and the growing interest in its pharmacological activities have led to further investigations. It is estimated that new compound is being discovered and described at a rate of one per day [4]. Thin-layer chromatography coupled with biological detection can be considered as a high-throughput, inexpensive and reliable procedure for screening plant extracts for the presence of potential drugs [5].

Because of the increasing demand for remedies and for authentication of the source material, it is vital to provide a single database containing information about authentic plant materials and their potential adulterants. The database should provide DNA barcodes for data retrieval and similarity search. In order to obtain such barcodes, several molecular methods have been applied to develop markers that aid with the authentication and identification of medicinal plant materials. The study of plant molecular determination will discuss the genomic regions and molecular methods selected to provide barcodes, available databases and the potential future of barcoding using next generation sequencing [6].

2. MATERIALS AND METHODS

2.1 Collection and Preservation of the Sample

Plant material was collected at Imugan Falls, Sta. Fe Nueva Vizcaya, Philippines. Imugan falls is a thirty-five-foot tall crystal clear waters resting up in the Caraballo Range falling into a shallow basin-like pool which flows all the way to Imugan River. The elevation above sea level is about 1685 meters.

Fresh young leaves of Kamiling was collected by using a knife and hand-picked with gloves. The sample was cleaned using fine brush and external moisture was wiped out with a dry cloth. It was placed in a container and then air dried condition for 5 days and kept for the preparation of the analysis of the phytochemicals. For the molecular identification, fresh leaves were put in a secured plastic bag and refrigerated in -80˚C freezer until the preparation for DNA extraction.

2.2 DNA Extraction, PCR amplification and Sequencing

DNA from the leaves of Kamiling was extracted using CTAB method by Murray and Thompson (1980) [7] with few modifications. The DNA was then checked by loading a 2 µl DNA in 1% agarose gel and run through electrophoresis (Enduro Gel XL). The DNA was run through PCR machine (2720 Thermal Cycler) using *mat*K primer pair (F:5'-

CCCRTYCATCTGGAAATCTTGGTTC-3' and R: 5'-GCTRTRATAATGAGAAAGATTTCTGC-3') and *rbc*L primer pair (F: 5'-ATGTCACCACAAACAGAGACTAAAGC-3' and R: 5'-TCGCATGTACCTGCAGTAGC-3'). 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 to amplify the *mat*K and *rbc*L region of each specimen. PCR components were made up of 2.5 µl of 10 x PCR Buffer, 1.5 µl of 25 Mgcl2, 1.25 µl of 10 DNTP mix, 1.13 µl of ITS 3D and ITS 4, 0.09 µl of Taq polymerase and 15.4 µl of dH20 which has a total volume of 25 µl together with 2 µl of DNA. Again the PCR products were loaded to agarose gel to check the quality of DNA and electrophoresis was carried out at 100 V for 30min. Samples with amplification were stored in the tubes, sealed with parafilm and was sent to 1st BASE Laboratory 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.

2.3 Phytochemical Profiling

The secondary metabolites present in the plant leaf extracts were determined using thin layer chromatography based on Aguinaldo et al. (2005) [4] TLC is less time consuming, low cost, and can be performed with less complicated technique it has a wide application in pharmaceutical analysis [8]. Also it has a wide application in identifying impurities in a compound. It can be used as a preliminary analytical method prior to HPLC. The dried sample was homogenized using blender. About 2 to 3 g of dried powdered plant material was defatted with 10 mL of hexane or petroleum ether and was heated over a water bath for 5 minutes. After which the solvent was then decanted and discarded. The defatted plant residue was treated with 10 mL of a mixture of chloroform and acetic acid, $CHCl_3$:CH_3COOH (99:1 v/v) and heat for 5 to 10 minutes over a water bath; the solution was then filtered and labelled the filtrate as Solution A. Resulting residue was treated with 10 mL of a mixture of chloroform, methanol and acetic acid, $CHCl_3$:CH_3OH:CH_3COOH (49.5:49.5:1) and heat for 5 to 10 minutes over a water bath; it was then filtered and the filtrate was labelled as Solution B. The residue was extracted with 10 mL of a mixture of methanol and water, CH3OH:H2O (1:1) and heat over a water bath for 5 to 10 minutes. The solution was then filtered and the filtrate was labeled the as Solution C. The plant residue was then discarded. All reagents and solvents used were analytical grade.

Thin layer plates (Merck Millipore) was cut into size of 1 inch by 2 inch. The plates were mark 0.5mm in both sides. To screen the plant extract using TLC, the chromatogram was developed with different solvent systems as suggested in Table 1 [4]. It was air dried and visualized with the following spray reagents as shown in Table 2 [4].

Table 1. Groups of plant constituents that would possibly be distributed in the different extracts

Extracting Solvents	Plant Constituents
Mixture of chloroform and acetic acid	**Solution A** Anthraquinones, phenols, flavonoids, steroids, coumarins
Mixture of chloroform, methanol and acetic acid	**Solution B** Flavonoid glycosides, cardenolides, anthraquinone glycosides, tannins, saponins, coumarins, indoles
Mixture of methanol and water	**Solution C** Flavonoid glycosides, anthraquinone glycosides, cardenolides, saponins, indoles, sugar, higher alcohols

Table 2. List of plant constituents, their visualizing agents and indication of a positive test

Constituents Tested	Spray Reagent	Observable results for positive test
Flavonoids Steroids	Antimony (III) chloride	Intense yellow to orange visible zone appear on spraying for glycosidic flavonoids fluorescent colors under UV365nm
Phenols Tannins Flavonoids	Potassium ferricyanide-ferric chloride	Blue spots
Alkaloids	Dragendorff's reagent	Brown- orange visible spots immediately on spraying colors aren 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
Higher alcohols Phenols Steroids Essential oils	Vanillin- sulfuric acid	Triterpenes and sterols appear mainly as blue- violet spots. Essential oil form zones with a wide range of colors.
Sugars	Naphthol- sulfuric acid	Blue spots

3. RESULTS

3.1 Molecular Identification

The Kamiling was amplified with the gene specific primer *mat*K and *rbc*L after the first two trials of amplification. To confirm the identity of the plant species, the DNA sequences of the amplified products were used for BLAST analysis. After the BLAST analysis, results both on *mat*K and *rbc*L primers showed that it was *Semecarpus australianensis*. However, the related nucleotide sequences identified using BLAST do not conform to the morphological characteristics and the distribution of Kamiling. The Kamiling was identified under the family Anacardiaceae, genus Semecarpus and named the species as *Semecarpus cuneiformis* identified by Blanco [10].

3.2 Phytochemical Profiling using TLC

Different extracting solvent was used to know the polarity of the plant constituents Table 1. Among the different extracts and various solvent system used, only in chloroform-methanol extract and in the solvent system of Toluene-acetone chloroform it develops. Upon spraying, Dragendorff's reagent, reveals that alkaloid is present in the plant leaf. Moreover, Borntrager reagent shows the occurrence of coumarins, anthraquinones, anthrones and phenols. Furthermore, vanillin-sulfuric acid, revealed the presence of higher alcohols, phenols, steroids and essential oils (Table 3).

Table 3. Phytochemical profiling of various fractions of *Semecarpus cuneiformis*

Test Solutions	Constituents Tested	Extracting Solvent Chloroform+Acetic Acid Solvent system (Toluene-acetone- chloroform)	Observable Results	RF value
Antimony III chloride	Flavonoids Steroids	-		1.342 cm 1.208 cm
Potassium ferricyanide-ferric chloride	Phenols Tannins Flavonoids	-		1.549 cm 1.341 cm 1.146 cm
Dragendorff's reagent	Alkaloids	+	Brown- orange visible spots	1.169 cm
Cardenolides	Cardenolides	-		1.157 cm
Methanolic potassium hydroxide (Borntrager reagent)	Coumarins Anthraquinones Anthrones Phenols	+	Anthraquinones give orange coloration Anthrones give yellow (UV365 nm) zones	1.864 cm 1.781 cm 1.492 cm 1.218 cm
Vanillin-sulfuric acid	Higher alcohols Phenols Steroids Essential oils	+	Blue- violet spots	1.960 cm 1.810 cm 1.388 cm 1.220 cm
Magnesium acetate	Anthraquinones	-		1.938 cm
Indoles	Indoles	-		2.036 cm

4. DISCUSSION

4.1 Molecular Identification

Conferring to all the present gathered data, the one showed 99% identity of the sample was identified as *Semecarpus australianensis*, but based on the previous literatures the *Semecarpus australianensis* can be found only in the Northern Terittory, Cape York Peninsula, and Wet Tropics of Queensland Australia, Torres Strait Islands, New Guinea, New Britain, Aru Islands and additional Pacific Islands (9), the said plant sample has no record in the Philippines species base on the identification of it under family Anacardiaceae [10, 11]. Kamiling was described and classified as *Semecarpus cuneiformis* based on the morphology and availability of the species in the vast areas of the country.

4.2 Phytochemical Profiling using TLC

Since wild toxic plant is often disregarded, by profiling *Semecarpus cuneiformis* it was found out that it may possibly cure and prevent various diseases based on the positive results recorded. The diverse pharmacological activities of anthrones, anthraquinones includes immunological adjuvant and wound healing, antimicrobial, antioxidant, laxative activities and modulation of apoptosis [12].

While coumarins are used as antifungal and anticoagulant reagents [13]. Plant material that contains phenolic compounds also has as antimicrobial and anti-inflammatory activity [14]. Whereas alkaloids have many pharmacological uses including, antihypertensive effects, anti-arrhythmic effects, antimalarial activity and anti-cancer actions. Furthermore, plant contains steroids that can be useful to decrease the amount of cholesterol in a body. And also, many

essential oils have antibacterial, antimicrobial, antiviral, anti-inflammatory, antiseptic, and antibiotic properties.

Since the plant material has a good source of secondary metabolites, this can be used in many aspects in pharmacology. As mentioned, alkaloids, anthraquinones and anthrones has apoptotic activity, S. *cuneiformis* might be one of the solution to discovery of another medication for cancer. Considering that there is a lot of antimicrobial, anti-inflammatory, antioxidant and anticoagulant product commercially, it still good to discover alternative source of material such as this plant which is neglected due to its toxicity effect. This research work that focused on phytochemical analysis shows the presence of the phytochemical constituents of *S. cuneiformis*. The phytochemical profiling was significant especially in the case of unharmed plants for the knowledge of their pharmacological activity.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 224-232

DOI: 10.21448/ijsm.450963

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

Effect of Pre-/ Postnatal Hypoxia on Pyruvate Kinase in Rat Brain

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Abstract: The effect of hypoxic hypoxia on the pyruvate kinase (PK) activity in the brain structures of white rats during ontogenesis in a comparative aspect has been studied. A clear dependence could be established in the increase of PK activity from an oxygen deficient state, the age of animals, the studied structure of the brain and the prolonged effect of hypoxia. Prenatal exposure to hypoxia has shown that the PK activity is not restored to the control value level in postnatal development. After postnatal exposure to hypoxia with increasing age in animals, the PK activity gets more resistant to the effect of stress-factors. The data analysis indicates that the increasing exhaustion of energy resources necessary for normal cell functioning makes an important contribution to the development of hypoxic state and the insufficiency of mitochondrial oxidative phosphorylation, the main energy forming system which underlies these disturbances. Energy-shortage, in its turn causes a variety of secondary negative metabolic alterations and gives rise to free radical oxidation in the cells. An explanation of the obtained results suggested can be considered as an evidence of the realization of the biological effect of hypoxia through the oxidative mechanism.

ARTICLE HISTORY

Received: 17 April 2018
Revised: 23 July 2018
Accepted: 27 July 2018

KEYWORDS

Albino rats, Age, Hypoxia, Brain, Ontogenesis, Pyruvate kinase (PK).

1. INTRODUCTION

The literature review shows that prenatal and postnatal hypoxia affects the cardiovascular system, the vegetative balance and the central nervous system (CNS). In addition, from all the stressful effects that the fetus can undergo during the period of intrauterine development, hypoxia leads to embryo toxic effects and various pathologies of development. This is due to the fact that during the period of intrauterine development, most of the damaging factors realize their effect in the mother-placenta-fetus system. Prenatal hypoxia plays a decisive role in delaying the maturation of the nervous system and the higher brain functions in children. The effects of prenatal hypoxia on the body depend on the severity of the effect, of individual tolerance and the period of intrauterine development. In this aspect, the period of intensive organogenesis (13-17 days of development) is one of the critical periods of embryogenesis [1]. In the postnatal period, hypoxia leads to the accumulation of damaged molecules in cells which contribute to the development of diseases and the premature aging of the organism [2,3].

Hypoxia accompanied by oxidative stress causes the pathology of the body which is displayed in the destruction of mitochondria, the activation of free radical processes and

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ISSN-e: 2148-6905 /© IJSM 2018

hypoxia of cells at all levels. In the course of normal metabolic reactions, active forms of oxygen are formed in the cells and their amount greatly increases during hypoxia [2]. Brain is hypersensitive to hypoxia [4,5]. A lot of enzymes are affected by hypoxia [6]. It was proven that the first response of the body to hypoxia is the synthesis of the factor HIF-1, which regulates the transcription of genes encoding some enzymes of glycolysis [7].At present, pyruvate kinase (PK; EC 2.7.1.40) is considered as a sensible and critical target for free radicals under hypoxic stress and a sharp increase was shown in PK activity [8-10].

The main objective aim of this paper is to study -in a comparative aspect- the activity of energy metabolism enzyme in the brain structures of rats exposed to pre- and postnatal hypoxia. The investigation of aged dynamics of PK activity in brain structures during hypoxia will bring some clarity into the mechanisms of redox–alterations in the brain. The study of the hypoxic effect on the energy metabolism of the brain makes it possible to evaluate the functional reserves of the organism in activities associated with changes in the conditions of external respiration or the gaseous environment [11,12].

2. MATERIAL AND METHODS

The experiments were conducted in 2 series on white nonlinear rats in mitochondrial (MF) and cytosolic fractions (CF) of orbital cortex (OC), limbic cortex (LC), sensorimotor cortex (SMC), hypothalamus (H) and cerebellum (C). In the first series of the experiments, 20 albino female rats were exposed; the experimental group included the progeny of females subjected to hypoxia during the organogenesis of E13-E17 days.

PK activity was assayed on 17 (period of maturation), 30 (period of weaning, completion of earlier period of postnatal ontogenesis) and 90 (reproductive period) days of postnatal ontogenesis. The first two periods are considered critical in postnatal ontogenesis.

In the second series of experiments, adult male rats were subjected to hypoxia at 3-month or P90 (mass 90-110 gr), 6-month or P180 (mass 130-150 gr), 12-month or P360 (mass 190-220 gr) of age. The pregnant and adult rats were exposed to hypoxia (for 20 minutes every day during 5 days in a special hyperbaric chamber with a volume of 0.012 m^3 into which gas mixture of 5% O_2 and 95% N_2 was supplied from gas cylinders, measured with a gas meter). In order to eliminate the effect of stress handling, an appropriate control group of animals of the same age was placed into the same hyperbaric chamber under normal oxygen content. The animals of both groups were sacrificed at the end of the experiments. The whole brains were quickly removed under hypothermia and then OC, SMC, LC, H and C were identified. MF and CF were removed with differential centrifugation [13]. PK activity was determined spectrophotometrically by the method of H.U. Bergmeyer (1975) [14]. Specific activity of PK was expressed in μM NADN/mg protein /1min. The protein contents of the samples were determined according to the method of Bradford [15] with bovine serum albumin used as a standard.

The experiments were conducted in accordance with bioethical principles and guide line documents, recommended by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, ETS-№170; 02.12.2005).The reliability of the differences was determined by the "t" Student test. The obtained data was processed by the method of variation statistics.

3. RESULTS AND DISCUSSION

In rats exposed to hypoxia prenatally during intensive organogenesis – one of the critical periods of intrauterine development, the time course of changes in PK activity - on P17, P30 and P90 days of postnatal ontogenesis was determined.

While in the OC of P17-day rats hypo activity was observed in the PK activity, by 3-month these indices exceeded the control value level by 4.5 times. In SMC, LC and H, in spite of decreasing noticeably by P17 day (45%, 54% and 70% correspondingly and relatively to the control value level), by P30 the PK activity increases by 2.5 times, but by P90 these indices have a tendency to decrease to the control value level.

As compared to the P17–day rats in P30-day ones PK activity in the brain structures increased by 1.2-2.5 times concerning to the controls. In 3-month-old rats, the PK activity changed unequally depending on the brain structures under study: in C, OC and SMC it increased by 1.2-4.5 times, while in LC it decreased. In H, it remained at the control value level. It should be noted that in C in all the periods of postnatal ontogenesis the PK activity was higher than the control value level (105%, 179% and 116%) ($p > 0.05$; < 0.001; < 0.05).

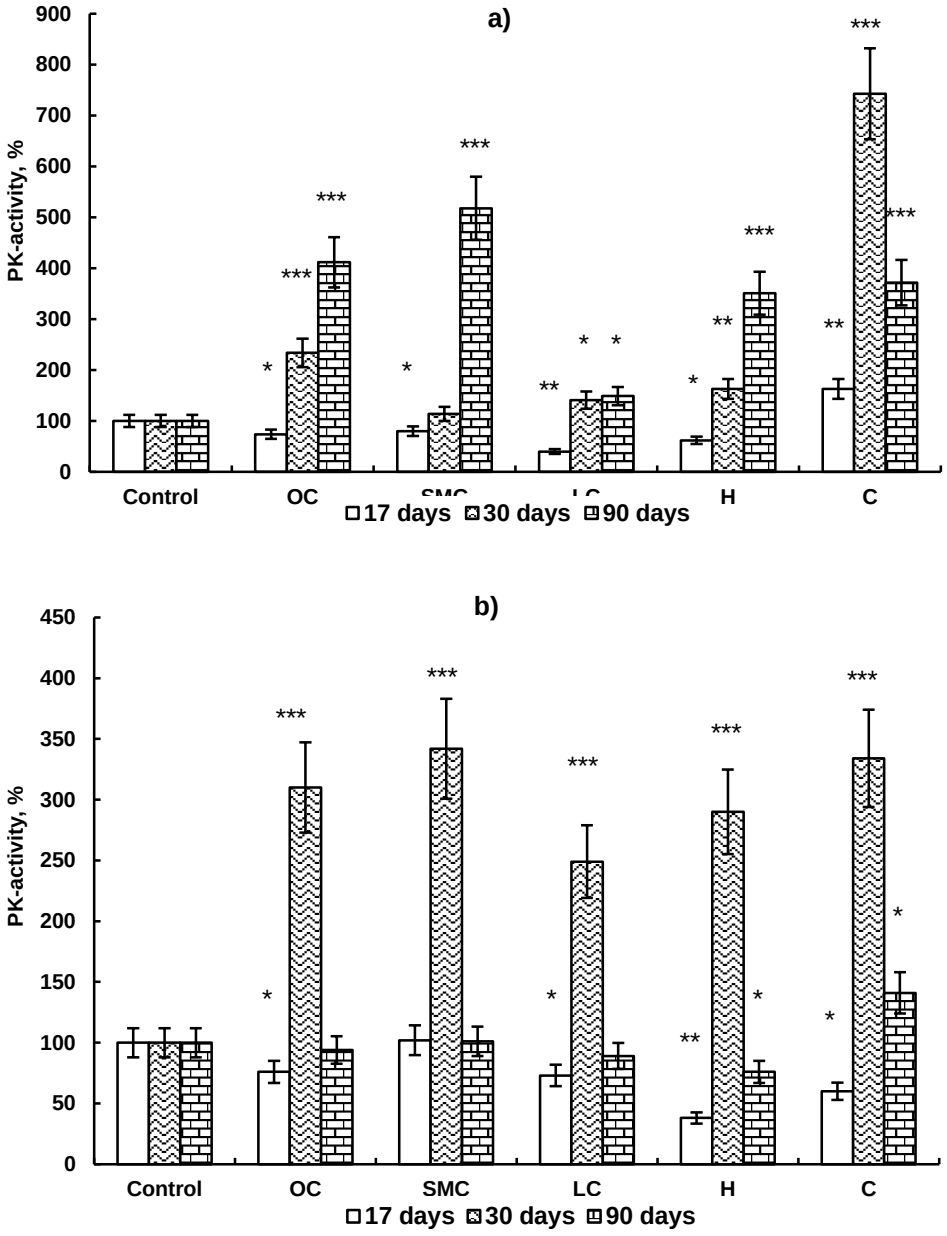

Figure 1. Time-relatedchanges in PK activity in: **a)** mitochondrial (MF) and **b)** cytosolic fractions (CF) of brain structures of rats exposed prenatally to hypoxia.

*- p<0.05; **- p<0.01; ***- p<0.001, relatively to the control value level.

In MF of brain structures of P17 days rats PK activity was lower than the control value level, with exception of C where it was higher than the control value level by 63% ($p<0.01$).With age the enzyme activity showed an increase by 1.3-5.0 times ($p<0.01$) in all the brain structures under study with the exception of C, where by P30 the PK activity increased by7.5, while on P90 it decreased by 3.8 times ($p<0.001$) (Figure 1a).

In the SF of all the brain structures with the exception of SMC the next picture was observed: the PK activity decreased by 1.2-3.0 times by P17, on P30 it greatly increased by 2.5-3.5 times and on 90 day it decreased to the control value level ($p<0.05$; <0.001) (Figure 1b). The PK activity in C made upon exception as well as increasing by 41% as compared to the control value level.

As known, hypoxic damage of the developing brain can lead to death or psychic and neural generative diseases with age [16].Organogenesis period of intrauterine development can be considered as the most susceptive to a number of stressful factors. In that period of development proliferation occurs in the brain, migration processes and maturation of neuroblasts. The impairment of the proceeding of these processes can be considered as an initial cause of changes in physiological and behavioral reactions and cognitive functions in later ontogenesis, as well [17].

Therefore the consequences of hypoxia endured by foetus prenatally in the course of intensive organogenesis on E13-E17 affects postnatal ontogenesis, which are in agreement with the data of other authors [18]. In the tissues and subcellular fractions of brain structures PK activity decreased by 1.2-3.0 times on P17, while on P30 it increased by 1.5-7.0 times. On P90 days PK activity increased compared to the control value level and only in cytosol it decreased and reached the control value level. The elevation in PK activity by 30-day and 3-month-old ones can be explained by the preservation in the brain tissue functioning of optimized energy supply, as well. The balance of 2-sygnal ways is affected by prenatal hypoxia and the results in the intensification of extracellular Ca^{2+}entry mechanisms and this disbalance is most displayed in the earlier periods of ontogenesis [19]. With age in the respiratory chain of mitochondria, reliable decreasing in oxidase activity of cytochrome C was revealed, which generates active forms of oxygen that damage macromolecules (DNA, protein and lipids) and leads to diseases and aging. In its turn, aging can be caused by 2 ways: physiological or pathological [3]. Free radical theory takes leading place among other theories [20]. According to the last theory oxidative stress development in the conditions of deficiency of antioxidant system, first of all, promotes aging of the brain [21].

The results, obtained during postnatal hypoxia, are of indubitable interest which points to the different reaction of PK activity to hypoxic effect.

The lowest indices in PK activity of 3-month-old animals were observed in SMC (below 100%) and in H, but in LC and C was hyperactivity of the enzyme (236% and 281% correspondingly) ($p<0.001$; <0.001). At the MF level all the indices, in spite of being below the control value level, the lowest activity in the PK was in M (30%), but the highest activity was in OC (94%). In SF of SMC and M the indices were reliably low ($p<0.01$); as compared to the other brain structures under study ($p<0.01$); in LC and H on the contrary, PK activity increased (122% and 129%, correspondingly; $p<0.01$; <0.01) (Figure 2).

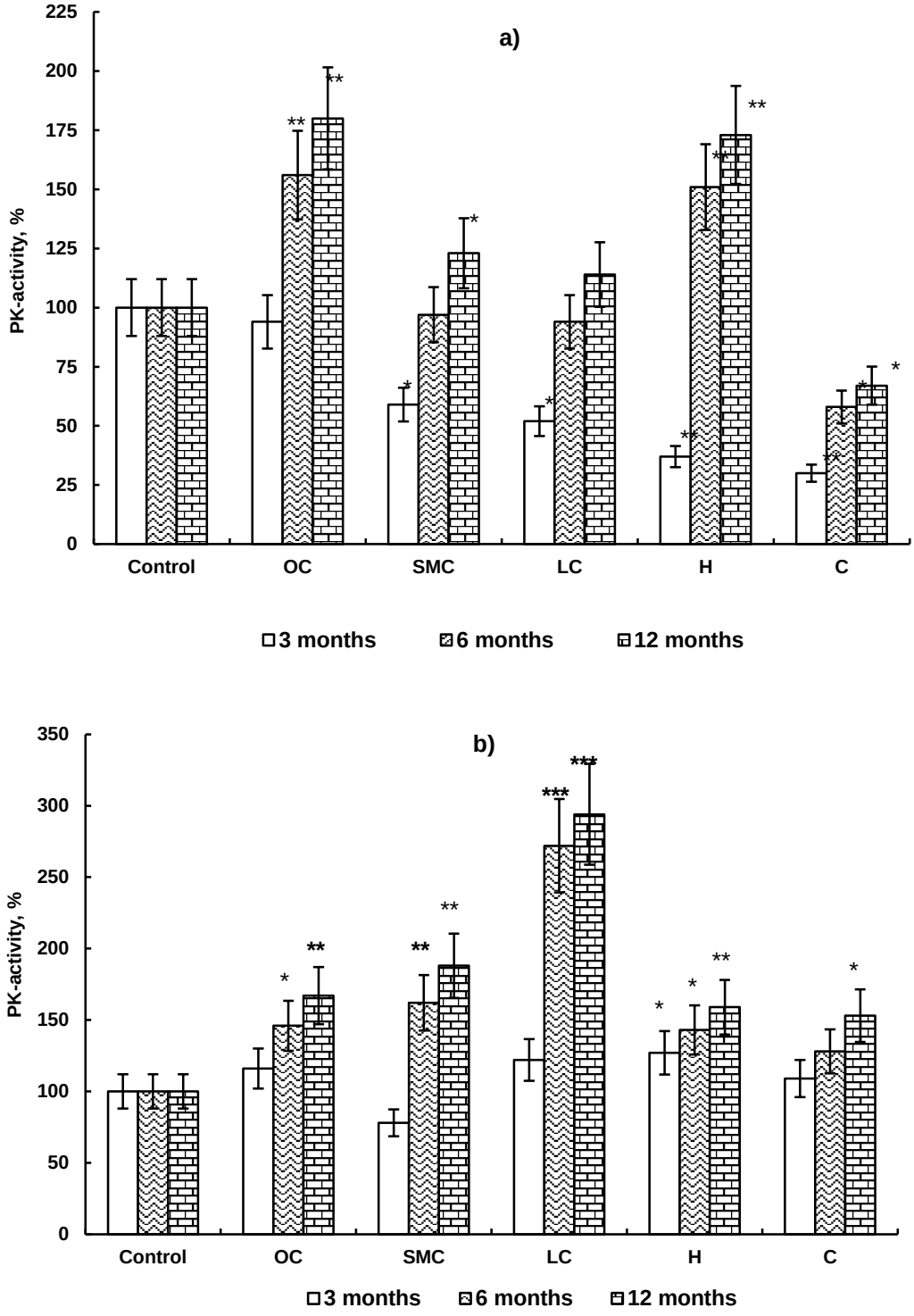

Figure 2. Time-related changes in PK activity in: a) mitochondrial (MF) and b) cytosolic fractions (CF) of brain structures of rats exposed to hypoxia in postnatal ontogenesis.

*- $p<0.05$; **- $p<0.01$; ***- $p<0.001$, relatively to the control value level.

Unlike this age group in six-month-old rats, PK activity at all levels of C was low compared to the other brain structures. In H, on the contrary, it increased in tissues and mitochondria (129 and 151%), while in SF of LC the enzyme activity increased by 2.7 times ($p<0.001$). It should be noted that in six-month-old rats in MF of C PK activity was considerably lower than the control value level (58%).

In the group of 12-month-old rats, acomparative resistance of PK activity to oxygen deficiency was revealed. The time-course of changes in PK activity both in tissues and subcellular fractions of brain structures was identical with the enzyme activity of six-month-old rats. This indicates that with age animals become more steady to the influence of exogenous factors and adaptive–compensatory mechanisms are more developed in these animals (Figure 2/a and 2/b). C in all the age groups turned out to be more sensitive to hypoxia.

There is no doubt that the mechanism to increase the activity of the enzyme in the brain during oxygen starvation is the enhancement of the glycolytic pathway of carbohydrate metabolism.

According to our results one can conclude that PK activity depends on the age, the brain structure under study and the subcellular fractions of this structure. In the OC, LC and H, the restoration of PK activity progresses more slowly than in SMC and C, which indicates that a definite level of metabolic processes corresponds to each functional state. The enzyme processes proceeding in the subcellular organoids of brain neurons allow enzyme adaptation in response to inadequate environmental situations both in brain and organism in general.

PK activity in spite of being at the same level in all the studied brain structures of the control group of animals, was fairly different in the experimental animals. One can assume that the influence of the products of breakdown on intracellular energy supply and the activation of biosynthetic processes in the brain, underlies the dynamics of changes in PK activity.

Under hypoxic conditions the disruption of the functional group of neurons that regulate the level of excitability of cortical neurons is one of the mechanisms of the development of pathological activity on the electro encephalo gramme (EEG) [22]. At the same time, it has been noticed, that based on a comparative analysis [23-25], it can be stated that the changes in the activity of the developing neurons under hypoxia lie in the same mechanisms that are typical for mature people. There are only some distinctive features [26,27]]. Also, the possible influence of this disturbance on the activity of enzymes of energy metabolism, including the PK of the brain, is not ruled out.

By comparing the data obtained in the two series of our experiments one can assume that the differences revealed in PK activity may be related to the individual features of the brain blood circulatory system in each age group. In most cases hypoxia leads to the development of deep disturbances in the metabolism of nervous cells which had a beneficial effect on the obtained results. However, in a number of cases adaptive-compensatory possibilities of the brain blood circulation was sufficient to cause considerable rebuilding in the metabolism and carry out temporal compensation of metabolic disturbances. The fact that PK activity was kept close to the control value levels in experimental animals can be a confirmation of this.

Energy metabolism in the brain differs from other tissues by its high reactivity and plays an important role in the adaptation of the functional state of the whole organism to stressful factors. The dynamics of changes in PK-activity in brain structures of rats exposed to hypoxia on E13-E17 days of intensive organogenesis, did not show restoration of PK activity up to the control indices on P17, P30 and P90 days ($p<0.01$; <0.001).The results show that hypoxia given to foetus during organogenesis leads to changes in the glycolysis process in the brain structures that bears an irreversible character.

The results of postnatal exposure to hypoxia on P90, P180 and P360 days showed that with increasing age, animals become more resistant to the effects of exogenous stress factors, indicating switching on more mature adaptive-compensatory mechanisms in these age groups ($p<0.01$).The highest resistance was observed in cortical structures. These data are considered as an evidence of therealization of thebiological effects of hypoxia through oxidative mechanism.

The study of the age-related changes in the PK-activity in the brain structures in response to hypoxia will allow understanding the mechanisms of redox–shifts in the brain, subjected to hypoxia in prenatal and postnatal periods of ontogenesis.

Though the progeny themselves were not exposed to hypoxia, thetransmission of the observed changes in the PK activity by epigenetically or other way from themothercan beconfirmed. Hypoxia exerts negative effect on synaptic apparatus of neurons in various cortical and subcortical structures of progeny brain [28].

According to the results of the hypoxia on P90, P180 and P360 days of postnatal ontogenesis one can conclude that with age the animals seems to be more steady to exogenous stressful factors that points to thegreater development of adaptive- compensatory mechanisms in these animals. An elevation in PK activity under hypoxia can be related to the brain ability to prevent metabolic disturbances in the mechanisms of regulation of biosynthetic and bioenergetic processes in nervous cells under stressful factors. The impact of pre- and postnatal hypoxia produces typical changes in the system of energy metabolism and in connection with its close interaction with functional-metabolic status of the organism, it is anecessary correction of theconsequences of energy-shortage in thebrain.

According to the authors, a short acute hypoxic effect on the body makes it possible to studythe compensatory-adaptive mechanisms to overcomegrowing hypoxemia [12]. If the hypoxic factor is not eliminated and the accumulation of acid metabolic products continues, then from the 7-10th minute of hypoxia, the tissue compensation mechanisms-thesecondary reactions of the body to oxygen deficiency are included in the body [29]. The reduction of oxygen in the blood inhibits cellular metabolism, reduces the formation of ATP and, consequently, the work of energy-dependent ion channels. The disruption of the operation of ion pumps and the transport of Na, K and Ca ions, in turn, leads to a change in the duration of the action potential [30]. At this time the secondary tissue mechanisms for the compensation of the organism in response to oxygen deficiency occur after the 7th minute of hypoxic exposure [29]]. Our results agree with these opinion.

Consequently the study shows the high sensibility of PK in brain structures to the influence of hypoxia in pre- and postnatal ontogenesis. The present work will be able to help with decisionsconcerningsome questions of energy supply in stressful conditions.

4. CONCLUSION

The data analysis indicates that increasing exhaustion of energy resources necessary for normal functioning of the cell makes an important contribution to the development of hypoxic state and the insufficiency of mitochondrial oxidative phosphorylation - the main energy forming system which underlies these disturbances. Energy-shortage, in its turn causes a variety of secondary negative metabolic alterations and gives rise to free radical oxidation in the cells. Owing to the high reactivity of free radicals, many components of the cell becomes the target of chemical lesions. The activation of lipid peroxidation processes leads to the modification or damaging the main functions of biological membranes. A distinctive range develops: oxygen deficiency disturbs energy metabolism and stimulates the free radical oxidation which by damaging the membranes of mitochondria makes deeper energy shortage in the cell.

Acknowledgement

The author gratefully acknowledges the generous financial support of Azerbaijan National Academy of Sciences, grant 0111AZ321 № 27.21:20.27.25.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 233-242

DOI: 10.21448/ijsm.401127

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

Analysis of Phytochemical Composition and Biological Activities of *Verbascum cheiranthifolium* var. *cheiranthifolium* stem and flowers

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Abstract: Within this study phytochemical composition, antioxidant and enzyme inhibitory activities of extracts obtained from stem and flower of *Verbascum cheiranthifolium* var. *cheiranthifolium* were analysed. Both of the extracts were detected as rich sources of phenolics (verbascoside and luteolin hexoside), various volatile and fatty acid compounds. Luteolin hexoside rich stem extract had pronounced FCR, FRAP and α-glucosidase inhibitory activities. Flower extract had high levels of ORAC assay and effectively suppressed activity of pancreatic lipase enzyme, which was rich in verbascoside compound. Phenolic compounds and volatile compounds present in the extracts might be the main contributors of antioxidant capacity and enzyme inhibitory activities of the stem and flower extracts. Pronounced antioxidant and enzyme inhibitory activities and rich bioactive composition determined in this study reveal that *Verbascum cheiranthifolum* extracts might be a good source for natural health attributing sources.

ARTICLE HISTORY

Received: 03 March 2018
Revised: 26 April 2018
Accepted: 04 August 2018

KEYWORDS

Antioxidant,
α-Glucosidase,
Pancreatic Lipase,
Phenolics,
Volatiles

1. INTRODUCTION

Verbascum species belong to Scrophulariaceae family are commonly known as mullein and compromise of approximately 250 taxa worldwide. Decoctions, infusions or poultice prepared from mullein species have been employed in folk medicine for their curative properties in the treatment a wide range of ailments such as asthma, haemorrhoids, rheumatic pain, earache, abdominal pain, eczema etc. [1-2]. Multiple species of mullein are commonly used as a plant based nutritional supplement and herbal tea [3].

Phytochemical compounds diversity and health attributing properties of mullein species were reported by multiple researchers. It was reported that *Verbascum* species contained a wide

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ISSN-e: 2148-6905 /© IJSM 2018

range of bioactive compounds such as saponins, glycosides, phenolics, steroids, alkaloids and polysaccharides [1], which were among the main contributors of *in vitro* and *in vivo* biological activities including antimicrobial, antimalarial, antioxidant, antiinflammatory, antinociceptive, antitumor, anticancer, cytotoxic, antiulcerogenic, antihepatotoxic, antitussive etc. [3-5].

Verbascum cheiranthifolium var. *cheiranthifolium* known as sığırkuyruğu has been extensively utilized by local people in Turkey. Though, there were multiple reports in scientific literature regards to chemical content and biological activities of *Verbascum cheiranthifolium* var. c*heiranthifolium*, those studies generally focused on selected plant parts (particularly leaf) and limited chemical composition and biological activities investigations. Therefore, we aimed to analyse extracts obtained from stem and flowers of *Verbascum cheiranthifolium* var. c*heiranthifolium* in the context of chemical composition, antioxidant capacity and enzyme inhibitory activities comprehensively.

Within this study, chemical composition was investigated via HPLC-MS/MS (for total and individual phenolics) and GC-MS (for volatiles and fatty acids). Antioxidant capacity was examined by performing complementary antioxidant methods including Folin-Ciocalteu reducing (FCR), Ferric reducing antioxidant power (FRAP) and Oxygen radical absorbance capacity (ORAC). Enzyme inhibitory activities of the extracts were measured towards selected enzymes (pancreatic lipase and α-glucosidase) isolated from mammalians.

2. METHOD

2.1. Plant Material

Verbascum cheiranthifolium var. c*heiranthifolium* (Scrophulariaceae) stem and flower samples with no apparent physical damage were collected from Konalga village, Çatak/Van city, in the Eastern Anatolia Region of Turkey, on August 12th, 2017 (GPS coordinates 37^{o} 51$^{'}$ 255$^{''}$N 043^{o} 09$^{'}$ 857"E). Plant materials were isolated in clean polythene bags and transferred to laboratory within a maximum of 2 h after harvest. The identity of plant material was confirmed at Van Pharmaceutical Herbarium, Pharmacy Faculty, Van Yuzuncu Yil University, Turkey and a voucher specimen was stored at the university's herbarium (Herbarium code: VPH-238; Collector code: MM204). The plant materials were properly cleaned from dust and contaminants by minimizing the loss of bioactive components and left at room temperature in the dark until dry. The plant materials were subsequently ground for a fine powder and stored at -20 °C until analysed.

2.2. Reagents

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Istanbul, Turkey) and were of analytical or HPLC grade. Acarbose was purchased as 'glucobay' from Bayer (Bayer, Turkey). Folin-Ciocalteu was purchased from Merck (Darmstadt, Germany).

2.3. Preparation of lyophilized extract

The ethanol-based lyophilized extracts were prepared as described previously [6]. Briefly, the ground plant material was mixed with a 20-fold volume of acidified ethanol (80% ethanol, 19% H_2O and 1% of 0.1% trifluoroacetic acid, v/v/v), shaken for 2 h at room temperature (22°C) and centrifuged for 20 min at 15320g (10000 rpm) at 4°C with the supernatant collected. The extraction was repeated one more time. The supernatants from the consecutive extractions were combined and the solvent evaporated under reduced pressure at 37°C using a rotary evaporator (Rotavapor R-205; Buchi, Switzerland). The derived fraction was dissolved in purified water and freeze-dried under a vacuum at -51°C to obtain a fine lyophilized powder.

2.4. Antioxidant capacity

2.4.1. Folin-Ciocalteu reducing capacity

Folin-Ciocalteu reducing capacities (Total phenolic content) of the extracts were determined as described previously [6] and the results were expressed as mg gallic acid equivalents per gram of dry weight of the lyophilized extract (mg GAE/g DW), based on Gallic acid standard curve and against a blank control. The analyses were conducted in triplicate.

2.4.2. Ferric reducing antioxidant power

Total reducing capacity was determined using the FRAP assay as described previously [6] and the reducing capacities of the extracts were expressed as µM of iron (Fe2+) per gram of dry weight of lyophilized extract (µM Fe^{+2}/g DW) based on an iron sulphate standard ($Fe2SO_4$) curve against a blank control. The analyses were conducted in triplicate.

2.4.3. Oxygen radical absorbance capacity

Oxygen radical scavenging capacity was determined using the ORAC assay as described previously [6] and antioxidant capacities of the samples were expressed as µM of trolox equivalent per gram of dry weight of lyophilized extract (µM T Eq./g DW) based on a trolox standard curve. The analyses were conducted in triplicate.

2.5. Inhibitory activities towards selected enzymes

2.5.1. α-Glucosidase inhibitory activity

The inhibition of α-glucosidase (obtained from intestinal acetone powders from rat) was determined was determined as described previously [6], using sucrose (2g of sucrose in 100 ml of maleic acid buffer) as a substrate. The relative α-glucosidase inhibition was calculated using the following formula: % Inhibition = [((ACB–AC) – (ASB–AS)) / (ACB –AC)] x 100, where AS and AC were the absorbance of sample and negative control, and where ASB and ACB were the absorbance of sample blank and control blank, respectively. The absorbance was measured at 505 nm using a Shimadzu 1601 spectrophotometer (Tokyo, Japan).

2.5.2. Pancreatic lipase inhibitory activity

The lipase inhibitory activity was assayed as described previously [6], using 4-methylumbelliferyl oleate (0.1 mM) as a substrate, with the exception of porcine pancreatic lipase (Sigma type II), which was prepared using a concentration of 0.085 g/ml. The relative lipase inhibition activity was calculated using the following formula: % Inhibition = (1- (FS-FSB) / (FC-FCB)) x100, where FS and FC were the values of samples and negative control measured fluorometrically at an emission wavelength of 460 nm and excitation of 320 nm with slit widths of 5 nm (POLARstar Omega, BMG Labtech, Germany), and where FSB and FCB were the fluorescence readings of sample blank and control blank, respectively.

2.6. Analysis of phenolic compounds

Identification and quantification of phenolic compounds by high liquid chromatography – diode array – mass spectrometry (HPLC-DAD-MS/MS) analysis were conducted as described previously [6]. The amount of total phenolic compounds detected at 280 nm were quantified as mg Gallic acid equivalents per gram weight of the extract (mg GA Eq./g DW) based on Gallic acid calibration curve (concentration range: 0.0125–0.5 mg/mL; r^2=1). Total phenolic compounds detected at 326 nm were calculated as mg of Chlorogenic acid equivalents per gram dry weight of the extract (mg CHA Eq./g DW) based on Chlorogenic acid calibration curve (concentration range: 0.0125–0.5mg/mL; r^2=1). Total phenolics content detected at 370 nm were quantified as mg Rutin equivalent per gram dry weight of the extract (mg Rutin Eq./g DW) based on the Rutin calibration curve (concentration range: 0.0125–0.5mg/mL; r^2=0.999).

2.7. Analysis of volatile and fatty acid compounds

Volatile compounds and fatty acids present in extracts were analysed by gas chromatography mass spectrometry (GC/MS) using a head space solid phase micro extraction and identified by the fragment ions and relative retention indices of their peaks with those of the MS library standards as described previously [7].

2.8. Data Analysis

The mean of results was calculated based on at least three independent evaluations (n=3) and the standard deviations (SD) were also calculated. IC-50 values were calculated from the corresponding dose inhibition curve according to their best-fit shapes based on at least four reaction points using Microsoft Excel. Statistical correlation analyses were performed using Graphpad Prism 5 (Graphpad Software, CA, USA), which were considered statistically significant when the $p<0.05$.

3. FINDINGS

3.1. Antioxidant and enzyme inhibitory activities

Table 1 presents antioxidant capacities and enzyme inhibitory levels of the extracts. Stem extract exhibited higher FCR and FRAP levels than that of the flower extract. On the contrary to FCR and FRAP, the ORAC value of flower extract was higher than that of the stem extract. With regards to enzyme inhibitory levels, both extracts showed mild inhibitory activities against α-glucosidase, which was lower than Acarbose (commercially used α-glucosidase inhibitor agent). However, the extracts particularly flower extract showed pronounced inhibitory activities against pancreatic lipase and showed high amounts of Orlistat equivalents (Table 1).

Table 1. Antioxidant and enzyme inhibitory activities

			Stem	Flower
Antioxidant	***activity***	FCR [1]	76.5±2.0a	68.3±1.2b
		FRAP [2]	1110.4±36.1a	490.0±3.5b
		ORAC [3]	4494.2±76.8b	5073.3±71.6a
Enzyme inhibitory	***activity***	α-Glucosidase inhibition		
		IC50 [4] (mg/ml)	2.15±0.06a	4.90±0.09b
		Acarbose Eq. (µmol/g DW)	21.46±0.58a	9.40±0.17b
		Pancreatic lipase inhibition		
		IC50 [4] (mg/ml)	1.81±0.03b	0.46±0.05a
		Orlistat Eq. (µmol/g DW)	1.52±0.02b	6.11±0.73a

Means with different letters in the same raw were significantly different at the level ($p< 0.05$); n=3.
[1] Total phenolics content (Folin-Ciocalteu values) – mg Gallic acid Equivalent/g DW,
[2] Ferric reducing antioxidant power – µ mol Fe^{2+}/g DW,
[3] Oxygen radical absorbance capacity - µ mol Trolox Equivalent/g DW.
[4] IC50-Half minimal inhibitory concentration.

3.2. Chemical Composition

Extraction yields (%), total phenolics and individual phenolic compounds were presented in Table 2. The flower extract yielded two times higher extraction than that of the stem. The total phenolic levels of extracts contained the highest amounts of phenolics at 326 nm. No any compounds were detected at 520 nm. Spectral characteristics of HPLC peaks (Figure 1)

revealed that phenolics were the dominating group of hydrophilic compounds. Mass spectrometric data confirmed these results (Table 2).

The major phenolic compound present in stem extract and second major compound of the flower extract had positively charged molecular ion ($[M+1]^+$) at m/z 449 and negatively charged molecular ion ($[M-1]^-$) at m/z 447, respectively and MS/MS fragments were at m/z 287 and 285, respectively. The neutral loss of 162 amu indicates the presence of hexoside unit. On the basis of molecular weight, fragmentation pattern and absorbance spectrum, this compound was tentatively identified as luteolin hexoside. This compound made up over 52 % of total phenolics (Table 2).

The dominated phenolic compound in the flower extract was identified as verbascoside based on m/z transition data and spectral and absorbance characteristics of HPLC peaks of the flower extract since it's negatively charged molecular ion ($[M-1]^-$) at m/z 623 and MS/MS fragments at m/z 461 (Table 2). This compound made up over 52 % of total phenolics of the flower extract. Additionally, the flower extract contained luteolin hexoside as the second major phenolic compound of contributing 9.6% of total phenolics (Table 2, Figure 1). Other phenolic compounds tentatively identified in both extracts based on m/z transition data were apigenin, chlorogenic acid, apigenin glucoside, quercetin hexoside and rutin at trace levels (Table 2).

Table 2. Mass spectrometric details and concentration of phenolic compounds

Yields and Total Phenolics			Stem	Flower
Yields (%)			14.0±0.8b	30.3±1.3a
Total Phenolics at 280 nm [1]			65.3±2.0b	84.1±0.4a
Total Phenolics at 326 nm [2]			88.6±0.7a	121.2±0.7a
Total Phenolics at 370 nm [3]			48.5±4.2a	32.9±2.2b
	MS/MS			
Individual phenolic compounds	$[M+1]^+/[M-1]^-$	Fragments		
Apigenin [4]	-/269	-/117	T	T
Chlorogenic acid [4, 3]	-/353	-/191	T	T
Apigenin glucoside [5]	-/431	-/269	T	T
Luteolin-7-O-glucoside [5,6]	449/447	287/285	46.4±0.2a	11.7±0.1b
Quercetin/hesperitin glucoside [5,6]	465/-	303/-	T	T
Quercetin rutinoside (Rutin) [4]	611/609	303/301	T	T
Verbascoside [4]	-/623	/461	T	63.4±0.1

All data represent the mean ± standard deviation of at least three independent experiments. T: traces (concentration < 2%); [1] Phenolics at 280 nm by HPLC (mg Gallic acid Equivalent/g DW); [2] Phenolics at 326 nm by HPLC (mg Chlorogenic acid Equivalent/g DW); [3] Phenolics at 370 nm by HPLC (mg Rutin Equivalent/g DW); [4] assignment confirmed with reference standard; [5] tentative assignment based on MS data only; [6] value is expressed as mg Luteolin Eq. /g DW.

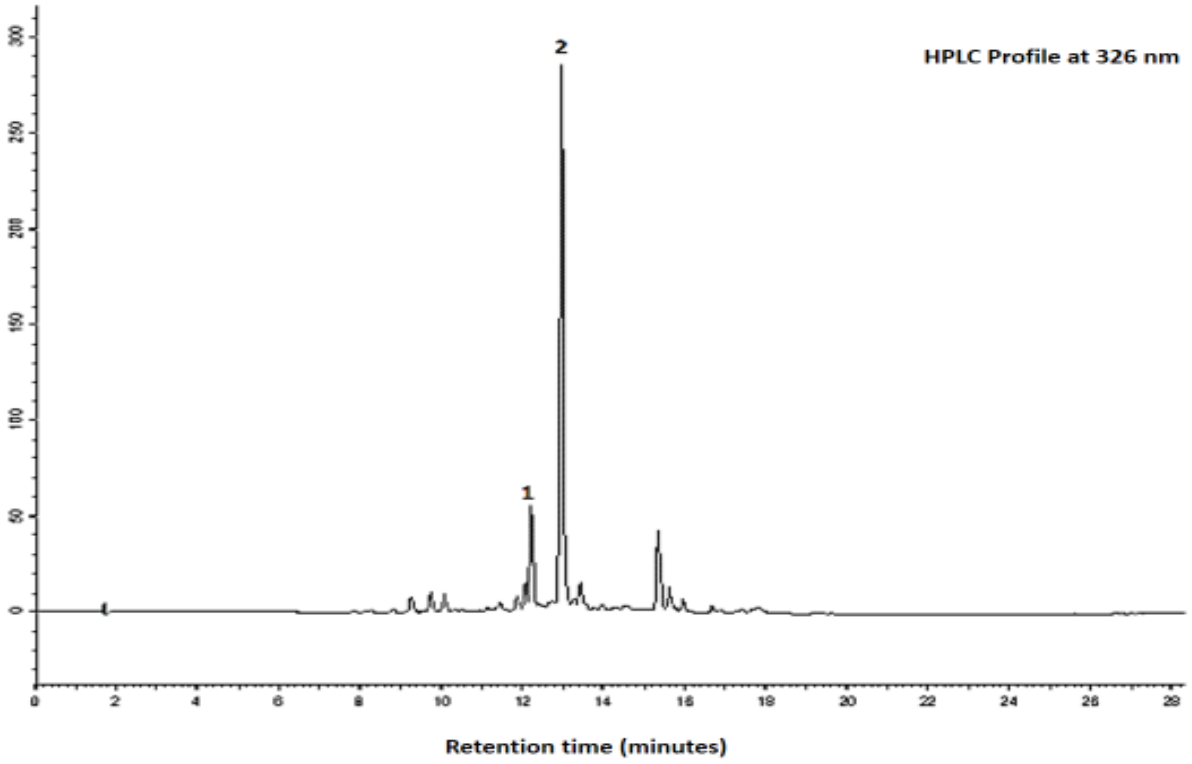

Figure 1. Representative HPLC chromatogram of extracts: (1) Luteolin-7-O-glucoside, (2) Verbascoside.

As presented in Table 3 and Figure 2, the extracts had a rich composition of volatile and fatty acids. GC-MS analysis revealed that nonanal, 2-undecenal, 2-decenal, palmitic acid ethyl ester and oleic acid ethyl ester were determined as the major volatile compounds of the extracts. Flower extract also contained 2,6-Di-tert butylphenol and 9,12-octadecadienoic acid ethyl ester compounds. The major volatile compound (compound 2) of the extracts produced fragment ions of 57, 70, 82, 98, 114 and 124 m/z (Figure 2, Table 3) and was tentatively identified as nonanal. Second major volatile compound contributed 21% of the stem and 16.8% of the flower extract, which produced fragment ions of 55, 70, 83, 97, 121 and 166 m/z. With regards to fatty acid compounds, the major compound was identified as palmitic acid ethyl ester, which produced 55, 88, 101, 115, 157, 241 and 284 m/z and had contributions of 17.2% and 9.3% for stem and flower extracts, respectively (Table 3, Figure 2).

Table 3. Volatile and fatty acid composition

No	Retention time	Name of compound	Molecular formula	Molecular mass	Fragment ions	Relative concentration (%)	
						Stem	Flower
1	16.3	Octanal	$C_8H_{16}O$	128	57, 69, 81, 84, 100, 110	11.12	2.92
2	19.4	Nonanal	$C_9H_{18}O$	142	57, 70,82, 98, 114, 124	23.85	17.46
3	21.6	Isophorone	$C_9H_{14}O$	138	54, 82, 95, 123, 138	T	2.83
4	21.7	2-Nonenal	$C_9H_{16}O$	140	55, 70, 83, 96, 111, 122	T	3.39
5	23.6	Safranal	$C_{10}H_{14}O$	150	51, 65, 91, 107, 121, 135, 150	ND	2.37
6	24.6	2-Decenal	C10H18O	154	55, 70, 83, 98, 110, 136	18.85	14.74

Table 3. Continuing

No	Retention time	Name of compound	Molecular formula	Molecular mass	Fragment ions	Relative concentration (%)	
7	26.9	2,4-Decadienal	C10H16O	152	67, 81, 95, 123, 152	T	4.41
8	27.7	2-Undecenal	$C_{11}H_{20}O$	168	55, 70, 83, 97, 121, 166	21.03	16.81
9	31.6	2,6-Di-tert-butylphenol	$C_{14}H_{22}O$	206	57, 74, 91, 163, 191, 206	T	3.85
10	40.2	2-Pentadecanone	$C_{18}H_{36}O$	268	58, 71, 85, 109, 124,	T	2.00
11	42.2	Palmitic acid methylester	$C_{17}H_{34}O_2$	270	55, 74, 87, 97, 143, 171, 199, 227, 270	4.13	2.1
12	43.6	Palmitic acid ethylester	$C_{18}H_{36}O_2$	284	55, 88, 101, 115, 157, 241, 284	17.23	9.32
13	48.5	Oleic acid ethylester	$C_{20}H_{38}O_2$	310	55, 69, 88, 97, 155, 180, 222, 264, 310	3.78	5.59
14	48.6	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	308	55, 67, 95, 164, 220, 263, 279, 308	T	9.90
15	49.1	Linoelic acid ethyl ester	$C_{20}H_{34}O_2$	306	55, 67, 79, 95, 108, 121, 261, 306	T	1.73

ND: not detected, T: traces.

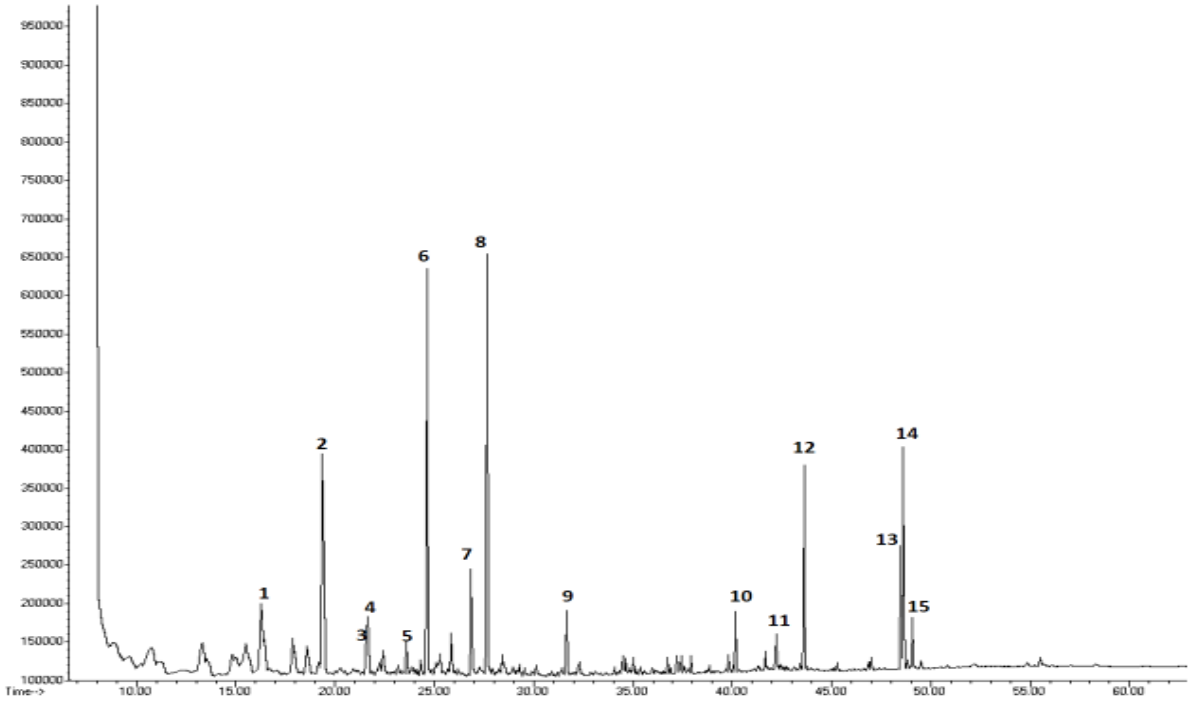

Figure 2. Representative GC-MS chromatogram of extracts: (1) Octanal, (2) Nonanal, (3) Isophorone, (4) 2-Nonenal, (5) Safranal, (6) 2-Decenal, (7) 2,4-Decadienal, (8) 2-Undecenal, (9) 2,6-Di-tert-butylphenol, (10) 2-Pentadecanone, (11) Palmitic acid methylester, (12) Palmitic acid ethylester, (13) Oleic acid ethylester, (14) 9, 12-Octadecadienoic acid, ethyl ester, (15) Linoleic acid ethyl ester.

4. DISCUSSION AND CONCLUSION

Natural sources are rich in physiologically active antioxidant compounds such as phenolics, glycosides, volatiles, fatty acids, polysaccharides, carotenoids and alkaloids, which able to prevent and / or minimize the negative effects of oxidative stress. Antioxidant capacity of plant-based extracts can be assessed via complementary antioxidant testing methods representing two basic antioxidant mechanisms; hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms. ORAC values can be used as reference antioxidant effectiveness since it is able to measure the radical chain breaking ability of antioxidants by monitoring the inhibition of peroxyl radical-induced oxidation. In order to distinguishing dominant mechanisms for different antioxidants, addition to ORAC assay, reducing based antioxidant assays such as; FRAP assay for acidic condition and Folin-Ciocalteu method for alkaline condition are suggested to reveal the complementary antioxidant potential of plant based extracts [8]. Therefore within this study, ORAC assay represented HAT mechanism and FCR and FRAP tests represented SET mechanism were utilized. Luteolin hexoside rich stem extract had higher antioxidant capacity in SET mechanism assays, while verbascoside rich flower extract had higher antioxidant capacity in HAT mechanism.

Enzyme inhibitors isolated from natural sources are recognised as natural preventative medicines without or with a minimum side effect [6]. The stem extract which luteolin hexoside was the major phenolic compound was more potent of α-glucosidase inhibition than that of the flower extract. This can be explained the amount of luteolin hexoside presence in the extracts since luteolin was reported as a potent α-glucosidase inhibitory agent among various phenolic compounds [9]. On the other hand, flower extract had pronounced pancreatic lipase inhibitory activity that of the stem extract, which can be linked to the presence of verbascoside- a potent oxygen radical suppressing agent [10-11]. Verbascoside was reported to show significant antioxidant activities from bitter tea (*Ligstrum purpurascens*), a popular beverage in southern China [12]. Moreover, it was reported that Verbascoside had high antioxidant, antihaemolytic activities, as well as enzyme inhibitory activities [13]. Koo and co-authors reported that Acteoside (Verbascoside) and its aglycones effectively scavenge 1,1-diphenyl-2-picrylhydrazyl and nitric oxide *in vitro* [14]. Biological activities of luteolin and its glycosides and several possible mechanisms of action have been elucidated including scavenging of ROS (Reactive Oxygen Species), transition metal chelation, reducing oxidative stress and inflammation, induction of apoptosis, lowering glucose level, reducing the uptake of glucose, protection against radiation and antiinflammatory action [15-17]. Volatile and fatty acid compounds present in the extracts might be the secondary contributors of antioxidant activities, since they were reported as weak antioxidant agents [18]. High extraction yields of the extracts indicate the presence of high amounts of hydrophilic compounds. HPLC results showed that the hydrophilic compounds were one of the major chemical compounds present in the extracts. With regards volatile and fatty acid compounds, both of the extracts had similar and rich volatile and fatty acids composition.

The stem and flower extracts had pronounced antioxidant and enzyme inhibitory activities and contained high amount of phenolic and volatile compounds. The utilization of *Verbascum cheiranthifolium* var. c*heiranthifolium* in folk medicine for a wide range of ailments treatment can be explained by significant and effective biological activities and phytochemical compounds diversity. Our findings presented in this study might help to researchers to conduct further studies on endemic mullein species in order to explore natural health attributing agents.

Acknowledgements

The authors are grateful to the Van Yuzuncu Yil University Grant Commission for providing financial assistance of the research (Project number: THD-2017-6381).

Conflict of Interests

Authors declare that there is no conflict of interests.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 243-251

DOI: 10.21448/ijsm.458827

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

Biological Activities of Wild Asparagus (*Asparagus acutifolius* L.)

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Abstract: *Asparagus acutifolius* L. (wild asparagus) is an edible plant species that is widely distributed throughout the Mediterranean. This study was designed to investigate the phenolic compounds, antioxidant, and cytotoxic activities of ethanol and water extracts of different parts (fruits and leaves) of *A. acutifolius*. The antioxidant activities of these extracts were analyzed using scavenging methods (DPPH and ABTS scavenging activity), the β-carotene/linoleic acid test system and the phosphomolybdenum method. Among the evaluations of the ethanol and water extracts of the different parts of *A. acutifolius*, the ethanol extract of the leaves was shown to have the highest amount of radical scavenging activities (DPPH, 0.383 mg/mL and ABTS, 0.145 mg/mL). Each extract of *A. acutifolius* exhibited strong antioxidant capacities with the β-carotene/Linoleic acid test system. For the phosphomolybdenum method, the antioxidant capacity of the extracts was in the range of 8.89-45.29 µg/mg and each extract exhibited high cytotoxic activities. The results will provide additional information for future studies on the biological activities of *A. acutifolius*, while also helping us to understand the importance of this species.

ARTICLE HISTORY

Received: 17 May 2018
Revised: 29 August 2018
Accepted: 04 September 2018

KEYWORDS

Asparagus acutifolius,
Antioxidant,
Cytotoxic,
Wild Asparagus.

1. INTRODUCTION

Not only plants used as food but due to their medical benefits they can also be used for the treatment of various diseases. Evaluating significance of their potential can therefore help in further understanding the medicinal value of these plants [1]. For this reason, medicinal or edible plants have been intensively investigated in recent years in order to find compounds that have antioxidant capacities, capable of protecting against a number of diseases [2].

The Asparagus genus includes more than 250 species that are of interest particularly in terms of their nutritional and medicinal use [3]. The genus is a valuable source of significant compounds and essential nutrients, oligosaccharides [4], vitamins and minerals [5]. Moreover, these plants contain flavonoids (mainly rutin) and phenolic compounds, both of which have powerful antioxidant properties [6]. One of the species belonging to the Asparagus genus is *Asparagus acutifolius* L. (wild asparagus), which is a native plant species commonly found throughout the Mediterranean [7]. *A. acutifolius* is an herbaceous, perennial, evergreen

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ISSN-e: 2148-6905 /© IJSM 2018

dioecious species with a tetraploid set of chromosomes [8, 9]. A wild edible plant, it has long been harvested and used in a variety of traditional cuisines throughout the Mediterranean [10]. *A. acutifolius* is rich in flavonoids and vitamin C, showing high antioxidant activity [11]. In western Anatolia, Turkey, the young shoots of this plant are consumed as vegetables and are added to dishes such as omelet and soups. Furthermore, *A. acutifolius* is considered to be a health-giving ingredient and is used in traditional medicinal treatments as a diuretic and antineuralgic [12-15].

Consequently, more research is required on the biological activities of this edible wild plant. We therefore consider that within the scope of this study *A. acutifolius* is a plant worthy of additional investigation. At the same time, while research exists on the antioxidant capacity and phenolic content of *A. acutifolius* spears [10, 15-18], to the best of our knowledge there is virtually no information on the biological activities of *A. acutifolius* fruit and leaves. With these aspects in mind, the objectives of the present study are to evaluate the antioxidant capacities, the cytotoxic activity and the total phenolic, flavonoid and tannin contents of the ethanol and water extracts of various parts from *A. acutifolius*.

2. MATERIAL AND METHODS

2.1. Plant Materials

Asparagus acutifolius L were collected from Çamlık, Denizli, Turkey, in June 2017. The taxonomic identification of the plant material was confirmed by Dr. Mehmet Çiçek, a plant taxonomist from the Biology Department of Pamukkale University, Denizli, Turkey. A voucher specimen (*Asparagus acutifolius* L; Herbarium No: 2017-99-1) was deposited at the private herbarium of M. Çiçek (PAU) at Pamukkale University (Denizli, Turkey).

2.2. Preparation of The Plant Extracts

The aerial parts (fruit and leaves) of *A. acutifolius* were air-dried in the dark and powdered at our laboratory. Twenty grams of each sample were weighed into Erlenmeyer flasks and then 200 mL of ethanol (96%) or water solvents were added to the samples. Extraction was carried out by shaking at 50 ^{o}C for 6 h in a temperature controlled shaker. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper, repeated twice and the solvent was evaporated at 40-50 ^{o}C using rotary evaporator (IKA RV10D, Staufen, Germany). All extracts were lyophilized (Labconco FreeZone, Kansas City, MO) and stored at -20 ^{o}C until use. In the manuscript, we used three-letter acronyms, the first signifying the plant, the second the solvent used and the third the part of plant (AEF: Ethanol extract of fruits of *A. acutifolius;* AEL: Ethanol extract of leaves of *A. acutifolius;* AWF: Water extract of fruits of *A. acutifolius* AWL: Water extract of leaves of *A. acutifolius*.All the experiments were done in triplicates.

2.3. Chemicals

β-carotene, Linoleic acid, 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Quercetin, Sodium phosphate, Gallic acid, methanol, chloroform and ethanol were purchased from Sigma-Aldrich. Butylated hydroxytoluene (BHT), Folin-Ciocalteu reagent and Tween 20 were purchased from Merck (Darmstadt, Germany). Other chemicals and solvents were of analytical grade.

2.4. Determination of Total Antioxidant Activity

2.4.1. β-carotene/linoleic acid method

The antioxidant activity of the plant extracts was determined according to the method of Amin and Tan [19]. One milliliter of β-carotene solution (0.2 mg/mL chloroform) was put into a round-bottom flask containing 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20. The chloroform was evaporated using a rotary evaporator. Then, the mixture was diluted with 100

mL of distilled water. The reaction mixture and one milliliter extracts (1 mg/mL) were placed in test tubes. The initial absorbances were immediately measured with a spectrophotometer at 470 nm. The reaction mixture was incubated at 50 ^{o}C for 2 hours and the absorbance of this mixture was measured again. The same process was repeated with BHT as a positive control. The total antioxidant activity (AA) was calculated as follows:

$$AA= [1- (A_{samp}-A_{co})/(A^{o}_{samp}-A^{o}_{co})] \times 100$$

(A_{samp} and $A_{co:}$ absorbance at the initial time of the incubation of samples and control, respectively and A^{o}_{samp} and A^{o}_{co}: absorbance in the samples and control at 120 min).

2.4.2. Phosphomolybdenum method

This was evaluated using the phosphomolybdenum method according to Prieto et al [20]. A reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate was prepared and the reagent solution (3 mL) and 0.3 mL extract were mixed. The reaction mixture was incubated at 95 ^{o}C for 90 min. The absorbances of the mixtures were measured at 695 nm using a spectrophotometer. The antioxidant activity of the extracts was expressed as equivalence of ascorbic acid.

2.5. Measurement of Radical Scavenging Activity

2.5.1. Free radical scavenging activity (DPPH)

The DPPH free radical scavenging method is an antioxidant assay based on electron transfer. The DPPH method is a quick and simple way for calculating antioxidants by means of spectrophotometry. The scavenging activity of *A. acutifolius* extracts on DPPH radicals was measured according to the method of Meriga et al [21]. Different concentrations (0.2-1.0 mg/mL) of the extracts (1 mL) were mixed with 4 mL of DPPH radical methanolic solution. The reaction mixture was kept in a dark room for 30 min. The absorbance measured at 517 nm. BHT was used as a control. Results were expressed as IC_{50} values. The values of IC_{50} denote the concentration of the sample that is required to scavenge 50% of DPPH free radicals.

2.5.2. ABTS radical cation scavenging activity

The ABTS method was conducted according to the procedure of Shalaby and Shanab [22] with slight modifications. The ABTS cations were produced by reacting 7mM ABTS stock solution with 2.45 mM potassium persulfate. The reaction mixture was kept in a dark room for 12-16 h before use. The absorbance (0.700±0.05) of the diluted reacting mixture and ethanol (1:1) were measured at 734 nm for the study of the extracts. The ABTS solution was mixed with 0.1 mL of the extracts (1 mg/mL). The absorbance of the solutions were read at 734 nm after 15 min. The results were expressed as IC_{50} values. Ascorbic acid was used as the positive control.

2.6. Determination of Total Phenolic, Flavonoid and Tannin Content

The total phenolic content was evaluated using the Folin-Ciocalteu method [23]. In this method, the extract (1 mg/mL) was mixed with 1 mL Folin-Ciocalteu reagent and 46 mL distilled water. After 3 min, 3 mL of 2% sodium carbonate (Na_2CO_3) solution was added. After keeping in the dark at room temperature for 2h, the absorbance of the samples was read at at 760 nm. The results were determined as mg of Gallic acid equivalents (mg GAE/g extract).

The total flavonoid content of the extracts was analyzed according to Arvouet- Grand et al. [24]. In this method, 1 mL of 2% $AlCl_3$ was mixed with the same volume of extract solution (2 mg/mL). After 10 min incubation at room temperature, the absorbance of the reaction mixtures were measured at 415 nm. The flavonoid content was calculated from the quercetin standard curve (mg QEs/g extract).

The tannin content was evaluated using the vanillin-HCL method of Broadhurst and Jones [25]. The test tubes were wrapped in aluminum foil. In these test tubes, the extract (1 mg/mL) was mixed with 3 mL vanillin reagent (4% vanillin in methanol) and concentrated hydrochloric acid. Then the reaction mixture was left standing for 15 min at 20ºC. Absorbance of the solution was measured at 500 nm. Tannin content was expressed as equivalents of catechin (mgCEs/g).

2.7. Cytotoxic Activity

The possible cytotoxic activity of different parts of *A. acutifolius* were evaluated using the Brine shrimp lethality bioassay [26]. The brine shrimps (*Artemia salina*) were hatched using *A. salina* eggs in a beaker, filled with air-bubbled sterile artificial seawater (3.8 g sea salt was dissolved in 100 ml water) and left to incubate under artificial light for 24-48 h at 28 ºC. In each experiment 0.5 mL of plant extract (2 mg/mL) was mixed with 4.5 mL of brine solution and the sample was tested at 1000, 500, 100, 50 and 10ppm. Following incubation, active nauplii free from egg shells were collected and used for assay. Ten nauplii were drawn through a glass capillary and placed into test tubes containing different concentration of extracts and the control tubes. The extracts and controls tubes were maintained under artificial light for 24 h at 28 ºC. For each concentration of the extract and control, the number of dead shrimps were counted and recorded using an overhead projector. Larvae were considered dead if no movement of the appendage was observed within 10 sec. To determine the LC_{50} values, the data was analyzed using the EPA Probit Analysis Program (version 1.5) [27]. In the toxicity evaluation of plant extracts, LC_{50} values lower than 1000 µg/mL are considered to be bioactive [26]. The experiments were conducted in a set of three tubes per concentration and the controls.

2.8. Statistical Analysis

All analyses were performed in triplicate and the results presented as mean ± SE (standard error). The results were analyzed using the MINITAB Statistical Package program. The variations between the different extracts were tested using Analyses of Variance (ANOVA) and a Tukey test was conducted to see how the groups differed from each other ($P<0.05$).

3. RESULTS AND DISCUSSION

3.1. β-Carotene / Linoleic Acid and Phosphomolybdenum Antioxidant Activity

In the present study, the potential of the plant to inhibit linoleic acid oxidation was evaluated using the β-Carotene/linoleic acid test system. The results showed that the ethanol extract of the fruit (AEF, 96.96±1.52) exhibited stronger antioxidant activity than the water extract of the fruit (AWF, 90.46 ± 0.72), the water extract of the leaves (AWL, 88.86 ±0.80) , the ethanol extract of the leaves (AEL, 82.41±0.25) and also standard antioxidant (BHT, 93.18±0.43) (Figure 1). Antioxidants effectively inhibit linoleic acid oxidation and minimize oxidation of the lipid components in the cell membranes [28]. All of the extracts analyzed in this study exhibited strong antioxidant properties and they appeared to reduce the oxidation of linoleic acid, a key concern for the food industry.

The antioxidant capacity of the extracts evaluated using the phosphomolybdenum method, were in the range of 8.89 - 45.29 µg/mg (Table 1). The results revealed that ethanol extract of the leaves showed the highest and the water extract of the fruit showed the lowest antioxidant activity. Among the extracts of *A. acutifolius* analyzed in the present study, there were significant differences between the ethanol extract of the leaves and the water extract of the fruit ($F_{3,20}$= 489.39 $p<0.001$), but no differences were found between the antioxidant capacities of the water extract of the leaves and the ethanol extract of the fruit. The strong antioxidant capacity of the ethanol extract of the leaves of *A. acutifolius* may be attributed to the presence of phenolic content.

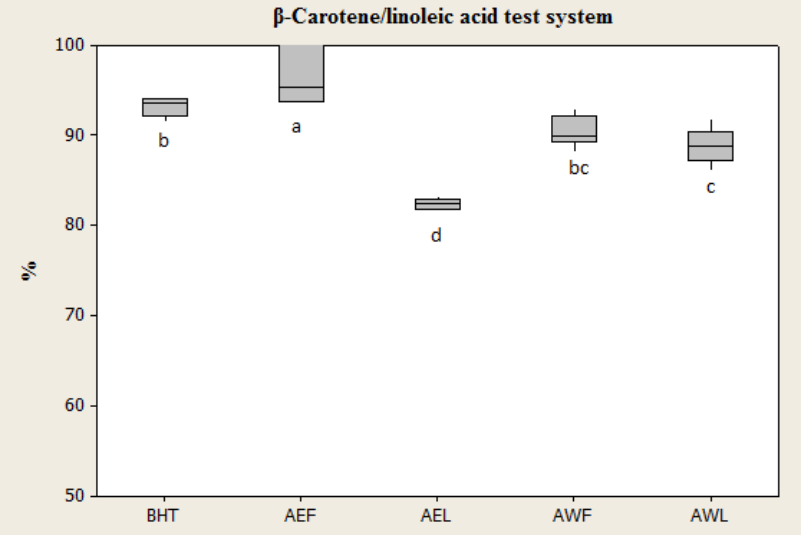

Figure 1. Antioxidant activity of *A. acutifolius* extracts

AEL: Ethanol extract of leaves of *A. acutifolius;* AEF: Ethanol extract of fruit of *A. acutifolius;* AWL: Water extract of leaves of *A. acutifolius* AWF: Water extract of fruit of *A. acutifolius;* BHT: Standard antioxidant; (different groups were shown with different letters on each boxplot)

Table 1. Antioxidant properties of *A. acutifolius*

Sample	Phosphomolybdenum (µg/mg)	DPPH (IC_{50}) (mg/mL)	ABTS (IC_{50}) (mg/mL)
AEL	45.29 ± 0.46^{a}	0.383 ± 0^{d}	0.145 ± 0.03^{cd}
AEF	16.61 ± 0.13^{b}	3.990 ± 0.17^{a}	1.815 ± 0.02^{a}
AWL	19.13 ± 1.12^{b}	0.799 ± 0^{c}	0.214 ± 0.04^{c}
AWF	8.89 ± 0.76^{c}	2.967 ± 0^{b}	0.838 ± 0.01^{b}
BHT	nt	0.033 ± 0^{e}	0.074 ± 0^{d}

AEL: Ethanol extract of leaves of *A. acutifolius;* AEF: Ethanol extract of fruits of *A. acutifolius;* AWL: Water extract of leaves of *A. acutifolius* AWF: Water extract of fruits of *A. acutifolius;* BHT: Standard antioxidant; nt: not tested

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

3.2. Radical scavenging activity (DPPH and ABTS)

DPPH is a stable free radical and the lower the absorbance of the antioxidant and DPPH reaction mixture is, the higher the free radical scavenging activity of the antioxidant [29]. All extracts tested in the present study showed a radical scavenging capacity and among the extracts analyzed the ethanol extract of the leaves exhibited the highest radical-scavenging activity. All extracts were significantly different both from each other and the BHT IC_{50} values ($F_{4,25}$= 545.37 $p<0.001$).

The ABTS scavenging capacity of the plant extracts was determined and the results are given in Table 1. The studied extracts showed scavenging activities in the range of 0.145-1.815 mg/mL. In addition, Zengin and Aktümsek [30] indicated that there was a correlation between the DPPH and ABTS methods. These results were in agreement with this study of Zengin and Aktümsek [30] and in the DPPH and ABTS assays, the ethanol extracts of the leaves showed the strongest radical scavenging activity. In addition, the ethanol extract of the leaves containing the highest amount of phenolic content. The phenolic content may be attributed to the DPPH and ABTS radical scavenging activity of the ethanol extract of the leaves. Our result confirmed the previous findings of Zengin and Aktümsek [30] and Uysal et al. [31].

3.3. Total Phenolic, Flavonoid and Tannin Contents

The total phenolic content in the ethanol and water extracts of the fruit and leaves from *A. acutifolius* ranged from 5.40 to 54.10 mgGAE/g (Table 2). As can be seen from Table 2, phenolic content was shown to be highest in the ethanol extract of the leaves, and to be lowest in the water extract of the fruit extract. The amount of phenolic content in the extracts varied according to the plant part and the solvent. These results were in accordance with the findings of Uysal et al. [31], these findings show that phenolic content may vary significantly between the different parts of the plants.

Table 2. Total flavonoid, phenolic and tannin content of *A. acutifolius*

Sample	Total phenolic content (mgGAEs/g)	Total flavonoid content (mgQEs/g)	Total tannin content (mgCEs/g)
AEL	54.10 ± 0.67^{a}	92.70 ± 0.21^{a}	37.72 ± 0.51^{a}
AEF	5.68 ± 1.25^{c}	13.05 ± 0.37^{c}	25.97 ± 0.17^{b}
AWL	24.92 ± 1.58^{b}	21.87 ± 0.12^{b}	25.97 ± 0.36^{b}
AWF	5.40 ± 0.62^{c}	11.07 ± 0.05^{d}	24.47 ± 0.17^{c}

AEL: Ethanol extract of leaves of *A. acutifolius;* AEF: Ethanol extract of fruit of *A. acutifolius;* AWL: Water extract of leaves of *A. acutifolius* AWF: Water extract of fruit of *A. acutifolius*

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

The total flavonoid contents in the *A. acutifolius* extracts were determined using the spectrophotometric method with aluminum chloride and the results varied from 11.07 to 92.70 mg QEs/g extract (Table 2).

In the present study, tannin content was detected using the vanillin-HCL method and these results were evaluated as catechin equivalents. The total tannins content of the *A. acutifolius* extracts are presented in Table 2 and vary from 24.47 - 37.72 mgCE/g. Phenolic compounds (such as phenolic acid, flavonoid and, tannin) are abundant in plants and have multiple biological effects including antioxidant activity. The determination of phenolic compounds in plants is very important in understanding their pharmacological properties and medicinal values. The results obtained in the present study showed that each extract of this plant had both a high phenolic content and a high antioxidant activity. This is an indication that the phenolic compounds present in the extracts are largely responsible for the antioxidant activity.

3.3. 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}. It has also been used for the detection of fungal toxins and pesticides [26, 32, 33] and universally as a test for detecting cytotoxic effects. Likewise, it is also frequently used in laboratories for the prescreening of plant extracts that have potential medicinal benefits, such as antimicrobial or antiparasitic [34-36]. In addition, the brine shrimp cytotoxic bioassay is highly sensitive to a variety of chemical substances [37] and only a small amount of sample is required [38].

The lethality of extracts are presented in Table 3 and show that the extracts possessed high cytotoxic activities against brine shrimp. The lethality of these extracts from *A. acutifolius* indicate the presence in this species of potent cytotoxic components, which require further investigation. The present study suggests the need for further investigations of this plant, in order to ascertain potential cytotoxic compounds.

Table 3. Cytotoxic activity of *A. acutifolius*

Sample	Cytotoxic activity (LC_{50}) (µg/mL)
AEL	160.523
AEF	312.028
AWL	236.454
AWF	310.324

AEL: Ethanol extract of leaves of *A. acutifolius;* AEF: Ethanol extract of fruit of *A. acutifolius;* AWL: Water extract of leaves of *A. acutifolius* AWF: Water extract of fruit of *A. acutifolius*

4. CONCLUSION

The results revealed in the present study show that the ethanol and water extracts of the fruit and leaves of this plant have antioxidant properties. They also show that the plant possesses rich phenolic, flavonoid and tannin contents. Furthermore, all extracts show LC_{50} values of less than 1000 µg/mL and these results indicate that these parts of the plant possess strong cytotoxic activities. The present study suggests that this plant could be considered as a source of natural agents for the food industry and pharmacological applications. Further investigation is required to isolate and identify the antioxidant, and cytotoxic components found in this plant.

Acknowledgements

We thank all the lab members of the Secondary Metabolites Laboratory. We also state that there is no conflict of interest among the authors.

Conflict of Interests

Authors declare that there is no conflict of interests.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 252-258

DOI: 10.21448/ijsm.459102

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

Electromagnetic radiation of low intensity as a factor of change of phenolic compounds content

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Abstract: The influence of low-intensity electromagnetic radiation (EMR) on the content of phenolic compounds (PC) in plants on different stages of ontogeny is discussed. A medicinal plant (Calendula officinalis L.) and agricultural crop Fagopyrum sagittatum G., diploid varieties and tetraploid varieties were chosen as objects of study. Microwave EMR in two frequency bands, 53.57-78.33 GHz (wide range mode) and 64.00-66.00 GHz (narrow range mode), has been selected to learn the physical effects on seeds, with a treatment exposure time of 20, 12, or 8 min. The experiments were conducted under laboratory and field conditions. Treated and control seeds were germinated by 100 pcs in a growth chamber on moistened filter paper at 22–24˚C in the dark and placed on the daylight from the third day. The level of PC in the seedlings was measured with the Folin-Ciocalteu reagent. It was found that in the EMR pretreated C. officinalis seedlings the level of PC is maximal on 14th day of germination. The wide mode treatment for 12 min results in a two-fold increase in the level of PC in juvenile shoots with respect to control. Different constitutional levels of phenolic compounds in the leaves of diploid and tetraploid varieties of F. sagittatum in the most critical phases of ontogeny were found. It is recommended to use EMR with a 12 min exposure time for C. officinalis. The narrow frequency mode with an exposure time of 20 min is preferable to use for most varieties of F. sagittatum.

ARTICLE HISTORY

Received: 28 March 2018
Revised: 29 June 2018
Accepted: 06 September 2018

KEYWORDS

Electromagnetic radiation of microwave range, Calendula officinalis *L.*, Fagopyrum sagittatum *G.*, Phenolic compounds.

1. INTRODUCTION

As a protection against various stressors plants use protective mechanisms associated with the synthesis and accumulation of specific metabolites, called secondary. Phenolic compounds (PC) are of great importance for the performance of this function. The properties of PC in the plant organism are numerous and varied. They are characterized by high physiological activity and antioxidant properties. Their content in the plant is determined by the type of plant, as well as by the conditions of growth, and, therefore, these substances are markers of stress conditions in plants [1, 2]. The influence of low-intensity electromagnetic radiation (EMR) on plants is studied by researchers from different countries for 50 years [3–12]. However, the mechanism of its action is not yet fully understood, but it has already

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ISSN-e: 2148-6905 /© IJSM 2018

been established that the activation of the antioxidant system of plants, including low- and high-molecular components, occurs during the treatment of EMR. Therefore, the study of the state of this system, and in particular low-molecular antioxidants, is important for understanding the mechanism of interactions of EMR with plants.

It is important for Belarus to look for optimal conditions and methods for cultivating these crops. There is a problem when growing marigold to obtain high-quality pharmaceutical raw materials, environmentally pure and containing a sufficient number of biologically active substances, as well as increasing crop yields. When buckwheat is grown in the conditions of Belarus, the issue of yield comes first. As it is known from literature, the level of phenolic compounds in a plant affects on the resistance to environmental factors and crop yields [13]. It is necessary to use stimulating factors to increase the agro technical characteristics of plants. For that purpose we choose the electromagnetic treatment of the millimeter range of low-intensity which has proved itself well on a number of vegetable crops both in Belarus and abroad.

2. METHOD

A medicinal plant *Calendula officinalis* L. ('Machroviy 2000', Belarus) and agricultural crop *Fagopyrum sagittatum* G. diploid varieties (´Kypava´ and ´Phenix´) and tetraploid varieties (´Alexandrina´, ´Anastasia´, ´Martha´, Belarus) were chosen as objects for the study.

Microwave electromagnetic radiation in two frequency bands has been selected to study the physical effects on seeds: Mode 1 (processing frequency 53.57-78.33 GHz – wide range) with 20 minutes of treatment exposure (R1), 12 minutes (R1.1), 8 minutes (R1.2) and mode 2 (64.00-66.00 GHz processing frequency – narrow range), with the same 20 minutes of treatment exposures (R2), 12 minutes (R2.1), 8 minute (R2.2). Seed treatment was carried out at the Institute for Nuclear problems of BSU in a laboratory microwave installation for seed treatment of various agricultural crops in a wide frequency range (37 to 120 GHz) with infinitely adjustable power from 1 to 10 mW [14].

The experiment was conducted in the laboratory and field conditions. In the laboratory treated and control seeds were germinated by 100 pcs in a growth chamber on moistened filter paper at a temperature 22˚C in the dark for 3 days. Next the germinating seeds were transferred to a room with natural light (24 °C, 12-hour photoperiod). The length and mass of the roots and seedlings have been evaluated. Preparation of extracts of all samples under study has been carried out according to the State Pharmacopoeia of the Republic of Belarus [15]. The level of PC was measured with Folin-Ciocalteu reagent at a wavelength of 730 nm [16] on spectrophotometer Agilent 8453. Calculation of the PC level has been carried out on a calibration curve, gallic acid was used as a standard. The range of concentrations of gallic acid was 0.10-1.0 g/l. The content of PC samples was expressed as equivalent to gallic acid in g/100 g of dry weight (DW). The experiments were performed in 3-fold biological and analytical replicates. Untreated seeds served as control. The results were processed using the statistical package M. Excel and Stadia 8.0. The figure and tables show the mean values of the definitions and their standard deviations.

3. RESULTS AND DISCUSSION

It is well known that the biosynthesis of PC in different phases of plant growth and development has an oscillatory nature. Plants can either grow PC synthesis or decrease their contents in the extreme conditions. So a decrease in temperature leads to an increase in the content of PC according to the literature [17]. Insufficient sunny days, violation of mineral nutrition and water regime also affect on the qualitative and quantitative composition of the PC [13, 18-19].

However, it is known that the correct selection of modes of EMR treatment contributes to an increase in the content of PC, especially under the laboratory conditions [20–21], but in

the field experiment the result is significantly offset by abiotic factors [22]. Accumulation of PC in raw materials may to increase, that we previously established on the plants of marigold in 2014–2015 [19], and to be at the control level 2016–2017 year (Table 1, on 60th day in the flowering phase).

Table 1. The content of PC in *C. officinalis* seedlings (on 7th day and on 14th day – laboratory conditions) and inflorescence (on 60th day – field experiment), g/100 g DW

Variant	Control	R1	R1.1	R1.2	R2	R2.1	R2.2
On 7th day	2.6±0.2	3.3±0.04	5.0±0.6	2.7±0.1	2.3±0.2	1.7±0.3	3.6±0.1
On 14th day	2.6±0.1	2.96±0.4	3.5±0.4	5.0±0.5	4.1±0.8	3.0±0.6	6.2±0.1
On 60th day	2.5±0.1	2.4±0.1	2.7±0.1	2.4±0.1	2.5±0.04	2.3±0.4	2.5±0.2

It was established that the influence of EMR on the PC accumulation in juvenile marigold seedlings under controlled conditions has a pronounced dependence from the exposure of EMR (Tabl. 1). An increase in the concentration of phenolic compounds in these seedlings after EMR treatment has been noted. Exposure during 8 min led to a maximum increase in the PC level. In 7th-day seedlings, the level of phenolic compounds was above the control value in R1 and R1.1 on 27 and 92%, respectively, and lower in R2 and R2.1 on 12 and 33% relative to control. An increase in the amount of PC after EMR treatment in 14th day plants of calendula was noted. The level of PC increases from 14 to 140% with respect to control, as much as it was in R1.2 and R2.2 to the 14th day. Shifts in the PC level under the influence of EMR were observed mainly in juvenile marigold plants, but they were practically absent at the time of flowering (Table 1).

It was noted that there is no direct relationship between the PC level and growth processes in the experiment with juvenile marigold sprouts (Table 1, Figure 1). The level of PC in control and R 1.2 coincide on the 7th day, but the growth processes of the experimental seedlings prevail over the control values. It was established that R1.2 has the highest morphometric parameters, R2.1 has the lowest PC level on 33% below the control values, but the growth rate was higher than the control values and the predominance of root growth has been observed. The predominance of growth and extension processes has been noted for the regimes of the first group because the parameters of root and sprout length have the maximum values. Activation of growth processes and an increase in the level of PC took place in the 14th day sprouts. Dependence of the growth rate and amount content of PC from the time of exposure has been noted for the regimes of the first group, maximum effect during treatment has been established through the 8 minutes (R1.2). It was noted for the second group of regimes (64-66 GHz) that stimulation of growth has been accompanied by an increase in the number of PC when EMR was processed for 12 minutes (R2.1).

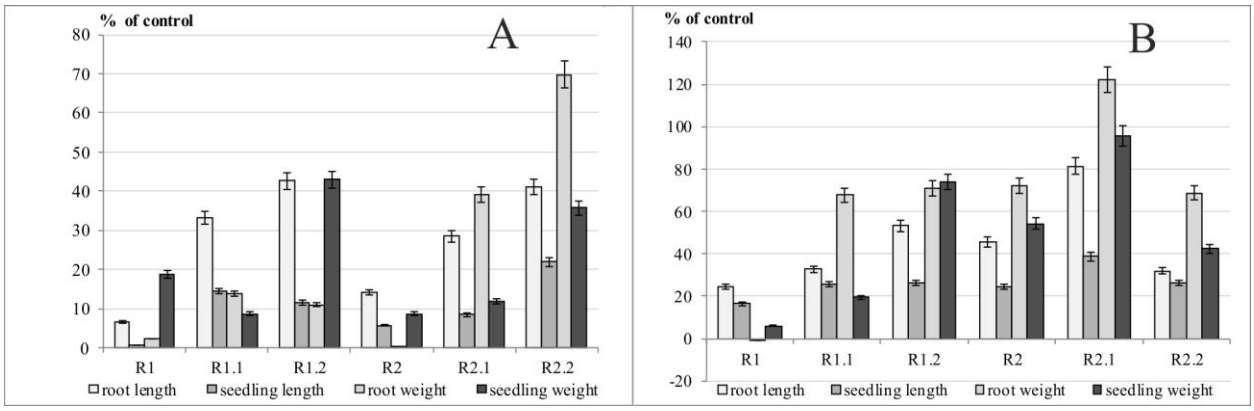

Figure 1. Effect of EMR on morphometric parameters of juvenile marigold plants: A – on the 7th day; B – on the14th day

Thus, it was found that an increase in content of PC has been occurred to the 14th day as a result of the influence of EMR. The dependence between the quantitative content of PC and exposure was established. Treatment by R1.2 led to an increase in 2 times in the level of PC in juvenile shoots with respect to control.

Different constitutional level of the PC in the leaves of diploid and tetraploid varieties of *Fagopyrym sagittatum* G. in the most critical phases of ontogenesis of plant development such as the initial stages (7th day) and the period of plants mass flowering (69th day) has been established during the research (Table 2).

Table 2. Influence of low-intensity electromagnetic radiation on accumulation of PC in leaves of di- and tetraploid varieties of *Fagopyrym sagittatum*, g/100 g DW

Variant	On 7th day			On 69 th day (mass flowering)		
	Control	R2	R2.1	Control	R2	R2.1
´Alexandrina´	7.4±0.4	5.1±0.3	2.9±0.2	12.2±0.7	12±0.5	11.3±0.6
´Anastasia´	6.6±0.4	7.8±0.4	9.3±0.5	11.4±0.6	10.8±0.5	12.7±0.6
´Martha´	5.9±0.03	8.6±0.4	7.8±0.4	11±0.6	11.2±0.1	11.2±0.5
´Kupava´	2.8±0.2	0.5±0.1	1.5±0.1	11±0.5	10.5±0.5	10.2±0.5
´Phenix´	2.2±0.1	2.7±0.1	1.6±0.1	8.9±0.4	11.3±0.6	1.6±0.1

It was noted that in all studied periods among the tetraploid varieties *Fagopyrym sagittatum* the control of ´Martha´ has the minimum content of these metabolites and ´Alexandrina´ has the maximum one.

It was revealed that under the influence of the EMR regimes the content of PC decreased with respect to the control with a reduction in the exposure time from 20 (R2) to 12 minutes (R2.1) on 30.7% and 60.7% respectively in ´Alexandrina´. ´Martha´ reacted differently to EMR regimes. The level of phenolic compounds was increased on 46.3% under the influence of R2 and on 31.8% under R2.1 on the initial stages, and there was practically no difference between control and experience on the 69th day. A different trend was noted in ´Anastasia´ so R2.1 significantly (on 41%) increased the level of PC at the initial stages and the stimulatory effect decreased to 11.1% to 69th day. It was noted that the reaction of ´Anastasia´ to R2 depended on the ontogenesis stage. There was an increase in the content of these metabolites on 18.2% on the 7th day and decrease on 5.3% was revealed to the period of mass flowering.

A specific reaction of diploid varieties of *Fagopyrym sagittatum* on the EMR regimes on the accumulation of phenolic substances in shoots and leaves has been noted. It was established

that R2.1 reduced the level of these compounds in both diploid varieties at the initial stages of vegetation on 44.4% (´Kupava´) and 27% (´Phenix´). It was noted that content of PC to 69th day in ´Kupava´ lowered to 6.84%, but in ´Phenix´ it increased on 82% relative to control on this date. It was noted that R2 sharply inhibited the accumulation of these metabolites on 83% in ´Kupava´ at the initial stages of ontogeny and at the period of mass flowering the differences were smoothed out. The level of phenolic compounds under the influence of R2 in ´Phenix´ increased on 23.4% and 27% on the 7th and 69th days of ontogenesis respectively.

Table 3. Effect of EMR regimes on the height of buckwheat plants of three tetraploid varieties on the 7th and 69th day of development

Variant	Length, cm	
	on 7th day	on 69th day
	´Alexandrina´	
Control	9.2±0.4	85.6±4.3
R2	9.4±0.3	82.5±4.1
R2.1	9.5±0.2	81.2±4.1
	´Anastasia´	
Control	6.5±0.2	89.4±4.5
R2	6.7±0.2	81.9±4.1
R2.1	5.5±0.2	86.3±4.3
	´Martha´	
Control	6.2±0.3	87.1±4.4
R2	5.7±0.3	83.0±4.2
R2.1	5.7±0.3	81.6±4.1

Almost everywhere there is no information about the influence character of PC on the nature of plant growth processes. Therefore, we attempted to assess the effect of EMR regimes on the height of plants on the 7th and 69th day of ontogeny and compare it with the level of PC (Table 2 and 3). It was noted that a significant decrease in PC under the influence of the EMR regimens with respect to control of ´Alexandrina´ practically didn't affect on the growth of seedlings at the initial stages of ontogenesis. It was revealed that in the period of mass flowering of plants a slight decrease in the level of these metabolites a little bit inhibited the growth of plants of this variety and especially in the case of R2.1 (It reduced on 5.2% compared to the control (Table 3)). A significant increase of PC (41%) under the influence of R2.1 was accompanied by a decrease in the intensity of the growth processes on the early stages on 15.4% in ´Anastasia´. The level of these compounds increased on 11.1% to the period of mass flowering, but the height of the experimental plants slightly differed from control one of ´Anastasia´. It was revealed that in ´Anastasia´ R2 had practically no effect on the length of shoots to the 7th day, whereas it inhibited the growth processes on 8% relative to control to the 69th day. It was found that a significant increase in the level of PC in the initial stages of ontogeny inhibited the growth processes during this period on 7.9% (R2) and 15.4% (R2.1), and to the period of mass flowering the magnitude of this effect decreases to 4,7% (R2) and 6.3% (R2.1) in plants of ´Martha´.

Thus, the shifts in the level of the phenolic compounds relative to the control were gradually reflected on the character of the growth processes, and the magnitude and direction of the deviations from the control values depended on the individual characteristics and the physiological state of the object under study.

4. CONCLUSION

Shifts in the accumulation of compounds of phenolic nature are one of the sides of the interaction mechanism of millimeter-wave electromagnetic radiation with plant objects. The consequences of seeds processing by EMR were observed for juvenile plants up to 14 days and by the time of flowering the after-effect was practically removed and had no significant effect on the accumulation of the phenolic compounds in all investigated objects. Variety-specific shifts were detected under the influence of EMR regimes in the accumulation of the phenolic substances in sprouts and leaves of di- and tetraploid varieties of buckwheat, which had an impact on the nature of the growth processes.

Change in the level of compounds of phenolic nature confirms the opinion that the EMR of the microwave range in the studied regimes is a small stress factor, and the responses that develop in the treated plants can be considered as phases of phytostress.

It was found that the maximum deviations in PC level are noted at the initial stages of ontogeny. The magnitude of the deviation is species- and variety specific. The amplitude of the differences between the control and experimental variants is reduced near the flowering period. Established changes in the content of PC under the influence of EMR allow to select the exposure time for the regulation of growth processes of cereals and medicinal crops for the purpose of using them for the industrial cultivation of these plants. Species - and variety-specific changes in the content of secondary metabolites under the influence of EMR allow to choose the exposure time for the regulation of the growth processes of cereals and medicinal crops for the purpose of using them in the industrial cultivation of these plants. It is recommended to use EMR in the exposure of 12 minutes (R1.1 or R2.1) for *Calendula officinalis.* It is better to use mode 2 with a time of exposure of 20 minutes for most varieties of *Fagopyrym sagittatum* and for ´Anastasia´ – R2.1 for 12 minutes duration of EMR.

Acknowledgements

The authors are grateful to Dr. Valentina N. Rodionova, Institute for Nuclear Problems of the Belarusian State University, for seed treatment.

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International Journal of Secondary Metabolite
2018, Vol. 5, No. 3, 259-269

DOI: 10.21448/ijsm.433681

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

Investigation of Chemical Composition, Antioxidant, Anticholinesterase and Anti-urease activities of *Euphorbia helioscopia*

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Abstract: In this study, chemical composition, antioxidant, anticholinesterase and anti-urease activities of the essential oil, hexane, acetone, methanol and water extracts of *Euphorbia helioscopia* were investigated. The chemical composition of the essential oil was analyzed by GC and GC/MS and *β*-cubebene (19.3 %), palmitic acid (12.2 %) and caryophyllene oxide (11.7 %) were identified as major compounds. The antioxidant activity of essential oil and extracts was performed by several methods such as β-carotene-linoleic acid, DPPH$^{\bullet}$, ABTS$^{\bullet+}$ radical scavenging, CUPRAC and metal chelating assays. The water extract showed higher antioxidant activity than BHA and α-tocopherol in β-carotene-linoleic acid, DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and CUPRAC assays with IC_{50}: 1.08±0.39, 15.57±0.48, 1.89±0.33 and 12.50±0.11 µg/mL values, respectively. Also, the anticholinesterase and anti-urease activities were tested against acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and urease enzymes, spectrophotometrically. The acetone extract (81.23±0.58 %) showed very close BChE inhibitory activity to galantamine. The hexane extract (96.97±0.36 %) of *E. helioscopia* showed higher anti-urease activity than thiourea (96.93±0.17 %) whereas the essential oil (91.37±0.26 %) indicated very close anti-urease activity to the standard.

ARTICLE HISTORY

Received: 13 June 2018
Accepted: 15 September 2018

KEYWORDS

Euphorbia helioscopia,
Essential oil,
Antioxidant activity,
Anticholinesterase activity,
Anti-urease activity

1. INTRODUCTION

Euphorbiaceae family is distributed in all parts of the world except the Antarctic continent with over 5000 taxa. *Euphorbia* species, the most well-known of this family, is represented by approximately 2150 taxa in the world, while there are 109 taxa in our country. Euphorbia species is known as 'Sütleğen' in Turkey and these species is characterized by milk latex tissues. In the folk medicine, *Euphorbia* species are used in the treatment of various diseases such as a migraine, gonorrhea, skin diseases, intestinal parasites and warts cures [1]. Until this time, it is seen that secondary metabolites such as terpenes, flavonoids, tannins, steroids, alkaloids, and lipids are isolated from *Euphorbia* species [2-5]. It has also been reported that

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ISSN-e: 2148-6905 /© IJSM 2018

Euphorbia species have bioactive properties such as anti-inflammatory, antiarthritic, antiviral, antitussives, antitumor, anti-allergy, anti-asthma and antioxidant [1, 6-7].

Alzheimer's disease (AD) is a neurodegenerative disorder with clinical features such as memory loss, speech impairment and visuospatial defect [8]. Lack of acetylcholine, tau protein hyperphosphorylation, β-amyloid (Aβ) aggregation, oxidative stress, and neuroinflammation are promising approaches used in the treatment of Alzheimer's disease [9]. Reactive oxygen species (ROS), the major source of oxidative stress, cause oxidation of proteins and lipids in the brain causing Alzheimer's disease (AD) [10]. The most important approach to the treatment of AD is the inhibition of the acetylcholinesterase enzyme which leads to the breakdown of acetylcholine. Today, many drugs derived from synthetic and natural sources are used in the treatment of AD. Natural drugs are more preferred because of the harmful and toxic effects of synthetic drugs [11]. For this reason, researchers are increasingly interested in researching new sources of natural medicines. Herbal medicines have given promising results among the natural sources due to the existence of volatile oils and secondary metabolites.

When the literature studies are examined, it is seen that researchers related to the essential oil composition and the extracts obtained from plants and their biological activities have gained more importance in recent years. According to our knowledge, there have been a limited number of reports about phytochemical contents and bioactivities of *E. helioscopia* in the literature. Therefore, in this study, we aimed to evaluate chemical composition, antioxidant, anticholinesterase and anti-urease activities of the essential oil and the hexane, acetone, methanol and water extracts of *E. helioscopia* with the total phenolic and flavonoid contents in details.

2. METHOD

2.1. Plant Material

The aerial parts of *E. helioscopia* were collected from Yusufeli, Artvin, Turkey in August, 2016. The voucher specimen has been deposited at the herbarium of Natural Products Laboratory of Muğla Sıtkı Koçman University.

2.2. Instruments and Chemicals

Bioactivity measurements were carried out on a 96-well microplate reader, SpectraMax 340PC^{384} (Molecular Devices, Silicon Valley, CA). The measurements and calculations of the activity results were evaluated by using Softmax PRO v5.2 software (Molecular Devices, Silicon Valley, CA). Chemical composition of the essential oils was performed using GC (Shimadzu GC-17 AAF, V3, 230V series gas chromatography, Japan) and GC/MS (Varian Saturn 2100T, USA).

Pyrocatechol, quercetin, *n*-hexane, methanol, ethanol, ferrous chloride, copper (II) chloride and ethylenediaminetetraacetic acid (EDTA) were purchased from E. Merck (Darmstadt, Germany). Butylatedhydroxyl anisole (BHA), α-tocopherol, β-carotene, polyoxyethylene sorbitan monopalmitate (Tween-40), linoleic acid, Folin–Ciocalteu's reagent (FCR), neocuproine, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2′-azino bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt (Ferene), acetylcholinesterase (AChE) from electric eel (Type-VI-S, EC 3.1.1.7, 425.84 U/mg, Sigma, St. Louis, MO), butyrylcholinesterase (BChE) from horse serum (EC 3.1.1.8, 11.4 U/mg, Sigma, St. Louis, MO),), urease [Type-III from Jack Beans, EC 232-656-0, 20990 U/g solid], 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), galantamine, thiourea, acetylthiocholine iodide, and butyrylthiocholine chloride were purchased from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade.

2.3. Isolation of the Essential Oil

The essential oil of dried aerial parts of *E. helioscopia* was extracted by hydrodistillation in a Clevenger type apparatus for 4 h. The oil was dried over anhydrous sodium sulphate and stored under + 4°C until analyzed.

2.4. Analysis of the Essential Oil

2.4.1. Gas chromatography (GC)

A Flame Ionization Detector (FID) and a DB-5 fused silica capillary non-polar column (30 m×0.25 id., film thickness 0.25 μm) were used for GC analyses. The injector temperature and detector temperature were adjusted 250 and 270°C, respectively. Carrier gas was He at a flow rate of 1.4 mL/min. Sample size was 1.0 μL with a split ratio of 20:1. The initial oven temperature was held at 60°C for 5 min, then increased up to 240°C with 4°C/min increments and held at this temperature for 10 min. The percentage composition of the essential oil was determined with GC solution computer program.

2.4.2. Gas chromatography–mass spectrometry (GC–MS)

An Ion trap MS spectrometer and a DB-5 MS fused silica non-polar capillary column (30 m×0.25 mm ID, film thickness 0.25 μm) were used for the GC–MS analyses. Carrier gas was helium at a flow rate of 1.4 mL/min. The oven temperature was held at 60°C for 5 min, then increased up to 240°C with 4°C/min increments and held at this temperature for 10 min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. Ion source temperature was 200°C. The injection volume was 0.2 μL with a split ratio of 1:20. EI–MS measurements were taken at 70 eV ionization energy. Mass range was from m/z 28 to 650 amu. Scan time 0.5 s with 0.1 inter scan delays. Identification of components of the essential oils was based on GC retention indices and computer matching with the Wiley, NIST-2005 and TRLIB Library as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and whenever possible, by co-injection with authentic compounds [12].

2.5. Extraction

The aerial parts of *E. helioscopia* were extracted separately with different solvents according to their increasing polarity: hexane, acetone, methanol at room temperature for 24 h and four times. Solvents were evaporated on a rotary evaporator to obtain hexane, acetone and methanol extracts. The remaining plant part was allowed to stand for one day with water at 80 °C. The water extract was obtained by lyophilisation using a freeze-drier. All extracts were stored at +4°C until analysis.

2.6. Antioxidant Activity

The total antioxidant activity of the essential oil and extracts was evaluated using β-carotene-linoleic acid test system as previously reported in the literature [13]. Radical scavenging activities were measured by DPPH free and ABTS cation radical scavenging assays [13]. Reducing powers were determined using CUPRAC assays [13]. Metal chelating activity on ferrous ions was determined using the method described in the literature [14].

2.7. Total Phenolic and Flavonoid Content

The phenolic content of extracts was stated as microgram of pyrocatechol equivalents (PEs) [15]. The phenolic contents were calculated according to the following equation that was obtained from standard pyrocatechol graph:

$$\text{Absorbance} = 0.0078\ [\text{pyrocatechol } (\mu g)] + 0.0623\ (r^2, 0.9992)$$

Measurement of flavonoid content of the extracts was based on the aluminum nitrate method and results were expressed as microgram of quercetin equivalents [16]. The flavonoid contents were calculated according to following equation that was obtained from the standard quercetin graph:

Absorbance=0.0412[quercetin (μg)] – 0.0662 (r^2, 0.9998)

2.8. Enzyme Inhibitory Activity

Anticholinesterase and anti-urease activities were measured using Ellman *et al.* [17] and Weatherburn [18] respectively.

2.9. Statistical Analysis

All data on antioxidant, anticholinesterase and anti-urease tests were the average of three parallel sample measurements. Data were recorded as mean ± S.E.M. Significant differences between means were determined by student's test, p values <0.05 were regarded as significant.

3. RESULTS AND DISCUSSION

3.1. Essential Oil Composition

GC-FID and GC/MS techniques were used to determine the chemical composition of the essential oil of *E. helioscopia*. The chemical composition of the essential oil, retention indices and relative percentage (%) of compounds are shown in Table 1. Thirty compounds, representing about 99.9 % of the essential oil of *E. helioscopia* were identified. *β*-cubebene (19.3 %), palmitic acid (12.2 %) and caryophyllene oxide (11.7 %) were identified as major compounds, respectively. Sesquiterpene hydrocarbons (34.8 %) were the most abundant compounds in the essential oil.

There is only one study about the essential oil composition of *E. helioscopia*. In that report, the chemical composition of the essential oil of *E. helioscopia* collected from Greece, was studied by Fokialakis *et al.* [19] and phytol (21.2 %), *β*-caryophyllene (10.0 %) and docosanoic acid methyl ester (8.1 %) were found as major compounds. Season, the geographical location and date of collection cause these differences in the chemical composition of the essential oil [20].

Table 1. Chemical composition of the essential oil of *E. helioscopia*

No	RI[a]	Compounds	Percentage %[b]	Identification methods[c]
1	1144	*Cis-β*-terpineol	0.4	Co-GC, MS, RI
2	1159	Terpinene-4-ol	0.3	Co-GC, MS, RI
3	1178	*α*-Terpineol	0.6	Co-GC, MS, RI
4	1189	Borneol	0.9	Co-GC, MS, RI
5	1290	Thymol	0.2	Co-GC, MS, RI
6	1299	Carvacrol	0.2	Co-GC, MS, RI
7	1359	*β*-Damascenone	0.6	MS, RI
8	1381	*β*-Bourbonene	0.5	Co-GC, MS, RI
9	1394	*β*-Cubebene	19.3	MS, RI
10	1424	*β*-Caryophyllene	1.6	Co-GC, MS, RI
11	1442	*τ*-Elemene	9.3	MS, RI
12	1458	*α*-Farnesene	1.7	MS, RI
13	1478	*τ*-Muurolene	1.6	MS, RI
14	1512	*δ*-Cadinene	0.8	MS, RI

Table 1. Continues

No	RI[a]	Compounds	Percentage %[b]	Identification methods[c]
15	1538	α-Cadinol	2.5	MS, RI
16	1576	Spathulenol	9.3	Co-GC, MS, RI
17	1580	Caryophyllene oxide	11.7	Co-GC, MS, RI
18	1720	Myristic acid	1.9	Co-GC, MS, RI
19	1833	Hexahydrofarnesly acetone	5.3	MS, RI
20	1851	Pentadecanoic acid	0.4	Co-GC, MS, RI
21	1942	Phytol	6.9	MS, RI
22	2001	Palmitic acid	12.2	Co-GC, MS, RI
23	2100	Heneicosane	1.9	MS, RI
24	2108	Linolenic acid	3.6	MS, RI
25	2120	Linoleic acid	1.6	Co-GC, MS, RI
26	2140	Octadecane, 3-ethyl-5-(2-ethyl butyl)	0.3	MS, RI
27	2200	Docasane	0.2	MS, RI
28	2225	Phytol acetate	2.2	MS, RI
29	2300	Tricosane	0.7	MS, RI
30	2400	Tetracosane	1.2	MS, RI
		Oxygenated monoterpenes	2.6	
		Sesquiterpene hydrocarbons	34.8	
		Oxygenated sesquiterpenes	23.5	
		Oxygenated diterpenes	9.1	
		Others	29.9	
		Total identified (%)	99.9	
		Total number of compounds	30	

[a] Retention indices on DB–5 fused silica column. [b] Percentage concentration. [c] Identification methods: Co-I: Co-injection: based on comparison with authentic compounds; MS: based on comparison with WILEY, ADAMS and NIST 08 MS databases; RI: based on comparison of calculated with those reported in ADAMS and NIST 08.

3.2. Total Phenolic and Total Flavonoid Contents

Phenolic and flavonoid compounds are responsible for the bioactive properties of the natural compounds. Phenolic compounds are of great interest to researchers because of their beneficial effects on oxidative stress-related diseases in addition to their antioxidant. Flavonoids scavenge many oxidation molecules such as singlet oxygen and other free radicals. Also, flavonoids prevent the formation of reactive oxygen species [21,22].

The calibration curve of pyrocatechol (0.0078[pyrocatechol (µg)] + 0.0623; r^2, 0.9992) was used to determine the total phenolic content and quercetin (0.0412[quercetin (µg)] – 0.0662; r^2, 0.9998) for the total flavonoid content. Table 2 presents the total phenolic and flavonoid contents of the different extracts of *E. helioscopia*. Among the extracts, water extract has the highest phenolic (161.20±0.98 µg PEs/mg) and flavonoid (11.22±0.05 µg QEs/mg) contents, followed by the methanol extract and the acetone extract. It is well known that phenolic compounds are secondary metabolites responsible for antioxidant activity. The water extract containing the highest amount of total phenolic and flavonoid compounds showed the highest antioxidant activity in all studied tests.

Maoulainine *et al.* [23] studied the total phenolic and flavonoid contents of the methanol and ethanol extracts of *E. helioscopia* and the highest phenolic and flavonoid contents were found in the methanol extracts (51.49±0.012 mg GAE/g dry weight and 11.38±0.004 mg

QE/dry weight, respectively). Total flavonoid contents of *E. helioscopia* collected from four different regions of Chongqing were investigated by Dan *et al.* [24] and contents of total flavonoids were found in the range of 5.485-5.742 mg/g and the results obtained are consistent with the literature. Total phenolic and flavonoid contents of the hexane, acetone and water extracts of *E. helioscopia* were determined for the first time in our study.

Table 2. Total phenolic and total flavonoid contents of the extracts of *E. helioscopia*[a]

		Total phenolic contents µg PEs/mg extracts[b]	Total flavonoid contents µg QEs/mg extract[c]
E. helioscopia	**Hexane**	10.40±0.26	1.01±0.01
	Acetone	44.98±0.58	3.93±0.17
	Methanol	55.17±0.83	5.36±0.52
	Water	161.20±0.98	11.22±0.05

[a] Values expressed are means ± S.E.M. of three parallel measurements. ($p<0.05$)
[b] PEs, pyrocatechol equivalents.
[c] QEs, quercetin equivalents.

3.3. Antioxidant Activity

The essential oil and the hexane, acetone, methanol and water extracts of *E. helioscopia* were screened for their antioxidant activity by using five methods, namely β-carotene-linoleic acid, DPPH free radical scavenging, ABTS cation radical scavenging, cupric-reducing antioxidant capacity (CUPRAC) and metal chelating activity. All of the extracts and the essential oil showed antioxidant activities in a dose-dependent manner. Table 3 shows the IC_{50} values of the extracts and standard compounds (BHA, α-tocopherol, and EDTA).

Table 3. Antioxidant activity of the essential oil and extracts of *E. helioscopia* by β-Carotene-linoleic acid, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, CUPRAC and metal chelating assays[a]

		Antioxidant Activity				
		β-Carotene-linoleic acid assay	**$DPPH^{\bullet}$ assay**	**$ABTS^{\bullet+}$ assay**	**CUPRAC assay**	**Metal chelating assay**
		IC_{50} (µg /mL)	**IC_{50} (µg /mL)**	**IC_{50} (µg/mL)**	**$A_{0.50}$ (µg/mL)[b]**	**Inhibition (%)[c]**
E. helioscopia	**Essential oil**	113.79±0.55	2.81±0.75[c]	21.45±0.43[c]	512.11±0.72	-[e]
	Hexane extract	579.81±0.71	4.57±0.97[c]	17.45±0.68[c]	295.23±0.89	29.40±0.27
	Acetone extract	2.33±0.16	90.18±0.22	17.15±0.19	53.03±0.49	19.85±0.31
	Methanol extract	1.14±0.21	16.43±0.67	15.10±0.82	18.20±0.35	13.51±0.12
	Water extract	1.08±0.39	15.57±0.48	1.89±0.33	12.50±0.11	79.31±0.51
Standards	**α-Tocophero**	2.10±0.08	37.20±0.41	38.51±0.54	66.72±0.81	*NT*[d]
	BHA	1.34±0.04	19.80±0.36	11.82±0.09	24.40±0.69	*NT*[d]
	EDTA	*NT*[d]	*NT*[d]	*NT*[d]	*NT*[d]	94.7±0.6

[a:] IC_{50} values represent the means ± SEM of three parallel measurements ($p< 0.05$).
[b]: $A_{0.50}$ values represent the means ± SEM of three parallel measurements ($p< 0.05$).
[c]: % inhibition of 200 µg/mL concentration of the essential oil and extracts of *Euphorbia helioscopia*.
[d]: NT: not tested.
[e]:- Not active.

In all studied methods, the highest antioxidant activity was found in the water extract and followed by the methanol extract. The water extract was found to be more active than BHA and α-tocopherol which used as standards in β-carotene-linoleic acid, $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and

CUPRAC assays with IC_{50}: 1.08±0.39, 15.57±0.48, 1.89±0.33 and 12.50±0.11 µg/mL values, respectively. The methanol extract exhibited higher antioxidant activity than BHA and α-tocopherol in β-carotene-linoleic acid, DPPH$^{\bullet}$ and CUPRAC assays with IC_{50}: 1.14±0.21, 16.43±0.67 and 18.20±0.35 µg/mL, respectively. Also, in ABTS$^{\bullet+}$ assay, the methanol extract (IC_{50}: 15.10±0.82 µg/mL) showed higher antioxidant activity than α-tocopherol (IC_{50}: 38.51±0.54 µg/mL).

In previous studies, antioxidant activity of the dichloromethane and methanol extracts of *E. helioscopia* were evaluated by thin layer chromatography (TLC) autographic assay method, using DPPH$^{\bullet}$ as spray reagent. At 100 µg concentration, when methanol extract appeared as a yellow spot against purple background, dichloromethane extract did not respond to DPPH$^{\bullet}$ [1]. In a report of Rauf *et al.* [25], DPPH$^{\bullet}$ scavenging effects of various extracts of *E. helioscopia* were determined and activity was decreased in order of methanol>ethyl acetate>ethanol>chloroform>hexane. In another study, antioxidant activity of aqueous, ethanol, petroleum ether, chloroform and methanol extracts of leaves, stem parts, and also latex of *E. helioscopia* were evaluated by using DPPH, TAC, FRAP, FTC and BCL assays. The highest antioxidant activity was found in latex and followed by the methanol extract of leaves in all the methods. The other extracts also showed significant antioxidant activity [26]. Antioxidant properties of the methanol and ethanol extracts of *E. helioscopia* leaves, flowers and stem were examined by using the DPPH$^{\bullet}$ scavenging assay by Maoulainine *et al.* [23]. The highest radical scavenging effect was observed in flowers methanol extract with IC_{50} value of 26.66±0.000 µg/mL. The results obtained in our study are similar to these findings.

When literature findings are examined, it is seen that DPPH$^{\bullet}$ scavenging assay was generally used for testing to antioxidant properties of *E. helioscopia*. In our study, antioxidant activities of the essential oil and the hexane, acetone, methanol and water extracts of *E. helioscopia* were evaluated by using β-carotene-linoleic acid, ABTS cation radical scavenging, cupric-reducing antioxidant capacity and metal chelating activity assays for the first time.

3.4. Anticholinesterase Activity

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of the essential oil and the extracts of *E. helioscopia* compared with that of galantamine were given in Table 4. The inhibitory activity of the extracts was divided into three separate classes; potent (>50 %), moderate (30-50 %), inactive or low (<30 %) activity [27]. According to this classification, when the hexane extract (47.49±0.19 %) of *E. helioscopia* showed moderate inhibitory activity against AChE, all other extracts showed low inhibitory activity against AChE. All extracts except methanol and water extracts of *E. helioscopia* were found to be potent inhibitors against BChE. Moreover, the acetone extract (81.23±0.58 %) of *E. helioscopia* exhibited very close BChE inhibitory activity to galantamine (82.23±0.67 %).

In previous studies, anticholinesterase activities of different *Euphorbia* species were investigated. Pisano *et al*. [28] evaluated cholinesterase inhibitory activities of aqueous and alcoholic extracts from leaves, stems, and flowers of *E. characias* and ethanol leaves extract showed fifty times more activity than galantamine. Cholinesterase inhibitory activities of the methanolic extracts of *E. denticulata* (flowers, leaf, stem, and mix of aerial parts) were studied by Zengin *et al.* [29] and the extracts were reported as medium-low activator for AChE and BChE. In a different study of *Euphorbia* species, *E. hebecarpa* is tested for AChE inhibitory activity and was inactive [30]. Cholinesterase inhibitory activities of the essential oil and hexane, acetone, methanol and water extracts of *E. helioscopia* were studied for the first time in this report.

Table 4. Anticholinesterase and anti-urease inhibitory activities of the essential oil and the extracts of *E. helioscopia*[a]

		Anticholinesterase Activity		Anti-urease Activity
		AChE assay	BChE assay	
E. helioscopia	**Essential oil**	18.68±0.16	50.85±0.67	91.37±0.26
	Hexane extract	47.49±0.19	54.32±0.92	96.97±0.36
	Acetone extract	8.74±0.14	81.23±0.58	54.17±0.92
	Methanol extract	6.97±0.07	20.88±0.15	70.94±0.32
	Water extract	0.67±0.02	9.87±0.31	19.32±0.18
Standards	**Galantamine**	80.41±0.98	82.23±0.67	*NT*[b]
	Thiourea	*NT*[b]	*NT*[b]	96.93±0.17

[a] : Inhibition % of 200 µg/mL concentration of the essential oil and the extracts of *E. helioscopia*
[b] : NT: not tested.

3.5. Anti-urease Activity

Since urease inhibitors can be used as potential drugs in the treatment of ulcer diseases, the discovery of new inhibitors is gaining in importance. Table 4 shows anti-urease activity by inhibition (%) at 200 µg/mL concentration. The hexane extract (96.97±0.36 %) of *E. helioscopia* showed higher anti-urease activity than thiourea (96.93±0.17 %) used as a standard. In addition, the essential oil (91.37±0.26 %) exhibited very close anti-urease activity to the standard.

Natural compounds isolated from *Euphorbia* species exhibiting anti-urease activity are reported in the literature. Ahmad *et al.* [31,32] and Lodhi *et al.* [33] isolated five diterpenes esters from *E. decipiens* inhibited urease enzyme and the first natural urease inhibitor was discovered by Ahmad *et al.* [31]. In a different study, the methanolic bark fraction of *E. umbellata* was reported to exhibit 78.6 % inhibition at 1024 µg/mL concentration [34]. In our study, the essential oil and all the extracts except the water extract were highly active, and these results are similar to previous studies on other *Euphorbia* species.

4. CONCLUSION

In this report, antioxidant, anticholinesterase and anti-urease activities of the essential oil and various extracts of *E. helioscopia* were determined with the total phenolic and flavonoid contents. Also, chemical composition of the essential oil was analyzed by GC and GC/MS and totally 30 compounds were determined. The water extract with the highest amount of total phenolic and flavonoid contents exhibited the highest antioxidant activities in all assays. Furthermore, the acetone extract exhibited very close BChE inhibitory activity to galantamine. In anti-urease activity test, when the hexane extract showed higher activity than thiourea, the essential oil exhibited very close activity to the standard. Consequently, *E. helioscopia* could become a source of potential bioactive compounds in pharmaceutical and food industries with strong enzyme inhibitory and antioxidant activities. However, further studies are needed for discovering of new natural bioactive compounds from these species.

Conflict of interest

The authors declare that they have no conflict of interest.

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