International Journal of Secondary Metabolite

IJSM
International Journal of Secondary Metabolite

e-ISSN: 2148-6905

http://dergipark.org.tr/en/pub/ijsm

IJSM

Volume: 7
Number: 4
2020

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

e-ISSN: 2148-6905

IJSM
International Journal of
Secondary Metabolite

Volume 7, Issue 4, 2020

Prof. Dr. Ramazan MAMMADOV
Editor
International Journal of Secondary Metabolite
&
Molecular Biology and Genetics,
Faculty of Science, Mugla Sıtkı Koçman University,
Mugla, Turkey

Phone : +90 252 211 1504
E-mail : ijsm.editor@gmail.com rmammad@yahoo.com

Founding Editör & Publisher : İzzet KARA
Frequency : 4 issues per year (March, June, September, December)
Online ISSN : 2148-6905
Website : https://dergipark.org.tr/en/pub/ijsm

Support Contact

Prof. Dr. İzzet KARA
Journal Manager & Founding Editor

Phone : +90 258 296 1036
Fax : +90 258 296 1200
E-mail : ikara@pau.edu.tr

hosted by Turkish JournalPark ACADEMIC

International Journal of Secondary Metabolite

Scope of International Journal of Secondary Metabolite is published 4 issues per year (starting from June 2018) and accepts English language manuscripts covering all areas of plant biology (medical aromatic plants. plant physiology, biochemistry, plant chemistry, allelopathy, plant hormones, secondary metabolites, plant biotechnology, antioxidant). International Journal of Secondary Metabolite welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Authors are required to frame their research questions and discuss their results in terms of major questions in plant biology. In general, papers that are too narrowly focused, purely descriptive, or broad surveys, or that contain only preliminary data or natural history, will not be considered. Contribution is open to researchers of all nationalities. The following types of article will be considered:

1. Research articles: Original research in various fields of botany will be evaluated as research articles.

2. Research notes: These include articles such as preliminary notes on a study or manuscripts on a plant physiology and new records.

3. Reviews: Reviews of recent developments, improvements, discoveries, and ideas in various fields of plant biology will be requested by the editor or advisory board.

4. Letters to the editor: These include opinions, comments relating to the publishing policy of the International Journal of Secondary Metabolite, news, and suggestions. Letters are not to exceed one journal page.

There is no submission or publication process charges for articles in IJSM.

IJSM is indexed in:

• TR Dizin
• Index Copernicus International
• MIAR
• DOAJ,
• EBSCO,
• SIS (Scientific Index Service) Database,
• JournalTOCs
• Google Scholar,
• idealonline
• ResearchBib
• CrossRef
• CAB Abstracts

Table of Contents

International Journal of Secondary Metabolite
2020, Vol. 7, No. 4, 229–236

https://doi.org/10.21448/ijsm.676985

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Antibacterial activity and essential oil composition of *Calendula arvensis* L.

Huseyin Servi[1,*], Cansu Vatansever[2], Ahmet Dogan[3], Vanya Ari Majeed[4],

[1]Department of Pharmaceutical Botany, Faculty of Pharmacy, Altınbaş University, Istanbul, Turkey
[2]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Altınbaş University, Istanbul, Turkey
[3]Department of Pharmaceutical Botany, Faculty of Pharmacy, Marmara University, Istanbul, Turkey
[4]School of Pharmacy, Faculty of Pharmacy, Altınbaş University, Istanbul, Turkey

Abstract: Essential oil composition and antibacterial activity of *Calendula arvensis* L. were investigated. The essential oil of aerial part was obtained through hydro-distillation using a Clevenger type apparatus with 0.38 (v/w) yield. The essential oil components were determined by GC-MS analyses. Thirty-six components were identified in the essential oil that represented 91.8 % of the oil. The major components of the essential oil were δ-cadinene (14.8 %), *epi*-cubebol (10.7 %), α-cadinol (8.5 %), cubenol (7.7 %), cubebol (7.2 %), 1-*epi*-cubenol (5.4 %) and ledene (5.1 %). Antibacterial activity of essential oil was observed against *Staphylococcus aureus* ATCC 29213, *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 by using a broth microdilution. The essential oil showed weak inhibitory activity against *E. coli* and *B. cereus* at 8 mg/mL. The oil didn't show any antibacterial activity against *S. aureus* and *P. aeruginosa*. These results revealed that the oil was rich in oxygenated sesquiterpenes as well as had a weak antimicrobial activity.

ARTICLE HISTORY

Received: January 20, 2020
Revised: March 09, 2020
Accepted: August 09, 2020

KEYWORDS

Calendula arvensis,
Essential oil,
Antibacterial activity,
GC-MS

1. INTRODUCTION

Calendula L. is a member of Calenduleae tribe of Asteraceae family. *Calendula* L. genus is illustrated in Turkey by three species which are *C. arvensis* M.Bieb., *C. officinalis* L., and *C. suffruticosa* Vahl [1]. *Calendula* species are used as an antipyretic and anti-inflammatory remedy in Italian folk medicine [2]. In European folk medicine, *Calendula* species are considered as an immune tonic that aids in prevention of sickness in winter [3]. *C. arvensis* is known by its local name as "Portakal nergisi" in Turkey and is used externally to treat varicose veins, eczema, fungus, warts and wounds [4]. *C. arvensis* has sedative, antibacterial, analgesic, lymphagogue, demulcent, choleretic, vulnerary anti-tumor, mild anodyne anti-inflammatory, antioxidants, anti-parasitic, antiviral, and antiseptic activities [5]. *C. officinalis* has immunostimulant, hepatoprotective, anti-HIV, antitumor, anti-inflammatory, spasmogenic and spasmolytic effects [6]. *C. suffruticosa* has mostly antioxidant and antifungal activity, especially its ethanolic extract [7]. Previously, the essential oils and hydrosol extract from aerial parts of *C. arvensis* L. were examined by using GC-FID and GC/MS. The main compounds were zingiberenol 1 (8.7-29.8%), eremoligenol (4.2-12.5%), β-curcumene (2.1-12.5%),

CONTACT: Hüseyin Servi ✉ servi@hotmail.com.tr Department of Pharmaceutical Botany, Faculty of Pharmacy, Altınbaş University, Istanbul, Turkey

ISSN-e: 2148-6905 /© IJSM 2020

zingiberenol 2 (4.6-19.8%) and (*E*, *Z*)-farnesol (3.5-23.4%). The antioxidant activity of essential oil and extract was checked by using DPPH, FRAP and β-carotene methods. The hydrosol extract showed high antioxidant activity. The antifungal activity of the essential oil and hydrosol extract was studied against *Penicillium expansum* and *Aspergillus niger*. The extract showed the highest inhibitory activity against *P. expansum* and *A. niger* [8]. The essential oil of aerial parts of *C. arvensis* L. was studied by GC-FID and GC-MS methods. The essential oil was obtained by hydrodistillation (HD) and microwave distillation (MD). Sesquiterpenes (HD: 30.5 % and MD: 23.4 %) and monoterpene compounds (HD: 26.3 % and MD: 24.3 %) were reported as the major groups. The main compounds were α-selinene (HD: 16.0 % and MD: 0.0 %), α-pinene (HD: 11.9 % and MD: 12.3 %), (*Z*)-α-santalol (HD: 8.2 % and MD: 7.4 %), λ-amorphene (HD: 0.0 % and MD: 8.0 %), (*Z*)-sesquilavandulol (HD: 4.8 % and MD: 0.0 %), 7-*epi*-silphiperfol-5-ene (HD: 2.6 % and MD: 3.7 %), viridiflorene (HD: 2.5 % and MD: 1.7 %) and β-pinene (HD: 1.8 % and MD: 2.4 %). The antimicrobial activities of the essential oils, hexane, ether and methanolic extracts of the *C. arvensis* L. were studied. The essential oil (HD) and methanolic extract had tolerable antibacterial activity against *Staphylococcus aureus* and *Bacillus cereus*. All the extracts were reported to have good antituberculosis activity against *Mycobacterium smegmatis* [9].

The first purpose of this research was to obtain essential oil from aerial parts of *C. arvensis* by using the hydrodistillation method as well as to determine antibacterial activity. And the second purpose was to determine the variation in the volatile oil composition of *C. arvensis* and to show that essential oil differences are related to geographical regions.

2. MATERIAL and METHODS

2.1. Plant Material

Plant material (natural) was collected in flowering periods from Istanbul (İkitelli-Başakşehir) province of Turkey in April 2017. Voucher specimen was deposited in the Herbarium of Marmara University (Voucher no: MARE20229), Turkey.

2.2. Isolation of the Essential Oil

The volatile oil of *C. arvensis* aerial parts (436 g) was obtained by Clevenger apparatus (3 h) with the hydrodistillation method. *C. arvensis* aerial parts produced 0.38% (v/w) essential oil yields. The oil was hold in amber vials under -20°C until analyzed.

2.3. Gas Chromatography/Mass Spectrometry Analysis

The GC-MS analysis was employed with an Agilent 5975C Inert XL EI/CI MSD system in EI mode. Essential oil of aerial part was kept in *n*-hexane was injected (1 µL) in split mode. The temperatures of the injector and MS transfer line were adjusted at 250˚C. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) and helium as carrier gas (1 mL/min) were utilized in GC/MS analyses. The temperature of oven was adjusted to 60˚C for 10 min. and increased to 220˚C at a rate of 4˚C/min. The temperature kept stable at 220˚C for 10 min. and then increased to 240˚C at a rate of 1˚C/min. Mass spectra were saved at 70 eV with the mass range *m*/*z* 35 to 425. The relative percentage quantities of the separated compounds were calculated from integration of the peaks in MS chromatograms (given in Figure 1). The analysis was realized in triplicate.

2.4. Identification of Essential Oil Components

The determination of volatile oil compounds was realized by comparison with their relative retention indices got by a series of *n*-alkanes (C5 to C30) to the literature [10-23] (given in Table 1 and Figure 1) and with mass spectra comparison to the in-house libraries (Wiley W9N11, NIST11).

2.5. Antibacterial Assay

Antibacterial activity of the essential oil was studied against *Staphylococcus aureus* ATCC 29213, *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922. The minimum inhibitory concentration (MIC) values were determined for the oil, on each organism by using microplate dilution method [24]. Stock solution of the oil (16 mg/mL) was prepared with %10 dimethyl sulfoxide (DMSO). Serial dilution of essential oil was done on 96-well microplates with using Mueller Hinton Broth (MHB). Bacteria were standardized in MHB according to McFarland No:0.5. Bacterial cultures were mixed with different concentrations of essential oils on microplates and were incubated 24 h at 37ºC. Minimum inhibitory concentrations (MIC: mg/mL) were detected at the minimum concentration where bacterial growth was not detected. All the experiments were performed in duplicate.

3. RESULTS and DISCUSSION

The essential composition from aerial parts of *C. arvensis* were analyzed by GC-MS. Thirty six compounds were identified comprising 91.8 ± 0.1% of the oil. The main compounds of the oil were δ-cadinene (14.8 ± 0.1%), *epi*-cubebol (10.7 ± 0.0%), α-cadinol (8.5 ± 0.1%), cubenol (7.7 ± 0.0%), cubebol (7.2 ± 0.0%), 1-*epi*-cubenol (5.4 ± 0.0%) and ledene (5.1 ± 0.0%). The essential oil composition is given in Table 1. The antibacterial activity of oil was studied against two Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*) and two Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) by using a broth microdilution assay. The essential oil showed weak inhibitory activity against *E. coli*, and *B. cereus* at 8 mg/mL. The essential oil did not show any antibacterial activity against *S. aureus* and *P. aeruginosa* (given in Table 2). Also, it has been suggested in previous studies that δ-cadinene, *epi*-cubebol, α-cadinol, cubenol, cubebol, 1-*epi*-cubenol and ledene have antibacterial activity [25-32]. Along with these compounds in the essential oils of *C. arvensis* may have contributed to the activity of the oil.

Zingiberenol 1 (8.7-29.8%), eremoligenol (4.2-12.5%), β-curcumene (2.1-12.5%), zingiberenol 2 (4.6-19.8%) and (*E*,*Z*)-farnesol (3.5-23.4%) were detected in higher quantity in the essential oil of *Calendula arvensis* from Algeria [8] while zingiberenol contained at a low amount in the current study. And other main compounds were not detected in the essential oil of current study.

According to a study from Trabzon, *C. arvensis* was reported to contain α-selinene, α-pinene, (*Z*)-sesquilavandulol, 7-*epi*-silphiperfol-5-ene, viridiflorene, and β-pinene as main compounds [9]. But these compounds did not detect in the essential oil of this study except for α-pinene (0.2%). The oil from Trabzon had monoterpene and sesquiterpene as major groups. In the present study, the oil from Istanbul has oxygenated sesquiterpenes as a dominant group and showed a dissimilar chemical profile from the previous study. The difference in the composition of both oils may be correlated with the geographical region, collection time and specific climate conditions. The oil from Trabzon showed moderate antibacterial activity against *S. aureus* and *B. cereus* but in the current study, the oil did not show activity against *S. aureus*. Both oil samples had activity against *B. cereus* at different MIC values. The antibacterial activity differences in previous research and present data could be related to different main compounds of oils.

Another study from Corsica-France had common main compounds with the current study. The main compounds of the oil from France were δ-cadinene (15.1%) and α-cadinol (12.4%) [33]. The similarities were observed between essential oil constituents of Corsica-France and Istanbul-Turkey plants. But there are quantitative dissimilarities in main compounds of both essential oils.

Table 1. Essential oil composition from aerial parts of *Calendula arvensis* L.

No	R.T. min.[1]	RRI[2]	RRI Lit.[3]	Compound	I [4] (%)	II (%)	III (%)	Average[5] (%)	SD[6]	Id.Met.[7]
1	8.67	1020	1032	α-Pinene	0.1	0.2	0.2	0.2	0.1	RI,MS
2	27.332	1461	1466	α-Cubebene	0.3	0.3	0.3	0.3	0.0	RI,MS
3	28.304	1488	1482	α-Longifolene	0.2	0.2	0.2	0.2	0.0	RI,MS
4	28.58	1496	1497	α-Copaene	0.5	0.5	0.5	0.5	0.0	RI,MS
5	29.906	1536	1544	α-Gurjenene	0.1	0.1	0.1	0.1	0.0	RI,MS
6	30.165	1544	1547	β-Cubenene	0.4	0.4	0.4	0.4	0.0	RI,MS
7	32.169	1605	1612	β-Caryophyllene	0.3	0.3	0.3	0.3	0.0	RI,MS
8	34.09	1669	1677	*epi*-Zonarene	1.3	1.2	1.3	1.3	0.1	RI,MS
9	34.448	1680	1687	α-Caryophyllene	0.5	0.5	0.5	0.5	0.0	RI,MS
10	35.195	1705	1707	Ledene	5.1	5.1	5.1	5.1	0.0	RI,MS
11	35.623	1720	1726	Bicyclosesquiphallendrene	0.2	0.2	0.2	0.2	0.0	RI,MS
12	35.793	1726	1726	Germacrene D	1.4	1.4	1.4	1.4	0.0	RI,MS
13	35.989	1733	1740	α-Muurolene	1.2	1.2	1.2	1.2	0.0	RI,MS
14	36.976	1768	1772	δ-Cadinene	14.9	14.8	14.8	14.8	0.1	RI,MS
15	37.261	1778	1783	β-Sesquiphallendrene	0.1	0.1	0.1	0.1	0.0	RI,MS
16	37.707	1793	1799	Cadina-1,4-diene	1.0	1.0	1.0	1.0	0.0	RI,MS
17	39.13	1846	1849	Calamenene	0.4	0.4	0.4	0.4	0.0	RI,MS
18	40.486	1896	1900	*epi*-Cubebol	10.7	10.7	10.7	10.7	0.0	RI,MS
19	41.275	1927	1955	Neophytodiene isomer	3.2	3.2	3.2	3.2	0.0	RI,MS
20	41.703	1943	1953	Palustrol	0.3	0.3	0.3	0.3	0.0	RI,MS
21	41.837	1949	1957	Cubebol	7.2	7.2	7.2	7.2	0.0	RI,MS
22	44.261	2045	2057	Ledol	3.0	3.0	3.0	3.0	0.0	RI,MS
23	44.821	2068	2080	Cubenol	7.7	7.7	7.7	7.7	0.0	RI,MS
24	44.962	2074	2080	1,10-di-*epi*-cubenol	3.1	3.1	3.1	3.1	0.0	RI,MS
25	45.114	2080	2088	1-*epi*-cubenol	5.4	5.4	5.4	5.4	0.0	RI,MS
26	45.333	2089	2092	β-Oplopenene	0.2	0.2	0.2	0.2	0.0	RI,MS
27	45.56	2098	2100	Viridiflorol	3.1	3.2	3.1	3.1	0.0	RI,MS
28	46.074	2120	2096	Sesquisabinene hydrate	2.8	2.9	2.8	2.8	0.1	RI,MS
29	46.305	2130	2131	Hexahydrofarnesyl acetone	0.4	0.4	0.4	0.4	0.0	RI,MS
30	46.462	2137	2135	Spathulenol	0.3	0.3	0.3	0.3	0.0	RI,MS
31	47.629	2187	2191	Zingiberenol	1.8	1.8	1.8	1.8	0.0	RI,MS
32	47.921	2199	2192	τ-Cadinol	2.6	2.6	2.6	2.6	0.0	RI,MS
33	48.169	2210	2219	δ-Cadinol	1.0	1.0	1.0	1.0	0.0	RI,MS
34	48.495	2225	2232	α-Bisabolol	0.4	0.4	0.4	0.4	0.0	RI,MS
35	48.975	2246	2255	α-Cadinol	8.4	8.5	8.5	8.5	0.1	RI,MS
36	59.374	2613	2622	Phytol	1.9	1.9	1.9	1.9	0.0	RI,MS
				Total	91.8	91.9	91.8	91.8	0.1	

[1]R.T: Retention time; [2]RRI: Relative retention time; [3]RRI Lit.: Relative retention time in the literature; [4]The analysis results; [5,6]The average % area of analysis with ± standard deviation (SD) (*n*=3); [7]Identification method.

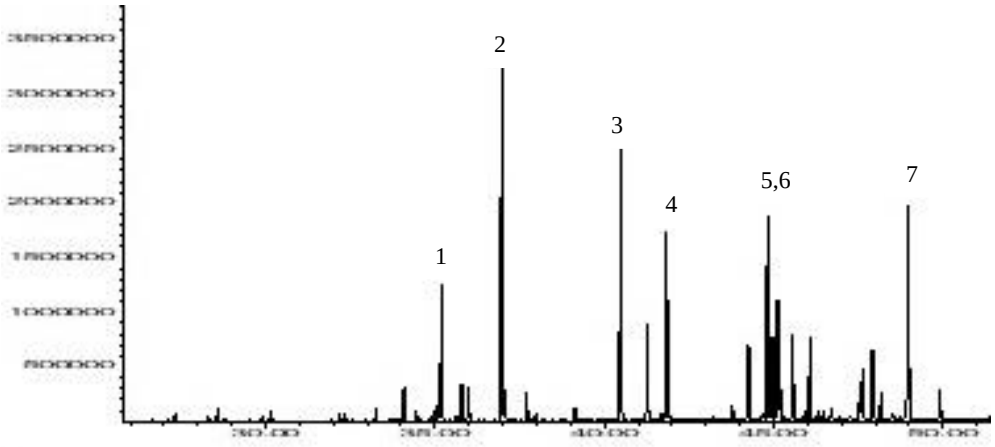

Figure 1. GC-MS Chromatogram of *Calendula arvensis* aerial parts essential oil. (**1:**Ledene; **2:**δ-Cadinene; **3:***epi*-Cubebol; **4:**Cubebol; **5:**Cubenol; **6:**1-*epi*-cubenol; **7:**α-Cadinol)

Table 2. Essential oil composition from aerial parts of *Calendula arvensis* L.

Bacteria	Essential oil (mg/mL)
Staphylococcus aureus	Not sensitive
Bacillus cereus	8 mg/mL
Escherichia coli	8 mg/mL
Pseudomonas aeruginosa	Not sensitive

4. CONCLUSION

The present study indicated that *C. arvensis* oil was rich in oxygenated sesquiterpenes and had antibacterial activity against *E. coli* and *B. cereus*. The oil did not show activity against *S. aureus* and *P. aeruginosa*. This result indicates a higher concentration of the oil is required to inhibit the growth of these bacteria. The essential oil of the current work is different from previous researches as quantitative and qualitative composition. The variations of essential oil ingredients and composition may be correlated with environmental factors such as temperature, humidity, and photoperiod. The quantitative composition of the essential oils can be related to plant age and harvesting time. Further studies on essential oil of *C. arvensis* are required to isolate main compounds of oil that are responsible for its antibacterial activities.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Orcid

Hüseyin Servi https://orcid.org/0000-0002-4683-855X

5. REFERENCES

[1]. Davis, P.H., Mill, R.R, Tan, K., Edmondson. J.R. (1982). Flora of Turkey and the East Aegean Islands. Edinburgh, Scotland: University Press.

[2]. De Tommasi, N., Conti, C., Stein, M.L., Pizza, C. (1991). Structure and in vitro antiviral activity of triterpenoid saponins from *Calendula arvensis*. *Planta Med*., *57*, 250-253. https://doi.org/10.1055/s-2006-960084

[3]. Lavagna, S.M., Secci, D., Chimenti, P., Bonsignore, L., Ottaviani, A., Bizzarri, B. (2001). Efficacy of *Hypericum* and *Calendula* oils in the epithelial reconstruction of surgical wounds in childbirth with caesarean section. *Il Farmaco*, *56*, 451-453. https://doi.org/10.1016/S0014-827X(01)01060-6

[4]. Tuzlacı, E., (2016). Türkiye Bitkileri Geleneksel İlaç Rehberi, İstanbul Tıp Kitabevi.

[5]. Abudunia, A.M., Marmouzi, I., Faouzi, M.E.A., Ramli, Y., Taoufik, J., El Madani, N., Ibrahimi, A. (2017). Anticandidal, antibacterial, cytotoxic and antioxidant activities of *Calendula arvensis* flowers.*J Mycol Med*., *27*, 90-97. https://doi.org/10.1016/j.mycmed.2016.11.002

[6]. Khalid, K.A., Da Silva, J.T. (2012). Biology of *Calendula officinalis* Linn.: focus on pharmacology, biological activities and agronomic practices. *Medicinal and Aromatic Plant Science and Biotechnology*. *6*, 12-27.

[7]. Ismahene, S., Ratiba, S., Miguel, C.M.D., Nuria, C. (2018). Phytochemical Composition and Evaluation of the Antioxidant Activity of the Ethanolic Extract of *Calendula suffruticosa* subsp. *suffruticosa* Vahl. *Pharmacogn J*., *10*, 64-70. https://doi.org/10.5530/pj.2018.1.13

[8]. Belabbes, R., Dib, M.E.A., Djabou, N., Ilias, F., Tabti, B., Costa, J., Muselli, A. (2017). Chemical variability, antioxidant and antifungal activities of essential oils and hydrosol extract of *Calendula arvensis* L. from western Algeria. *Chem Biodivers*., *14*, 1-14. doi: https://doi.org/10.1002/cbdv.201600482

[9]. Tosun, G., Yayli, B., Arslan, T., Yasar, A., Karaoglu, S. A., Yayli, N. (2012). Comparative essential oil analysis of *Calendula arvensis* L. Extracted by hydrodistillation and microwave distillation and antimicrobial activities. *Asian J Chem*., *24*, 1955-1958.

[10]. Başer, K.C., Özek, T., Demirci, B., Kürkçüoğlu, M., Aytaç, Z., Duman, H. (2000). Composition of the essential oils of *Zosima absinthifolia* (Vent.) Link and *Ferula elaeochytris* Korovin from Turkey. *Flavour Frag J*., *15*, 371-372. https://doi.org/10.1002/1099-1026(200011/12)15:6<371::AID-FFJ919>3.0.CO;2-Z

[11]. Kirimer, N., Tabanca, N., Özek, T., Tümen, G., Baser, K.H.C. (2000). Essential oils of annual *Sideritis* species growing in Turkey. *Pharm Biol*., *38*, 106-111. https://doi.org/10.1076/1388-0209(200004)3821-1FT106

[12]. Gören, N., Demirci, B., Başer, K.H.C. (2001). Composition of the essential oils of *Tanacetum* spp. from Turkey. *Flavour Frag J*., 16, 191-194. https://doi.org/10.1002/ffj.976

[13]. Başer, K.H.C., Demirci, B., Tabanca, N., Özek, T., Gören, N. (2001). Composition of the essential oils of *Tanacetum armenum* (DC.) Schultz Bip., *T. balsamita* L., *T. chiliophyllum* (Fisch. & Mey.) Schultz Bip. var. *chiliophyllum* and *T. haradjani* (Rech. fil.) Grierson and the enantiomeric distribution of camphor and carvone. *Flavour Frag J*., *16*, 195-200. https://doi.org/10.1002/ffj.977

[14]. Demirci, B., Baser, K.H.C., Dadandi, M.Y., (2006). Composition of the Essential Oils of *Phlomis rigida* Labill. and *P. samia* L. *J Essent Oil Res*., *18*, 328-331. https://doi.org/10.1080/10412905.2006.9699103

[15]. Kıvçak, B., Akay, S., Demirci, B., Başer, K. (2004). Chemical composition of essential oils from leaves and twigs of *Pistacia lentiscus*, *Pistacia lentiscus* var. *chia*, and *Pistacia terebinthus* from Turkey. *Pharm Biol*., 42, 360-366. https://doi.org/10.1080/13880200490519677

[16]. Ahmed, A., Choudhary, M.I., Farooq, A., Demirci, B., Demirci, F., Can Başer, K.H. (2000). Essential oil constituents of the spice *Cinnamomum tamala* (Ham.) Nees & Eberm. *Flavour Frag J., 15,* 388-390. https://doi.org/10.1002/1099-1026(200011/12)15:6<388::AID-FFJ928>3.0.CO;2-F

[17]. Gürbüz, İ., Demirci, B., Franz, G., Başer, K.H.C., Yeşilada, E., Demirci, F. (2013). Comparison of the volatiles of *Daphne pontica* L. and *D. oleoides* Schreber subsp. *oleoides* isolated by hydro-and microdistillation methods. *Turk J Biol*., 37, 114-121. https://doi.org/10.3906/biy-1204-20

[18]. Demirci, B., Hüsnü Can Başer, K., Yıldız, B., Bahçecioğlu, Z. (2003). Composition of the essential oils of six endemic *Salvia* spp. from Turkey. *Flavour Frag J., 18,* 116-121. https://doi.org/10.1002/ffj.1173

[19]. Viljoen, A.M., Demirci, B., Başer, K.H.C., Potgieter, C.J., Edwards, T.J. (2006). Microdistillation and essential oil chemistry-a useful tool for detecting hybridisation in *Plectranthus* (Lamiaceae). *S Afr J Bot*., *72,* 99-104. https://doi.org/10.1016/j.sajb.2005.05.003

[20]. Polatoğlu, K., Arsal, S., Demirci, B., Başer, K.H.C. (2015). DPPH scavenging, PRAP activities and essential oil composition of edible *Lathyrus ochrus* L.(Cyprus Vetch, Luvana) from Cyprus. *J Oleo Sci*., *64,* 309-314. https://doi.org/10.5650/jos.ess14173

[21]. Demirci, F., Demirci, B., Gürbüz, İ., Yeşilada, E., Başer, K.H.C. (2009). Characterization and biological activity of *Achillea teretifolia* Willd. and *A. nobilis* L. subsp. *neilreichii* (Kerner) Formanek essential oils. *Turk J Biol*., *33,* 129-136. https://doi.org/10.3906/biy-0808-1

[22]. Polatoglu, K., Demirci, F., Demirci, B., Gören, N., Baser, K.H.C. (2010). Essential oil composition and antibacterial activity of *Tanacetum argenteum* (Lam.) willd. ssp. *argenteum* and *T. densum* (Lab.) Schultz Bip. ssp. *amani* Heywood from Turkey. *J Oleo Sci*., *59,* 361-367. https://doi.org/10.5650/jos.59.361

[23]. Tümen, G., Başer, K.H.C., Demirci, B., & Ermin, N. (1998). The essential oils of *Satureja coerulea* Janka and *Thymus aznavourii* Velen. *Flavour Frag J., 13,* 65-67. https://doi.org/10.1002/(SICI)1099-1026(199801/02)13:1<65::AID-FFJ695>3.0.CO;2-Q

[24]. İşcan, G., Kirimer, N., Kürkcüoğlu, M., Başer, H.C., Demirci, F. (2002). Antimicrobial screening of *Mentha piperita* essential oils. *J Agr Food Chem*., *50,* 3943-3946. https://doi.org/10.1021/jf011476k

[25]. Yonzon, M., Lee, D.J., Yokochi, T., Kawano, Y., Nakahara, T. (2005). Antimicrobial activities of essential oils of *Nepal*. *J Essent Oil Res*., *17*(1), 107-111. https://doi.org/10.1080/10412905.2005.9698846

[26]. John, A.J., Karunakaran, V.P., George, V., Pradeep, N.S., Sethuraman, M.G. (2007). Constituents and antibacterial activity of the essential oils from the leaves and fruits of *Pittosporum viridulum*. *J Essent Oil Res*., *19*(6), 591-593. https://doi.org/10.1080/10412905.2007.9699338

[27]. Prakash, O., Rajput, M., Kumar, M., Pant, A.K. (2010). Chemical Composition and Antibacterial Activity of Rhizome Oils From *Hedychium coronarium* Koenig and *Hedychium spicatum* Buch-Ham. *Journal of essential oil bearing plants, 13(2),* 250-259. https://doi.org/10.1080/0972060X.2010.10643819

[28]. Buitrago, A., Rojas, J., Rojas, L., Velasco, J., Morales, A., Peñaloza, Y., Díaz, C. (2015). Essential oil composition and antimicrobial activity of *Vismia macrophylla* leaves and fruits collected in Táchira-Venezuela. *Nat Prod Commun*., *10*(2), 375-377. https://doi.org/10.1177/1934578X1501000244

[29]. Yiğit Hanoğlu, D., Hanoğlu, A., Güvenir, M., Süer, K., Demirci, B., Başer, K.H.C., Özkum Yavuz, D. (2017). Chemical composition and antimicrobial activity of the essential oil of *Sideritis cypria* Post endemic in Northern Cyprus. *J Essent Oil Res*., *29*(3), 228-232. https://doi.org/10.1080/10412905.2016.1251503

[30]. Magina, M.D., Dalmarco, E.M., Wisniewski, A., Simionatto, E.L., Dalmarco, J.B., Pizzolatti, M.G., Brighente, I.M. (2009). Chemical composition and antibacterial activity of essential oils of *Eugenia* species. *J Nat Med*., *63*(3), 345-350. https://doi.org/10.1007/s11418-009-0329-5

[31]. Kumar, S., Javed, M.S., Kumar, P.K.R., Kumar, R. (2018). Chemical Composition, *in vitro* Antibacterial, And Antioxidant Activity of Essential Oil From Leaves of *Ajuga parviflora* Benth. *Asian J Pharm Cline Res*., *11*(2), 57-61. https://doi.org/10.22159/ajpcr.2018.v11s2.28529

[32]. Mulyaningsih, S., Sporer, F., Reichling, J., Wink, M. (2011). Antibacterial activity of essential oils from *Eucalyptus* and of selected components against multidrug-resistant bacterial pathogens. *Pharm Biol*., *49*(9), 893-899. https://doi.org/10.3109/13880209.2011.553625

[33]. Paolini, J., Barboni, T., Desjobert, J.M., Djabou, N., Muselli, A., Costa, J. (2010). Chemical composition, intraspecies variation and seasonal variation in essential oils of *Calendula arvensis* L. *Biochem Syst Ecol*., *38*, 865-874.

International Journal of Secondary Metabolite
2020, Vol. 7, No. 4, 237–246

https://doi.org/10.21448/ijsm.714108

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

RP-HPLC-UV Analysis of the Phenolic Compounds, Antimicrobial Activity Against Multi-Drug Resistant Bacteria and Antioxidant Activity of Fruit and Seed of *Diospyros lotus* L.

Zeynep Akar[1,*], Aylin Karakurt[2], Funda Okumus[2], Sedanur Cinemre[2], Azer Ozad Duzgun[1,3], Bulent Akar[4], Zehra Can[5]

[1]Department of Genetics and Bioengineering, Faculty of Engineering and Natural Sciences, Gümüşhane University, Gümüşhane, Turkey
[2] Department of Biotechnology, Institute of Natural Sciences, Gümüşhane University, Gümüşhane, Turkey
[3]Medicinal Plants, Traditional Medicine Practice and Research Center, Gümüşhane University, Gümüşhane, Turkey
[4]Department of Food Engineering, Faculty of Engineering and Natural Sciences, Gümüşhane Universty, Gümüşhane, Turkey
[5]Department of Emergency and Disaster Management School of Applied Sciences, Bayburt University, Bayburt, Turkey

Abstract: The object of the present study was to investigate the antioxidant, antimicrobial activity and phenolic compounds of fruit and seeds of *Diospyros lotus* L. The fruits consumed by humans as nutrients were harvested from the plants that grow naturally in the province of Trabzon-Akçaabat, Eastern Black Sea Region of Turkey. The antioxidant activities of fruit and seeds of *Diospyros lotus* were determined by using four methods (% DPPH radical scavenging activity, FRAP antioxidant power determination, CUPRAC reducing antioxidant activity and total phenolic content (TPC) in five different proportion of methanol-water extracts. All extracts of fruit and seeds of *D. lotus* analyzed through whole antioxidant analysis methods showed significant antioxidant activity. In addition, antimicrobial activity of fruit and seeds extracted with DMSO was determined against seven standard bacteria and three multi-drug resistance clinical strains. Although fruit extracts did not have the antimicrobial activity against bacteria, seeds showed antimicrobial effect to both standard strains (Gram-negative and Gram-positive) and antibiotic resistance clinical isolates (*Klebsiella pneumoniae* carrying bla_{KPC}, *Acinetobacter baumannii*, harboring bla_{OXA-23} gene and resistance to colistin, and multi-drug resistant *Pseudomonas aeruginosa*). MICs' value of plant seed extracts for standard strains was 0.75-25 mg/mL and antibiotic resistant clinical bacteria were 12.5 and 25 mg/mL. Additionally, phenolic compounds in methanol extracts of fruit and seeds were also determined in by HPLC using 19 standards. Gallic acid and chrysin phenolic compounds were the major phenolic compounds in fruit and seeds, respectively.

ARTICLE HISTORY

Received: April 03, 2020
Revised: July 03, 2020
Accepted: November 06, 2020

KEYWORDS

Antioxidant,
Diospyros lotus,
Multi-drug Resistant Bacteria,
Phenolic Compounds

CONTACT: Zeynep AKAR ✉ zeynep_iskefiyeli@hotmail.com Department of Genetics and Bioengineering, Faculty of Engineering and Natural Sciences, Gümüşhane University, Gümüşhane, Turkey

ISSN-e: 2148-6905 /© IJSM 2020

1. INTRODUCTION

While organisms continue their cellular metabolic functions in the usual way, at the same time, the oxygen required for aerobic respiration, which is one of the elements of usual metabolic functions, can cause serious cell damage during oxidation. In other words, oxygen in the cell can produce reactive oxygen species (ROS) that occurs free radicals, while it produces energy with oxidative phosphorylation [1]. Increased production of ROS causes oxidative stress that is the cause of many diseases such as diabetes, cancer, epilepsy, cardiovascular conditions and inflammatory diseases [2,3]. For example, in 2018 year, new cancer cases of 18.1 million were detected in the world, 9.6 million of which resulted in death [4]. There are antioxidant defense systems in order to halt the progression of these diseases in the body. The antioxidants effect chain reactions with free radicals that occur in the cell for various reasons and damage the cell and affect the that they initiate. Antioxidants, one of the components of the defense system, react with free radicals and affect the formation of chain reactions the excess in diseases caused by free radicals the increased the tendency towards natural foods with strong antioxidant properties [5,6].

Not only free radicals but also infections show a significant effect on the development of some diseases in humans and animals. Especially infections were started to be controlled with the exploration of antibiotics in the later 19th century and early 20th century. For 60 years, antibacterial drugs have been considered a panacea to cure infections and have been used extensively regardless of the source and type of infection. In addition, misuse of antibiotics, such as skipping dose, overuse and low dose antibiotic intake, has potentially promoted antibiotic resistance to the bacteria rather than eliminating the infection [7-11]. The type of infection has become a life-threatening problem. Therefore, in recent times, interest has been raised to the researches for the development of new antimicrobial substances from various organisms to combat antibiotic resistance. Although new antibiotics against bacteria are commonly obtained from prokaryotic organisms (bacteria) and some eukaryotic organisms (microorganisms, plants, and various animals); especially microorganisms and plants are the main source of them [12]. Secondary metabolites obtained from plants exhibit antimicrobial effect as well as some pharmacological effects (antioxidative, anticancer and anti-inflammatory activities and prevent coronary heart disease, anti-atherosclerotic and hepatoprotective) on human health [10,13]. The usage of plants in the cure of various diseases has a long history. Up to the present time, 35000-70000 species of plant have been screened for medical use. Particularly, plants with ethnopharmacological have been the main sources for drug discovery [14]. Among these plants, *Diospyros lotus* L. fruits are used in traditional medicine but also consumed as food. The fruits are used as antiseptic, antitumor, astringent, antidiabetic and in treating diarrhea, dry cough, febrifuge, hypertension [15]. In addition, its seeds show sedative effect [16].

Diospyros genus in the Ebanaceae family contains approximately 200 species. *D. lotus* is one of the four species that have a pomological value among these species. The species is a deciduous tree and its maximum heights 15 m. It spreads naturally in the Northeast and South regions of Turkey. The fruit is a globose shape which is about from 1.5 to 2.0 cm in diameter and immature and ripe fruits are yellow and bluish-black color, respectively [17-19]. Some studies were performed to determine both the biological activities and the chemical composition of the *D. lotus* [15,16,20-24]. In addition, although there are some studies on the biological activity [25] and chemical composition of *D. lotus* collected from Turkey [17,26-29], any studies have not been conducted to determine the antimicrobial activity against multi-drug resistant bacteria and antioxidant activity of the fruit and seed portion of *D. lotus*.

Fruit and seed of *D. lotus* grown in Turkey's Eastern Black Sea Region in the present study were aimed to determine the antioxidant activity with different methods and antimicrobial

activity against three Gram-positive, four Gram-negative bacterial strains and three multi-drug resistant bacteria. Also it was determined that the chemical composition of the fruit and seed.

2. MATERIAL and METHODS

2.1. Chemicals and Reagents

The chemicals and reagents used in the study were obtained from various companies, for example; Trolox®, 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,9-dimethyl-1,10-phenanthroline (neocuproine), anhydrous iron (III) chloride ($FeCl_3$) were purchased from Sigma-Aldrich. Copper (II) chloride ($CuCl_2$), acetic acid (≥99.8 %), ammonium acetate, HPLC grade methanol (≥99.9 %) and ethanol (≥99.5 %), were supplied from Merck. Folin-Ciocalteu reactive and HPLC standards Sigma-Aldrich, HPLC Elite LaChrom Hitachi, Japan. Evaporator IKA-Werke, Staufen, Germany. Syringe filter RC-membrane, 0.45 µm.

2.2. Preparation of Plant Extraction

The fruits of the *D. lotus* were harvested in the Akçaabat district of Trabzon province in December 2017. The seeds (S) from fruit (F) of the plant were separated and both fruit and seeds are dried at room temperature. The dried fruits and seeds are milled in Waring Commercial Blender device.10 g of fruit powder and seed powder were weighed, separately and extracted in five different percentages of 100 mL methanol-pure water solutions (Table 1).

Thus, the extracts were mixed with a magnetic stirrer for 2 hours and were successively filtered through Whatman filter papers to obtain clear solutions. The extracts were stored in a cool, dark place at room temperature until analysis. In addition, in order to determine the antimicrobial activity, DMSO extracts were prepared for both fruits and seeds as in preparation of methanol-water extracts.

Table 1. Solvent ratios for fruit and seed extraction

Fruit Samples	% Methanol	% Water	Seed Samples	% Methanol	% Water
F1	100	-	S1	100	-
F2	75	25	S2	75	25
F3	50	50	S3	50	50
F4	25	75	S4	25	75
F5	-	100	S5	-	100

2.3. Plant Extraction for HPLC

Solvents of the methanol extracts of the fruit and seeds of the plant were evaporated with a rotary evaporator device at 40 °C. The residue dissolved in 10 mL pH 2 water was extracted, for the phenolic compounds, three times with 15 mL of diethyl ether then, three times with 15 mL of ethyl acetate consecutively. Organic phase was picked up in the same flask and evaporated till drying under reduced pressure in a rotary evaporator at 40 °C. Then the residues were weighed and dissolved with 2 mL of methanol for HPLC analysis. This solution was filtered with 0.45µm Whatman nylon filter and analyzed by HPLC-UV [30].

2.4. Determination of Antioxidant Activity

2.4.1. DPPH Radical Scavenging Activity

DPPH radical cleaning test is one of the most preferred methods in the determination of the antioxidant activity of the substances. The method developed by Cuendet et al. [31] was modified and applied to extracts. Firstly, a 100 µM methanolic DPPH• solution was mixed in the magnetic stirrer for 30 min and then a DPPH• reagent solution was prepared. Both the sample mixture and the reagent blank 3 were run in parallel. After 50 min, the absorbance values

of the tubes mixed with DPPH reagent were determined at 517 nm and % inhibition (DPPH• cleaning) values were calculated using the following formula.

% Inhibition (radical cleaning power) = [(A_{DPPH} - A_{Sample}) / A_{DPPH}] X 100

A_{DPPH} : Absorbance value of the DPPH solution

A_{Sample} : Absorbance value of the sample extract

2.4.2. Ferric Reducing Antioxidant Power (FRAP)

The antioxidant assay method was based on the principle of measuring the absorbance of the Fe^{2+} -TPTZ (2,4,6-tris (2-pyridyl) -s-triazine complex in 595 nm [32]. The FRAP reagent consists of mixture of 10mM TPTZ in 40mM HCl and 20mM $FeCl_3.6H_2O$ and 300mM acetate buffer (pH 3.6), in 1:1:10 ratio respectively. The calibration curve was created using the Trolox standard in different volumes (1000-500-250-125-62.5 µM). 50 µL of the plant extracts and standard Trolox solutions were vortexed with FRAP reagent (1.5 mL) and were kept at room temperature for 20 minutes. Then the values of absorbance were read at 595 nm against pure water. In addition, the absorbance of reagents and sample blanks was measured and these values were subtracted from the mean values of the three studied parallel samples. Antioxidant activity of FRAP was calculated based on Trolox calibration graph and expressed as TEAC, µM (Trolox equivalent antioxidant capacity). In the evaluation of the results, the high TEAC values of the samples also indicate high FRAP and therefore high antioxidant capacity.

2.4.3. Cupric Reducing Antioxidant Capacity (CUPRAC)

In CUPRAC method, 2,9-dimethyl-1,10-phenanthroline (Neocuproine-Nc) was used with Cu (II) to form Copper (II) -neocuproine complex [Cu (II) -Nc] [33]. The antioxidant capacity was determined with the reduction of the Cu(II)-Nc to Cu(I)-Nc chelate that has maximum absorbance at 450 nm[33]. Antioxidant standard Trolox® was studied at six different concentrations (0.03125-0.625- 0.125-0.25-0.5-1-mg/mL) for plotting calibration curve. After pipetting, tubes were vortexed and kept in darkness for 30 minutes. Absorbance values of the solutions in each tubes transferred to plastic cuvettes were read at 450 nanometers. The results were compared with Trolox®, a standard antioxidant with a high reduction potential and were expressed as Trolox® equivalent antioxidant capacity (µM TEAC).

2.4.4. Total Phenolic Content (TPC)

The total phenolic content of the fruit and seed of the *D. lotus* was determined by modified Slinkard and Singleton [34]. method with use of Folin-Ciocalteu reagent. Firstly, 50 µL of the sample solution was diluted with 2.5 mL of distilled water, then 250 µL of 0.2 N Folin-Ciocalteu reagent and 750 µL of Na_2CO_3 (7.5%) was added and vortexed. The prepared tubes were incubated for 2 hours at room temperature and the absorbance values at 765 nm were determined. The amount of phenolic compounds in the samples was determined based on function of the line obtained from calibration graph of gallic acid standart (1000-500-150-125-62.5 µg/mL) in six different concentrations as gallic acid equivalent (GAE (µg/mL)).

2.5. Analysis of Phenolic Compounds by HPLC

The phenolic contents of the methanol extracts of the fruits and seed of *D. lotus* were determined by using the Elite LAChrom Hitachi, Japan HPLC with a UV-Vis detector. In total, nineteen of phenolic compounds standards (caffeic acid, catechin, chrysin, daidzein, epicatechin, ferulic acid, gallic acid, hesperetin, luteolin, myricetin, *p*-coumaric acid, 4-hydroxybenzoic acid, pinocembrin, protocatechuic acid, resveratrol, rutin, syringic acid, *t*-cinnamic acid and CAPE (caffeic acid phenethyl ester)) were used. The extracts were injected into an inverted phase C18 column (150 mm x 4.6 mm, 5 µm; Fortis). The mobile phase was formed within mixing of solvents A (2% AcOH in water) and B (acetonitrile/water in 70:30 ratio) which was sonicated before stirring and continuously degassed by the built-in HPLC

system. The temperature of the column was fixed at 30 °C and 20 µL of extracts were injected. The mobile phase was composed of water (2% AcOH) and acetonitrile/water (70:30) filtered using a 0.45µm nylon filter (Whatman, Maidstone, The United Kingdom) degassed in a sonicator for 30 min. The mobile phase flow rate was 0.75 mL/min, and the elution in the gradient mode occurred as follow: 0 min 5% B in A; 8 min 15% B in A; 10 min 20% B in A; 12 min 25%B in A; 20 min 40%B in A; 30 min 80%B in A; 35 min 5% B in A; 50 min 5%B in A. Phenolic profile was determined according to Can et al. [30].

2.6. Determination of Minimum Inhibition Concentration

The minimum inhibitory concentration (MIC) of the plant extract against to 3 Gram-positive (*Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes* ATCC 19615 and *Staphylococcus aureus* ATCC 25923) and 4 Gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 43288, *Yersinia pseudotuberculosis* ATCC 911) and antibiotic resistant strains (*Acinetobacter baumannii*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) were determined with the use of the liquid microdilution method. DMSO was used as a solvent for extracts. The extracts concentration are 100 mg/mL (serial two-fold dilution was carried out) obtained from the fruit and seed of the plant. Ampicillin used as control. MIC values of the DMSO fruit and seed extracts were determined in the 96-well plates and triplicate.

3. RESULTS and DISCUSSION

3.1. Antioxidant Activity of *D. lotus* Extract

The antioxidant activities of fruits and seeds of *D. lotus* plant were investigated by using four different antioxidant determination methods in methanol-water extracts at different percentages. In the DPPH radical scavenging method, while F1 (100% methanol) had the highest antioxidant activity value with 79.04 and the lowest activity was measured as 59.94 for F4 sample (25% methanol- 75% water (Table 2). In the extracts of the seeds, the highest antioxidant activity was measured as 85.63 in S1 extract (100% methanol) as in fruits. However, the lowest activity was determined with 64.17 in S2 extract (75% methanol- 25% water) (Table 2).

Table 2. The antioxidant activities of methanol and water extracts in different percentage of fruits and seeds of *D. lotus* plant

Samples	TPC (GAE µg/mL)	FRAP (µM TEAC)	CUPRAC (µM TEAC)	DPPH (%)
F1	416.51 ±0.05	250.00 ± 0.01	0.05 ± 0.06	79.04 ± 0.05
F2	306.82 ±0.01	347.60 ± 0.03	0.04 ± 0.01	72.24 ± 0.07
F3	289.82 ±0.11	163.60 ± 0.10	0.03 ± 0.01	66.44 ± 0.08
F4	124.12 ±0.08	43.64 ± 0.01	0.02 ± 0.07	59.94 ± 0.10
F5	105.91 ±0.01	7.270 ± 0.02	0.01 ± 0.01	66.54 ± 0.08
S1	569.81 ±0.03	190.60 ± 0.02	0.27 ± 0.02	85.63 ± 0.04
S2	659.80 ±0.04	471.50 ± 0.05	0.58 ± 0.06	64.17 ± 0.09
S3	632.61 ±0.01	215.51 ± 0.12	0.32 ± 0.02	69.78 ± 0.07
S4	521.00 ±0.10	71.82 ± 0.03	0.22 ± 0.02	70.77 ± 0.07

*Different letters in the same column indicate significantly different at the 5% level ($p < 0.05$) among the results.

Similar results were obtained in FRAP, CUPRAC and TPC antioxidant detection methods. In all three methods, F2 (75 % methanol- 25 %) and F5 (100 % water) showed the highest and lowest antioxidant activity respectively for both seed extracts and fruit extracts. It

was determined that the antioxidant activity of the seed extracts was higher than the fruit extracts in all antioxidant determination methods.

In addition, the antioxidant activity of the extracts, which are generally 100 % water in the solvent, was found to be lower than the others. Except for the DPPH test, antioxidant activity was higher in fruits and seeds in 75 % methanol- 25 % water and 50 % methanol- 50 % water extracts for all three methods. In some studies conducted to determine DPPH radical scavenging activity values of *D. lotus* plant fruits (methanolic extract) grown in different regions of the world, determined values were significant [20,21,23]. Moreover, Lozzio et al. [20] also found high antioxidant activity using FRAP and ABTS methods.

3.2. Phenolic Compounds in *D. lotus* Extract

While five phenolic acids; gallic acid > ferulic acid >protocatechuic acid>syringic acid >*t*-cinnamic acid were determined in methanolic fruit extract (Table 3) eight phenolic compound; gallic acid> chrysin>CAPE> catechin>caffeic acid>*p*-coumaric acid >protocateuic acid>*t*-cinnamic acid were identified in the seed extract (Table 3). It was observed that the seed parts were richer in terms of phenolic content as well as antioxidant activity. The results support the antioxidant results. As it is seen in the Table 3, gallic acid, protocatechuic acid and *t*-cinnamic acid were present in both fruit and seed. While gallic acid was measured in the highest quantity with 234.74 µg/g in fruit extract and it was second with 1065.54 µg/g in seed extract among the determined phenolic compounds. However, although the chrysin and CAPE was not present in fruit extracts, they reached quantitatively highest abundance with 1086.09 and 768.2 µg/g in seed extract respectively.

Table 3. HPLC analysis of phenolic composition of *D. lotus*

HPLC standards	Fruit (µg extract/g)	Seed (µg extract/g)
Gallic acid	234.7	1066
Protocatechuic acid	28.13	44.43
4-hydroxybenzoic acid	nd	nd
Catechin	nd	343.2
Caffeic acid	nd	121.4
Syringic acid	15.53	nd
Epicatechin	nd	nd
p-coumaric acid	nd	68.27
Ferulic acid	48.44	nd
Rutin	nd	nd
Myricetin	nd	nd
Resveratrol	nd	nd
Daidzein	nd	nd
Luteolin	nd	nd
t-cinnamic acid	6.36	21.42
Hesperetin	nd	nd
Chrysin	nd	1087
Pinocembrin	nd	nd
CAPE	nd	768.2

nd: non detected

Ayaz et al. [27] searched phenolic content of *D. lotus* fruits at different times during the development period of fruit in GC-MS and reported that eight phenolic acids (salicylic acid, 4-hydroxybenzoic acid, vanillic acid, gentistic acid, 3,4-dihydroxybenzoic acid, syringic acid, *p*-coumaric acid, gallic acid). Gallic acid and syringic acid were also detected and gallic acid showed the highest amount the same as our study. Gao et al. [16] also used ten phenolic acid standards in HPLC analysis of phenolic compounds in different extracts of *D. lotus*. Seven of

these standards (caffeic acid, ferulic acid, gallic acid, myricetin, *p*-coumaric acid, protocatechuic acid and rutin) were common with the present study. Similarly, rutin was not detected, while gallic acid was detected in the highest abundance [16]. Gallic acid, a phenolic compound of plant origin, is a powerful antioxidant [35]. In addition, it also shows the effects of antimutagenic, antitumor, antibacterial anti-inflammatory [35,36]. Rashed et al. [24] reported that among seven phenolic compounds obtained from *D. lotus* fruits, gallic acid was the most active compound against HIV-1 and inhibited HIV-1_{IIIB} replication with EC_{50} value of 6.09 µg/mL. Again, the plant phenolic compounds were used against human cancer cells and gallic acid exhibit the highest anticarcinogenic effect against colorectal adenocarcinoma (IC50 2.6 µg/mL) and lung large cell carcinoma (IC50 4.66 µg/mL) [20]. Moghaddam et al. [23] conducted a study on the seed of this plant to determine its biological activity. However, the phenolic content of seed has not been studied. Although chrysin was present in some plants, honey, and propolis [37] it was first detected in the *D. lotus* plant in this study. It was also measured at the highest level among the identified phenolics in the seed. Chrysin is an important natural flavonoid having many biological activities such as aromatase inhibitor, antioxidant, anti-cancer, antiviral activities and anti-inflammatory effects [38,39].

3.3. Antimicrobial Activity

DMSO extract of plant seed has inhibited the growth of studied bacteria but DMSO extract of plant fruit did not have antimicrobial activity against bacteria. MICs of plant seed extracts were found to be between 0.75-25 mg/mL. Extract of plant seed showed very potent activity against *E. coli* and *S. pyogenes* with 0. 75 mg/mL MIC. MIC values of plant seed extract for *Bacillus subtilis* and *P. aeruginosa* were 12.5 mg/mL. Also, it was determined that the DMSO extract of plant seed had a lower MIC value (25 mg/mL) against *S. aureus*, *Y. pseudotuberculosis* and *Proteus vulgaris*. MIC values of plant extract against antibiotic resistant clinical bacteria were 12.5 and 25 mg/mL. DMSO extract of the plant seed was determined to be the highest MIC (12.5 mg/mL) against *K. pneumonae* carrying bla_{KPC}, *A. baumannii* harboring bla_{OXA-23} gene and resistance to colistin, and multi-drug resistance *P. aeruginosa*. MIC value of the plant seed extract was found to be 25 mg/mL against NDM type metallo beta lactamase harboring *K. pneumonia* and multi-drug resistance *E. coli*.

4. CONCLUSION

Methanol and water extracts in different percentages of *D. lotus* plant fruits and seeds showed antioxidant activity in all antioxidant methods. Significantly, the content of phenolic compounds in plants contributes antioxidant activity. Among the phenolic compounds detected in this study, the highest measured gallic acid (in fruit and seed) and chrysin (only seed) may be determinant antioxidant activity. Chrysin flavonoid, which has high biological activity, can be studied in more detail in both naturally grown and cultivated *D. lotus* seeds. Antibiotic-resistant strains, especially multidrug-resistant isolates, are a serious threat to public health. The spread of beta-lactamases that hydrolyze antibiotics among bacteria has become a worldwide problem. To combat antibiotic resistance, many scientists are looking for both synthetic and natural products around the world. In this study, we show the antibacterial activity of plant seed extracts against multi-drug resistant strains that cause difficulties in the clinic.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Orcid

Zeynep Akar https://orcid.org/0000-0001-9262-8070
Aylin Karakurt https://orcid.org/0000-0002-4755-6669
Funda Okumus https://orcid.org/0000-0002-2494-7050
Sedanur Cinemre https://orcid.org/0000-0001-9556-9678
Azer Ozad Düzgün https://orcid.org/0000-0002-6301-611X
Bülent Akar https://orcid.org/0000-0002-1421-374X
Zehra Can https://orcid.org/0000-0002-7156-4941

5. REFERENCES

[1]. Halliwell, B., Gutteridge J.M.C. (1990). Role of Free Radicals and Catalytic Metal Ions in Human Disease: An Overview. *Method Enzymol.*, *186*, 1-85. https://doi.org/10.1016/0076-6879(90)86093-b

[2]. Panayiotidis, M. (2008). Reactive Oxygen Species (ROS) in Multistage Carcinogenesis. *Cancer Lett.*, *266*(1), 3-5. https://doi.org/10.1016/j.canlet.2008.02.027

[3]. Ferlay, J., Autier, P., Boniol, M., Heanue, M., Colombet, M., Boyle, P. (2007). Estimates of the Cancer Incidence and Mortality in Europe in 2006. *Ann. Oncol.*, *18*(3), 581-592. https://doi.org/10.1093/annonc/mdl498

[4]. Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D.M., Piñeros, M., Znaor, A., Bray, F. (2019). Estimating the Global Cancer Incidence and Mortality in 2018: GLOBOCAN Sources and Methods. *Int. J. Cancer*, *144*(8), 1941-1953. https://doi.org/10.1002/ijc.31937

[5]. Ames, B.M., Shigenaga, M.K., Hagen, T.M. (1993). Oxidants, Antioxidants, and the Degenerative Diseases of Aging. *P. Natl. Acad. Sci. USA.*, *90*, 7915-7922. https://doi.org/10.1073/pnas.90.17.7915

[6]. Valle, I., Álvarez-Barrientos, A., Arza, E., Lamas, S., Monsalve, M. (2005). PGC-1α Regulates the Mitochondrial Antioxidant Defense System in Vascular Endothelial Cells. *Cardiovasc. Res.*, *66*(3), 562-573. https://doi.org/10.1016/j.cardiores.2005.01.026

[7]. Li, B., Webster, T.J. (2018). Bacteria Antibiotic Resistance: New Challenges and Opportunities for Implant-Associated Orthopedic Infections. *Journal of J. Orthopaed. Res.*, *36*(1), 22-32. https://doi.org/10.1002/jor.23656

[8]. WHO (World Health Organization). (2014). Antimicrobial Resistance: Global Report on Surveillance 2014, World Health Organization: Geneva, Switzerland. ISBN: 978924156474 8.

[9]. Kardas, P., Devine, S., Golembesky, A., Roberts, C. (2005). A Systematic Review and Meta-Analysis of Misuse of Antibiotic Therapies in the Community. *Int. J. Antimicrob. Ag.*, *26*(2), 106-113. https://doi.org/10.1016/j.ijantimicag.2005.04.017

[10]. Górniak, I., Bartoszewski, R., Króliczewski, J. (2019). Comprehensive Review of Antimicrobial Activities of Plant Flavonoids. *Phytochem. Rev.*, *18*(1), 241-272. https://doi.org/10.1007/s11101-018-9591-z

[11]. Tanrıkulu, G.İ., Ertürk, Ö., Yavuz, C., Can, Z., Çakır, H.E. (2017). Chemical compositions, antioxidant and antimicrobial activities of the essential oil and extracts of Lamiaceae family (*Ocimum basilicum* and *Thymbra spicata)* from Turkey. *Int. J. Second. Metab.*, *4*(3-2), 340-348. https://doi.org/10.21448/ijsm.373828

[12]. Balouiri, M., Sadiki, M., Ibnsouda, S.K., (2016). Methods For in Vitro Evaluating Antimicrobial Activity: A Review. *J. Pharm. Anal.*, *6*(2), 71-79. https://doi.org/10.1016/j.jpha.2015.11.005

[13]. Akar, Z., Küçük, M., Doğan, H. (2017). A New Colorimetric DPPH• Scavenging Activity Method with No Need for a Spectrophotometer Applied on Synthetic and Natural Antioxidants and Medicinal Herbs. *J. Enzym Inhib. Med. Ch.*, *32*(1), 640-647. https://doi.org/10.1080/14756366.2017.1284068

[14]. Veeresham, C. (2012). Natural Products Derived From Plants as a Source of Drugs. *J. Adv. Pharm. Technol. Res.*, *3*(4), 200-201. https://doi.org/10.4103/2231-4040.104709

[15]. Uddin, G., Rauf, A., Siddiqui, B.S., Muhammad, N., Khan, A., Shah, S.U.A. (2014). Anti-Nociceptive, Anti-Inflammatory and Sedative Activities of the Extracts and Chemical Constituents of *Diospyros lotus* L. *Phytomedicine*, *21*(7), 954-959. https://doi.org/10.1016/j.phymed.2014.03.001

[16]. Gao, H., Cheng, N., Zhou, J., Wang, B., Deng, J.J., Cao, W. (2014). Antioxidant Activities and Phenolic Compounds of Date Plum Persimmon (*Diospyros lotus* L.) Fruits. *J. Food Sci. Technol.*, *51*(5), 950-956. https://doi.org/10.1007/s13197-011-0591-x

[17]. Ayaz, F.A., Kadioglu, A. (1999). Fatty Acid Compositional Changes in Developing Persimmon (*Diospyros lotus* L.) Fruit. *New Zeal. J. Crop Hort.*, *27*, 257-261. https://doi.org/10.1080/01140671.1999.9514104

[18]. Yildirim, N., Ercisli, S., Agar, G., Orhan, E., Hizarci, Y. (2010). Genetic Variation among Date Plum (*Diospyros lotus*) Genotypes in Turkey. *Genet. Mol. Res.*, *9*(2), 981-986.

[19]. Yılmaz, B., Genç, A., Çimen, B., İncesu, M., Yeşiloğlu, T. (2017). Characterization of Morphological Traits of Local and Global Persimmon Varieties and Genotypes Collected From Turkey. *Turk. J. Agric. For.*, *41*(2), 93-102. https://doi.org/10.3906/tar-1611-27

[20]. Loizzo, M.R., Said, A., Tundis, R., Hawas, U.W., Rashed, K., Menichini, F., Frega, N.G., Menichini, F. (2009). Antioxidant and Antiproliferative Activity of *Diospyros lotus* L. Extract and Isolated Compounds. *Plant Food. Hum. Nutr.*, *64*(4), 264-270. https://doi.org/10.1007/s11130-009-0133-0

[21]. Nabavi, S.M., Ebrahimzadeh, M.A., Nabavi, S.F., Fazelian, M., Eslami, B. (2009). In Vitro Antioxidant and Free Radical Scavenging Activity of *Diospyros lotus* and *Pyrus boissieriana* Growing in Iran. *Pharmacogn. Mag.*, *5*(18), 122-126.

[22]. Uddin, G., Rauf, A., Siddiqui, B.S., Shah, S.Q. (2011). Preliminary Comparative Phytochemical Screening of *Diospyros lotus* Stewart. *Middle East J. Sci. Res.*, *10*(1), 78-81.

[23]. Moghaddam, A.H., Nabavi, S.M,. Nabavi, S.F., Bigdellou, R., Mohammadzadeh, S., Ebrahimzadeh, M.A., (2012). Antioxidant, Antihemolytic and Nephroprotective Activity of Aqueous Extract of *Diospyros lotus* seeds. *Acta Pol. Pharm.*, *69*, 687-692.

[24]. Rashed, K., Zhang, X.J., Luo, M.T., Zheng, Y.T., (2012). Anti-HIV-1 Activity of Phenolic Compounds Isolated From *Diospyros lotus* Fruits. *Phytopharmacology*, *3*(2), 199-207.

[25]. Saral, S., Ozcelik, E., Cetin, A., Saral, O., Basak, N., Aydın, M., Ciftci, O. (2016). Protective Role of *Diospyros lotus* on Cisplatin-Induced Changes in Sperm Characteristics, Testicular Damage and Oxidative Stress in Rats. *Andrologia*, *48*(3), 308-317. https://doi.org/10.1111/and.12448

[26]. Ayaz, F.A., Kadıoğlu, A., Hayırlıoğlu, S. (1995). Determination of Some Mono-and Disaccharides in The Fruits of *Diospyros lotus* L. Using Gas Chromatography. *Turk. J. Bot.*, *19*, 493-495.

[27]. Ayaz, F.A., Kadıoğlu, A., Reunanen, M. (1997). Changes in Phenolic Acid Contents of *Diospyros lotus* L. During Fruit Development. *J. Agr. Food. Chem.*, *45*(7), 2539-2541. https://doi.org/10.1021/jf960741c
[28]. Ayaz, F.A., Kadıoğlu, A. (1998). Nonvolatile Acid Composition during Fruit Development of *Diospyros lotus* L. *Turk. J. Bot.*, *22*(2): 69-72.
[29]. Glew, R.H., Ayaz, F.A., Millson, M., Huang, H.S., Chuang, L.T., Sanz, C., Golding, J.B. (2005). Changes in Sugars, Acids and Fatty Acids in Naturally Parthenocarpic Date Plum Persimmon (*Diospyros lotus* L.) Fruit during Maturation and Ripening. *Eur. Food Res. Technol.*, *221*(1−2), 113-118. https://doi.org/10.1007/s00217-005-1201-9
[30]. Can, Z., Baltas, N. (2016). Bioactivity and Enzyme Inhibition Properties of *Stevia rebaudiana*. Curr. *Enzym Inhib.*, *12(2)*, 188-194. https://doi.org/10.2174/157340801266 6160402001925
[31]. Cuendet, M., Hostettmann, K., Potterat, O., Dyatmiko, W. (1997). Iridoid Glucosides with Free Radical Scavenging Properties from *Fagraea blumei*. *Helv. Chim. Acta*, *80*(4), 1144-1152. https://doi.org/10.1002/hlca.19970800411
[32]. Benzie, I.F.F., Strain, J.J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Anal. Biochem.*, *239*(1), 70-76. https://doi.org/10.1006/abio.1996.0292
[33]. Apak, R., Güçlü, K., Özyürek, M., Karademir, S.E. (2004). Novel Total Antioxidant Capacity Index for Dietary Polyphenols and Vitamins C and E, Using Their Cupric Ion Reducing Capability in The Presence of Neocuproine: CUPRAC Method. *J. Agr. Food Chem.*, *52*(26), 7970-7981. https://doi.org/10.1021/jf048741x
[34]. Slinkard, K., Singleton, V.L., (1977). Total Phenol Analysis: Automation and Comparison with Manual Methods. *Am. J. Enol. Viticult.*, *28*(1), 49-55.
[35]. Yen, G.C., Duh, P.D., Tsai, H.L. (2002). Antioxidant and Pro-Oxidant Properties of Ascorbic Acid and Gallic Acid. *Food Chem.*, *79*(3), 307-313. https://doi.org/10.1016/S0308-8146(02)00145-0
[36]. Chanwitheesuk, A., Teerawutgulrag, A, Kilburn J.D., Rakariyatham, N. (2007). Antimicrobial Gallic Acid from *Caesalpinia mimosoides* Lamk. *Food Chem.*, *100*(3), 1044-1048. https://doi.org/10.1016/j.foodchem.2005.11.008
[37]. Samarghandian, S., Afshari, J.T., Davoodi S. (2011). Chrysin Reduces Proliferation and Induces Apoptosis in the Human Prostate Cancer Cell Line Pc-3. *Clinics*, *66*(6), 1073-1079. https://doi.org/10.1590/S1807-59322011000600026
[38]. Mani, R, Natesan, V. (2018). Chrysin: Sources, Beneficial Pharmacological Activities, and Molecular Mechanism of Action. *Phytochemistry*, *145*, 187-196. https://doi.org/10.1016/j.phytochem.2017.09.016
[39]. Salimi, A., Pourahmad, J. (2018). Role of Natural Compounds in Prevention and Treatment of Chronic Lymphocytic Leukemia. Polyphenols: Prevention and Treatment of Human Disease, 2nd ed.; Watson R.R., Preedy, V.R., Zibadi, S. Eds,; Academic Press: Cambridge, MA, USA, 2018, Volume 1-2, pp 195-203. ISBN: 9780128130094

International Journal of Secondary Metabolite
2020, Vol. 7, No. 4, 247–252

https://doi.org/10.21448/ijsm.718254

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

A Study on Cotton (*Gossypium* sp.) Nectar Production in Uzbekistan

G'ulom Hamidov[1], Qodirjon Maxsudov[1,*], Shahzoda Voxobova[1], Dilyora Akramova[1]

[1]Department of Biology, Natural Sciences Faculty, Fergana University, Fergana, Uzbekistan

Abstract: Given the importance of cotton varieties in beekeeping, we have been able to determine the nectar and honey yield per hectare for several years Cotton nectaries produce highly concentrated carbohydrates, nectarines, amino acids and fatty acids. Unlike other types of plants. In different varieties of cotton, there are 4 types of nectaries (intra-cup, bract, sub-bract, and leaf), and the number and concentration of nectarium are different for all types. Nectar productivity of fine fiber varieties is significantly higher than that of medium fiber varieties. Nectar is an important nutrient source for insects and pathogens due to its high sugar content. Several methods of nectar determination were used to determine the number of angles of cotton varieties planted in the country, such as "tube" and "washing." On average, honey yield of these varieties is 50-60 kg per hectare. It is understood from the research results that cotton varieties have high honey and nectar productivity in Uzbekistan environment.

ARTICLE HISTORY

Received: April 03, 2020
Revised: July 03, 2020
Accepted: September 20, 2020

KEYWORDS

Cotton varieties,
Nectar,
Nectar Concentration,
Test Method,
Washing Method

1. INTRODUCTION

Cotton (*Gossypium* sp.) is an important source of feed for beekeeping farms in Uzbekistan. Because currently the main source of beekeeping honey is cotton fields, along with natural flora. However, the data on the cotton crop is not quite complete. Also, many new varieties of cotton have been released lately and the amount of wreaths has not been determined. Therefore, this issue is of great importance both in cotton growing and beekeeping. Despite many scientific observations and experiments, so far there is no consensus on the amount of cotton flower juice in science. It is known that nectar extinction is a complex biological process that is influenced by climatic conditions of the year and by anthropogenic factors. Although cotton has been planted since ancient times, its "originality" has only been relatively recent. At first it was not known whether cotton produced nectar or not. Beekeeping was observed later in the cotton flowers. It is determined by the yield of the flower [1- 3].

Kaziev's research shows that the yield of cotton in the conditions of Azerbaijan in 1964 is 26-37 kg. [4]. According to SG Minkov [5], in South Kazakhstan each flower produces honey 8-11 mg and its honey yield is from 11 to 33 kg per hectare. Also, the amount of cotton wool

CONTACT: Qodirjon Maxsudov ✉ makhsudov79@inbox.ru Department of Biology, Natural Sciences Faculty, Fergana University, Fergana, Uzbekistan

ISSN-e: 2148-6905 /© IJSM 2020

was calculated in Bulgaria and Egypt. According to their data, in each of these countries each flower produces 8-12 mg from rose. Their honey yield ranges from 20 to 30 kg [6].

Cotton nectaries produce highly concentrated carbohydrates, nectarins, amino acids and fatty acids [7, 8]. Nectar is an important nutrient source for insects and pathogens due to its high sugar content [9-12]. Cotton nectar attracts insects beneficial for pollination and protection, which enable the plants to achieve greater reproduction [13].

2. MATERIAL and METHODS

Objects of the research are the following cotton varieties: cotton "Namangan-77", created by VA Avtonomov, M. Saidakhmedov, A. Egamberdiev and others at the Scientific Research Institute of Agrotechnologies of Crop Breeding and Breeding. The average growth period is 100-120 days and included in the State Register since 2012. Sh.Namazov, N.Khojambergenov and others at the Sulton Scientific Research Institute of Crop Breeding, Seed Breeding and Agro-Technology. The ripening period is 115-120 days on average and has been included in the state register since 2011. "Andijan 35" was developed by T.Komilov and others at the Andijan scientific experimental station of the Scientific- Research Institute of Crop Breeding, Seeding and Breeding Agrotechnology. The ripening period is 122–255 days on average and is included in the 2006 State Register.

Several methods of nectar determination were used to determine the number of angles of cotton varieties planted in the country, such as "tube" and "washing." The tube technique is simple and easy to use and has been used by many people. The number of angles determined by the tube method is as follows. Glass tubes of 0.1-0.3 mm in diameter and 4.5 cm in length are manufactured. They are weighed on accurate weights and weighed. These tubes are transmitted between plant florets and extracted from the juice. It will be weighed again. Of course, the number of flowers and what candies are derived. The amount of sugar in the rose (in percentage) is determined by the refractometer. The second method is the “washing” method where distilled water is poured into specially prepared tubes (40 cm^3). These vials are given a certain amount of flowers. The filtrate is shaken for 20-25 minutes when the juice from the flowers is transferred to the vial. The filtrate is analyzed by the Hagedorn and Jensen [14] method in the chemical laboratory.

3. RESULTS and DISCUSSION

Cotton blooming in Uzbekistan lasts from early July until late autumn, and slower flowering lasts from mid-July to late August. Vegetation buds begin to open at 8-9 am, slopes begin at 10 am and last until the evening. As you know, all of cottons produces nectar. According to our observations, the intensity of the hernia separation lasts from the morning to the middle of the day. In a study, Gilliam et al. [15]. Floral nectar from 3 cultivars of field-grown cotton, 9 cultivars of cotton grown in a greenhouse, 5 cultivars of citrus, saguaro cactus, and prickly pear cactus were examined for bacteria, yeasts, and molds by plating on selective microbiological media. No microbes were isolated from nectar of any cotton flowers. Of 23 samples of citrus nectar, 3 contained a few gram-negative rod-shaped bacteria. Nectar from prickly pear cactus contained no microbes, although nectar from saguaro cactus contained a few bacteria (gram-negative rods, gram-positive rods, and gram-positive cocci). These results are discussed in relation to possible antimicrobial substances in nectar as well as the origin of the microflora of honey bees.

To our knowledge, the amino acids in the floral nectar of cotton (*Gossypium* spp.) have not been determined, although Clark and Ulkefahr [16], using paper chromatography, failed to find amino acids in *Gossypium* extrafloral nectar. However, Mound [17] detected two ninhydrin-positive substances on paper chromatograms of extrafloral nectar from *G.*

barbadense L., and Hanny and Elmore [18], using both gas and thin layer chromatography, found 24 free amino acids in extrafloral nectar of *G. hirsutum* L. Yokohama [19], without giving data, stated that she found a high concentration of amino acids in extrafloral nectar of *G. hirsutum*.

Given the importance of cotton varieties in beekeeping, we have been able to determine the nectar and honey yield per hectare for several years (Table 1). It was found that the amount of nectar in each flower ranges from 8-10 to 30-36 mg. The amount of honey per hectare is 30-50 kg and honey yield is 15-20 kg to 30-40 kg.

Table 1. Nectar productivity of different medium fiber varieties.

No	Cotton varieties	The amount of nectar per flower (mg)	Nectar productivity kg/hectares	Honey product kg/hectares
1	Bukhora–102	26.10 ± 3.40	33.47	24.85
2	Andijan-36	11.75 ± 1.34	35.60	16.20
3	C-8286	10.32 ± 1.16	30.04	15.71
4	C-6524	9.66 ± 1.13	25.92	16.10
5	C-9063	12.66 ± 1.81	33.04	21.10

From the above, it can be seen from the fact that mid-fiber varieties are an important nectar-honey source for beekeeping farms. Therefore, all cotton plantations should be used on time.The nectar extraction of the pellets of cotton (Table 2) differs from each other. As shown in the table, the most widespread of medium-fiber varieties are produced by shellfish (74%), while others range from 3% to 14%. At the same time, the abdominal cavity accounted for 84%, and at least the leaflet (1.18%).

Table 2. Sugar yield of different types (%)

No	Part	Sugar contents (mg)	
		Medium fiber varieties	Varieties of fine fibers
1	Inside the flowerpot	25.9 (74%)	54.90 (84%)
2	Underneath the skull	3.15 (9%)	6.94 (11%)
3	In front of the flowerpot	5.08 (14%)	2.00 (3%)
4	On the leaf	1.12 (3%)	1.18 (2%)
5	Total	35.25 (100%)	65.02 (100%)

The most productive of cotton candy is the pearl inside the turquoise. Based on this, we have identified the dynamics of the wreath, which is separated by the apple tree in the flowerpot: (Table 3). Cotton plants present a relevant model system for comparing the defence strategies against herbivores [20]. While a lot of research has been carried out about the biochemical mechanisms underlying VOC emission in cotton plants and their associated benefşçşal interactions with insects [21], very little is known about the role of nectar [22]. Results of Llandres's study showed that wild cotton varieties produced a greater quantity of nectaries and this nectaries enhanced the diversity of natural enemies. The amount of nectar excreted by the entrails also varies. Accordingly, the number of bees that visit them varies. In another study, Özkök and Silici [20] performed biological analyses of different honey samples (chestnut, citrus, clover, cotton and sunflower). According to their results, cotton honey has 38.99 ± 5.08 mg GAE/100 g sample total phenolic content, and also it posses 139.95 ± 4.52 mg AAE/g sample antioxidant activity and %6.67 ± 0.42 antiradical activity [23]. As noted earlier, nectar and honey yield of fine fibers is much higher than that of medium-fiber varieties. It is reported that the honey yield of the previously planted and cultivated fine-fiber varieties in the country

ranges from 43.68 kg to 100 kg/ha. The most productive varieties are Bukhara-1, Termez-7. The S-6040, Surxon-2 can be cited. On average, honey yield of these varieties is 50-60 kg per hectare.

Table 3. The process of separation of the wreath by the giraffe inside the turquoise

Time	Air temperature (^{O}C)	Varieties					
		Namangan-77		Sulton		Andijan-35	
		Nectar content (mg)	Nectar concentration (%)	Nectar content (mg)	Nectar concentration (%)	Nectar content (mg)	Nectar concentratio n
7	24	1.80 ± 0.21	20	0.30 ± 0.06	15	1.33 ± 0.18	43
9	27	12.32 ± 3.12	22	2.22 ± 0.45	30	3.66 ± 0.48	43
13	36	46.00 ± 7.18	30	8.50 ± 1.19	42	7.33 ± 1.10	50
15	35	11.00 ± 2.71	35	10.00 ± 1.31	45	13.66 ± 3.14	53
17	35	7.03 ± 0.94	45	10,50 ± 1,32	47	12 ± 3.12	52
19	32	7.00 ± 0.94	45	8.00 ± 1.12	47	9.20 ± 1.29	53
Aver		10.18	31.71	6.24	37.54	5.02	47

4. CONCLUSION

In conclusion, it is important to note that cotton varieties sown in all regions of Uzbekistan are an important source of nutrition for beekeeping farms. Cotton nectar attracts insects beneficial for pollination and protection, which enable the plants to achieve greater reproduction. According to our observations, the intensity of the hernia separation lasts from the morning to the middle of the day. At the same time, timely pollination of cotton varieties with bees is an important factor in increasing their productivity.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Orcid

G'ulom Hamidov https://orcid.org/0000-0002-0599-7562
Qodirjon Maxsudov https://orcid.org/0000-0002-1345-3936
Shahzoda Voxobova https://orcid.org/0000-0002-5203-3483
Dilyora Akramova https://orcid.org/0000-0002-1827-4304

5. REFERENCES

[1]. Layok, V. D. (1941). *Kulturnıye medonosı Uzbekstana i rol pçelv povışenii ix urojaynosti* (Tekst)/pod red. Akad. R.R.Şedreva-Taşkent: Uzgiz. pp. 68.
[2]. Eskov, E. K. (1992). *Etologiya medonosnoy pçelı*. Moskova, Kolos, pp. 336.
[3]. Hamidov, G. H. (1977) *Beekeeping and cotton growing*. Tashkent, pp. 132.
[4]. Kaziev, T. I. (1964). *Problems of nectar secretion in cotton and the role of honey bees in increasing its yield*. Baku, Azerb. Gos. İzdat. pp. 215.
[5]. Minkov, S. G. (1957). Nectar productivity of cotton in South-Kazakh region. *Pchelovodstvo*, *34*(12), 35-40.
[6]. Radoev, L. (1963). *Studies on bee pollination and honey productivity of cotton*. In 19th Internatl. Apic. Cong. Proc. Liblise, Czechoglovaciya. pp.99.
[7]. Bulgakova, L. L. (1978). *Medonosnaya rastitelnost pribrejnoy zonı srednogo teçeniya peki Sırdarı*. Avtoreferat dis. Kand. Biol. Nauk. Taşkent, pp. 19.

[8]. Anton, S., Komon, J. E., Denisow, B. (2017). Floral nectary, nectar production dynamics and chemical composition in five nocturnal Oenothera species (Onagraceae) in relation to loral visitors. *Planta*, *246*, 1051-1067. https://doi.org/10.1007/s00425-017-2748-y

[9]. Heil, M. (2011). Nectar: Generation, regulation, and ecological functions. *Trends in Plant Science*, 16, 191–200. https://doi.org/10.1016/j.tplants.2011.01.003

[10]. Chalcoff, V. R., Aizen, M. A., & Galetto, L.(2006). Nectar concentration and composition of 26 species from the temperate forest of South America. *Annals of Botany*, 97, 413–421. https://doi.org/10.1093/aob/mcj043

[11]. Knopper, L. D., Dan, T., Reisig, D. D., Johnson, J. D., & Bowers, L. M. (2016). Sugar concentration in nectar: A quantitative metric of crop attractiveness for refined pollinator risk assessments. *Pest Management Science*, 72, 1807–1812. https://doi.org/10.1002/ps. 4321

[12]. Nicolson, S.W. (2007). *Nectar consumers*. In S. W. Nicolson, M. Nepi, & E. Pacini (Eds.), Nectaries and nectar. Dordrecht, The Netherlands: Springer. pp. 289–342. ISBN 978-1-4020-5937-7

[13]. Gonzalez-Teuber, M., Silva-Bueno, J.C., Heil, M., & Boland, W. (2012). Increased host investment in extrafloral nectar (EFN) improves the efficiency of a mutualistic defensive service. *PLoS One*, *7*, e46598. https://doi.org/10.1371/journal.pone.0046598

[14]. Hanes, C. S. (1929). An application of the method of Hagedorn and Jensen to the determination of larger quantities of reducing sugars. *Biochem J.*, *23*(1), 99-106. https://doi.org/10.1042/bj0230099

[15]. Gilliam, M., Moffet, J. O., Kauffeld N. M. (1983). Examination of floral necta of citrus, cotton, and Arizona desert plants for microbes. *Apidologie*, *14*(4), 299-302. https://doi.org/10.1051/apido:19830403

[16]. Clark, E. W., Lukefahr, M. J. (1956). A partial analysis of cotton extrafloral nectar and its approximation. *Journal of Economic Entomology*, *49* (6), 875-876. https://doi.org/10.10 93/jee/49.6.875

[17]. Mound, L. A. (1962). Extrafloral nectaries of cotton and their secretions. *Emp. Cotton Grow. Rev*., *39*, 254-261. https://doi.org/10.1093/jxb/34.2.103

[18]. Hanny, B. W., Elmore, C. D. (1974). Amino acid composition of cotton nectar. *Agr. Food Chem.*, *22*, 476-478. https://doi.org/10.1021/jf60193a038

[19]. Yokohama, V. Y. (1978). Relation of seasonal changes in extrafloral nectar and foliar protein and arthropod. *Environmental Entomology*, *7*(6), 799–802. https://doi.org/10.109 3/ee/7.6.799

[20]. Llandres, A.L., Verdeny-Vilalta, O., Jean, J., Goebel, F. R., Seydi, O., Brévault, T. (2019). Cotton extrafloral nectaries as indirect defence against insect pests. *Basic and Applied Ecology*, *37*, 24-34. https://doi.org/10.1016/j.baae.2019.05.001

[21]. Hagenbucher, S., Olson, D. M., Ruberson, J. R., Wäckers, F. L., Romeis, J. (2013). Resistance mechanisms against arthropod herbivores in cotton and their interactions with natural enemies. *Critical Reviews in Plant Sciences*, *32* (6), 458-482. https://doi.org/10.1080/07352689.2013.809293

[22]. Grasso, D. A., Pandolfi, C., Bazihizina, N., Nocentini, D., Nepi, M. S. (2015). MancusoExtrafloral-nectar-based partner manipulation in plant-ant relationships. AoB Plants, *7*. https://doi.org/10.1093/aobpla/plv002

[23]. Özkök, D., Silici, S. (2018). Effects of crystallization on antioxidant property of honey. *Journal of Apitherapy*, *3*(2), 24-30. https://doi.org/10.5455/ja.20180607113134

International Journal of Secondary Metabolite
2020, Vol. 7, No. 4, 253–265

https://doi.org/10.21448/ijsm.648072

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Investigation of Some Biological Activities of Extracts *Centranthus longiflorus* subsp. *longiflorus*

Elif Ayar[1], **Nezahat Kandemir**[2,*], **Sevket Kandemir**[2], **Umut Celikoglu**[3], **Onder Idil**[4]

[1]Institute of Science and Technology, Amasya University, Amasya, TURKEY
[2]Department of Mathematics and Science, Faculty of Education, Amasya University, Amasya, TURKEY
[3]Department of Chemistry, Faculty of Science, Amasya University, Amasya, TURKEY
[4]Department of Pre-school Education, Faculty of Education, Amasya University, Amasya, TURKEY

Abstract: In this study, the effects of above and below-ground extracts of *Centranthus longiflorus* subsp. *longiflorus* plant, commonly found in Turkey, on antioxidant, antimicrobial and DNA damage were evaluated. Plant extracts were prepared by applying three different solvents (hexane, methanol and ethanol). The antimicrobial activity tests of the extracts were performed using four different standard strains and one yeast. DPPH, total phenolic content calculation and CUPRAC methods were applied for antioxidant activity studies. Additionally, the effects of plant extracts on DNA damage were investigated using pBR322 plasmid DNA. According to the data obtained, especially the below-ground hexane (MIC value:375µg/mL) extract showed more antimicrobial activity than other plant extracts, and it was found to be more effective Gram negative bacteria. The highest antioxidant activity was determined in extracts prepared with above (IC_{50} value of methanol extract:4.5mg/mL) and below-ground (IC_{50} value of methanol extract:5.7mg/mL) methanol. The above (93,9 µg GAE/mL) and below-ground (96.9 µg GAE/mL) methanol extracts were seen to have high total phenolic content. It has also been observed that above-ground hexane and methanol extracts have no effect on pBR322 plasmid DNA, but other extracts affect pBR322 plasmid DNA in the direction of degradation or deformation. Especially, the extracts of the above and below-ground ethanol had the effect of completely eliminating the open ring form. Therefore, it was concluded that this taxon could be widely used in the treatment and prevention of oxidative stress-related diseases in the future.

ARTICLE HISTORY

Received: December 18, 2019
Revised: February 25, 2020
Accepted: December 06, 2020

KEYWORDS

Antimicrobial,
Antioxidant,
Centranthus longiflorus,
pBR322 plasmid DNA

1. INTRODUCTION

Centranthus DC. genus belongs to Caprifoliaceae family and there are 3 species called *C. ruber* (L.) DC, *C. longiflorus* Stev. and *C. calcitrapa* (L.) Dufr. in Turkey [1]. *C. longiflorus* subsp. *longiflorus* Stev. is a herbaceous, rhizomatous, perennial plant with 70-200 cm in length. It distributes in rocky slopes, bushes and heavy soils at altitudes of 0-2300 m. The flowers are

CONTACT: Nezahat Kandemir ✉ nezahatkndmr@gmail.com Amasya University, Faculty of Education, Department of Mathematics and Science, Amasya, Turkey

ISSN-e: 2148-6905 /© IJSM 2020

dark pink. In Turkey, it spreads widely in Northern, Southern and Central Anatolia regions and is called as red valerian. The above and below-ground parts of this plant are used as sedative and anxiolytic [2]. Also, it has a high invasive species property for meadow.

Medicinal plants in the world are natural antioxidant sources as they contain some secondary metabolites such as phenolic acids, flavonoids, cinnamic acid derivatives, coumarins, tocopherols and tannins [3-5]. Therefore, they are preferred as they prevent or treat many diseases in many parts of world. Free radicals play a role in the development of many diseases. Antioxidants protect the human body against diseases caused by free oxygen derivatives. Valepotriates**,** iridoids, patrinoside, kanokoside A, oleanolic acid, valerianic acid, sitosterol, chlorogenic and caffeic acid, essential oils, saponins, resins, alkaloids and coumarins are isolated from above and below-ground parts of *Centranthus longiflorus* subsp. *longiflorus*. So that, some researchers indicated that it could be used in the treatment of diseases related to oxidative stress in the future [4-11].

Secondary metabolites with antimicrobial properties are grouped as phytochemical alkaloids, terpenoids, essential oils, phenolics, lectins-polypeptides and polyacetylenes by Cowan [12]. On the other hand, Okunade and Elvin-Levis [13] classified them as alkaloids, flavones, polyphenols, steroids, terpenoids, peptides, chromones, saponins, phenols, coumarins and chalcones. Some phenols and alkaloids are the most important groups of plant-derived antimicrobial agents [14, 15]. Secondary metabolites show the antimicrobial mechanism affecting the proteins and ion channels in the outer membrane of microorganisms. In particular, phenolic compounds inhibit different mechanisms in bacterial growth and are effective in the treatment of diseases caused by resistant strains [16]. DNA cleavage is an event that takes place in the cell throughout the natural process with enzymatic or oxidation processes. Besides, the goal of many anticancer drugs is to interact with DNA and trigger the cell apoptosis [17]. Therefore, the interaction of plant extracts and metal complexes with DNA is very important for the discovery of new drugs. Accordingly, we aimed to determine antioxidant, antimicrobial activities and effects on DNA damage of below and above-ground extracts of *C. longiflorus* subsp. *longiflorus* which are used traditionally in Turkey, in different organic solvents.

2. MATERIAL and METHODS

2.1. Collection and Identification of Plants Samples

Plant samples were collected during the flowering periods from the vicinity of Tortum Lake (Erzurum) and between Tortum-Oltu in 2014. The taxonomic description is based on Güner et al. [1] and Richardson [18]. The above and below-ground parts of plant samples were cut into small pieces and dried in the shade. Dried plant samples were stored in cloth bags until analyzed. Later, dried samples were milled and used for chemical analysis. Some of the plant samples also were made herbarium samples and deposited in the research laboratory of Faculty of Education, Amasya University, Turkey.

2.2. Preparation of Extracts

The below and above-ground parts of the subspecies were separated and extracted with Soxhlet apparatus by using different organic solvents. For this purpose, 100 g of plant samples were taken into Soxhlet cartridges and extracted with hexane, ethanol and methanol for 8 h [19]. The organic solvents evaporated under reduced pressure and the resulting extracts were stored at -20 °C until analysis.

2.3. Antioxidant Assay

2.3.1. Total Phenolic Compound Activity

The amount of phenolic compounds of the plant extracts were determined as the gallic acid equivalent (GAE) with the method determined by Singleton and Rossi [20]. 100 µl of the

extract solution (1 mg/mL) were completed with methanol to 2.3 mL and 50 µl of Folin-Ciocalteu reagent was added. After 3 min, 150 µl of 2% (w/v) Na_2CO_3 solution was added and incubated for two hours at room temperature. The absorbance of the samples was read at 760 nm (Thermo Scientific Genesys 10S UV-VIS Spectrophotometer) against the blank, which did not contain a test sample. The results were expressed as µg(GAE)/mL (extract).

2.3.2. DPPH Free Radical Scavenging Activity

The antioxidant activities of the plant samples were evaluated by testing the DPPH free radical scavenging activity. Butylated hydroxy anisole (BHA) was used as a standard antioxidant. For this purpose, 50 µl of different concentrations (3-10 mg/mL) of plant extracts were incubated with 2850 µl of DPPH solution ($6x10^{-5}$ M) in dark and at room temperature for 30 minutes. At the end of this period, the absorbance was measured 517 nm against the blank sample [21]. The DPPH % was calculated according to formula (I) and the results were expressed as IC_{50} value. IC_{50} refers to the concentration of plant at the moment when half of the DPPH amount was scavenging.

$$\text{Inhibition \%} = (A_{DPPH\cdot} - A_{sample}) / A_{DPPH\cdot} x100 \quad \text{(I)}$$

Respectively, A_{DPPH} refers to the DPPH radical in the absence of plant extract and A_{sample} refers to the DPPH radical in the presence of plant extract absorbance (at 517 nm).

2.3.3. Cu (II) Reducing Activity (CUPRAC)

In order to determine the antioxidant capacity of Trolox equivalent (TEAC), Cu (II) reduction activity test was performed by CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method. 1 mL of 10^{-2} M $CuCl_2$, 1 mL of $7.5x10^{-3}$ M neocuproine and 1 mL of 1 M NH_4Ac were placed in a test tube, respectively. These plant extracts were put into tubes at certain concentrations (10-100 µg/mL) and diluted with dH_2O to 4.1 mL. After the tubes were kept closed for 30 minutes at room temperature, the absorbance values at 450 nm were measured. Results were expressed as trolox equivalent (µmol trolox/mg extract) antioxidant capacity ($TEAC_{CUPRAC}$) utilizing formula (II) [22].

$$TEAC_{CUPRAC} = A_{(plant\ extract)} / A_{(Trolox)} \quad \text{(II)}$$

$A_{(plant\ extract)}$ and $A_{(trolox)}$ in Formula (II) refer to the molar absorption coefficient for plant extract and trolox, respectively.

2.4. Antimicrobial Activity

Antimicrobial activity was performed using the MIC (Minimum Inhibitory Concentration) method [23]. Microorganisms were obtained from Ondokuz Mayıs University. Gram positive (*Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 7064), Gram negative (*Escherichia coli* W3110, *Pseudomonas aeruginosa* ATCC 27853) and a yeast (*Candida albicans* ATCC 10231) were used. Stock solutions of the used extracts were prepared at a concentration of 40 mg/mL. Extracts were dissolved in DMSO. The last tube without bacterial growth was determined as MIC value. MIC values obtained in the study were shown as µg/mL.

2.5. DNA Interaction

Determination of the plant extracts effect on plasmid DNA (pBR322) was made according to agarose gel electrophoresis method [24]. For this purpose, 1% Agarose gel was prepared in TBE (1X) buffer. 120 µg/mL plant extracts were interacted with 0,5 µg/mL pBR322 plasmid DNA at 37^0C for 2 hours. After incubation samples were mixed with 6X loading dye and loaded on 1 % agarose gel. Electrophoresis was carried out 100 v for 80 min. The gel, then, was stained with EtBr (Ethidium Bromide) and the bands were imaged with the aid of the UV transilluminator (Cleaver Clear View).

3. RESULTS and DISCUSSION

Saponins, flavonoids, phenols and tannins are among the secondary metabolites in plants and play an important role in anticancer and antioxidant activities [25-27]. The iridoids, fatty acids and 16 phenolic compounds are isolated from the above and below ground parts of *C. longiflorus* subsp. *longiflorus* [6-8, 11]. For this, the above-ground and root parts of this taxon are used in the prevention and treatment of diseases such as soothing, antispasmodic, anticholytic, familial hypercholesterolemia, coronary artery disease and colon cancer in traditional Turkish medicine [10, 28]. On the other hand, scientists have stated that methanol is widely used and it is an effective solvent for the extraction of antioxidants [29, 30].

3.1. Total Phenolic Compounds

The standard graph was prepared using gallic acid in order to calculate the total phenolic content and shown in Figure 1. The phenolic contents of above and below-ground extracts were given in Table 1. When total phenolic results of above and below-ground extracts of *C. longiflorus* subsp. *longiflorus* were examined, above and below-ground methanol extracts were seen to have high total phenolic content. Especially above and below-ground hexane extracts had the lowest total phenolic content compared to other extracts of this subspecies. For this, above and below-ground methanol extracts of studied subspecies may be preferred as natural antioxidant sources in the future.

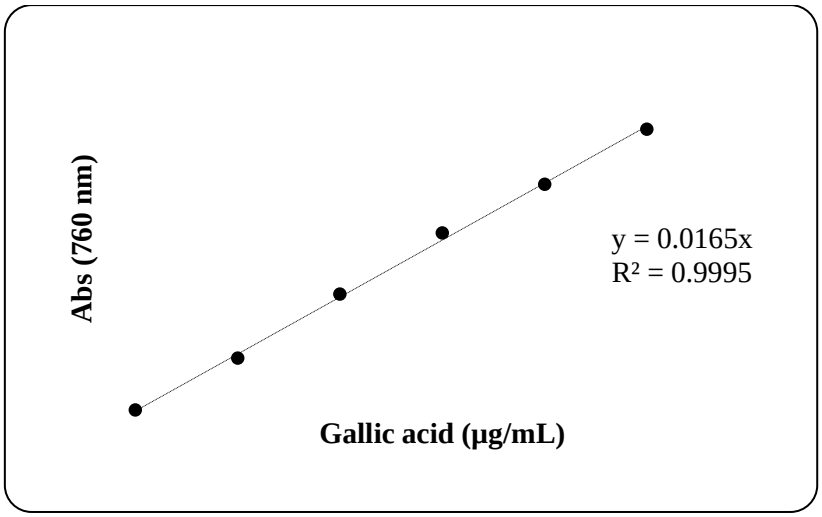

Figure 1. Gallic acid standard graph

3.2. Free Radical Scavenging Analysis

The free radical scavenging activity results of the above and below-ground extracts were given in Figure 2 and in Table 2. According to these results, IC_{50} values of above and below-ground hexane extracts could not be calculated since they do not have radical extinguishing capabilities. In particular, IC_{50} value of the above and below-ground methanol extracts had the highest antioxidant capacity. This situation is related to the high concentration of phenolic compounds in the above and below-ground methanol extracts. Antioxidant activity occurs due to the natural polyphenolic compounds present in plant extracts [31]. The amount of polyphenolic compounds in plants is important, since plants containing high polyphenolic compounds will be important antioxidant sources. We think that above and below-ground methanol extracts of *C. longiflorus* subsp. *longiflorus* may be used as an antioxidant source.

Table 1. Phenolic contents of plant extracts

Plant Extracts	Phenolic contents (µg GAE/mL)
Above-ground hexane	21.8
Above-ground ethanol	24.2
Above-ground methanol	93.9
Below-ground hexane	18.2
Below-ground ethanol	38.8
Below-ground methanol	96.9

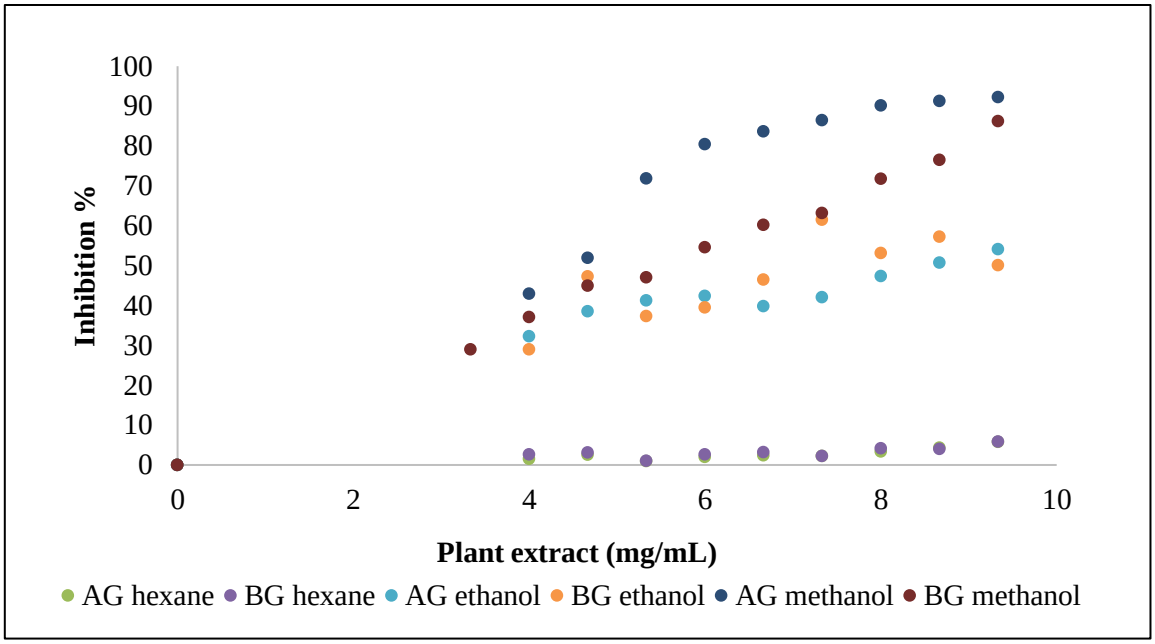

Figure 2. DPPH radical trapping activity. AG: Above-Ground, BG: Below-Ground

Table 2. IC_{50} values of plant extracts

Plant Extracts	IC_{50} (mg/mL)
BHA	0.019
Above-ground hexane	*
Above-ground ethanol	8.6
Above-ground methanol	4.5
Below-ground hexane	*
Below-ground ethanol	7.3
Below-ground methanol	5.7

*: IC_{50} values of hexane extracts could not be calculated

Similar results have been reported in antioxidant studies of *Centranthus longiflorus* subsp. *longiflorus.* Namely, Zengin et al. [32] used phosphomolybdenum, free radical scavenging activity and metal chelating activity tests for antioxidant activity of *C. longiflorus* subsp. *longiflorus* and *Cerinthe minor* subsp. *auriculata* (Ten.) Domac extracts. The extracts of both plants were prepared with methanol, ethyl acetate and aqueous. Plant samples of *C. longiflorus* subsp. *longiflorus* and *Cerinthe minor* subsp. *auriculate* collected from Ankara and Afyon vicinity. In particular, *C. longiflorus* subsp. *longiflorus* extracts prepared with methanol were determined to show higher antioxidant activity. Zengin et al. [32] reported that *C. longiflorus* subsp. *longiflorus* and *Cerinthe minor* subsp. *auriculata* could be valuable in the

development of new drug formulations and the preparation of new food supplements. In a study on *C. longiflorus* growing in Lebanon, three different tests (DPPH, H_2O_2 and iron chelating) were applied to plant extracts prepared with aqueous and methanol. The tests showed that antioxidant activity in *C. longiflorus* extracts were 80%, 70% and 50%, respectively [4]. In our study, it was determined that above and below-ground extracts especially prepared with methanol had significant antioxidant activity. Briefly, it is possible to think that the above and below-ground extracts prepared with methanol of this subspecies contain more polyphenols compounds than other extracts. Also, Aliyazıoğlu et al. [11] put forward that methanol extracts of *C. longiflorus* showed strong antioxidant activity. In short, our antioxidant results are consistent with antioxidant results of Rammal et al. [4], Aliyazıcıoğlu et al. [11], Zengin et al. [32]. This is due to the use of the same organic solvent and similar antioxidant tests in four studies. Similar antioxidant results were reported in *Gagea fibrosa* (Desf.) Schulte & Schultes fil.-leaves methanol extract and *Romulea ramiflora* Ten subsp. *ramiflora*-bulb methanol extract by Mammadov et al. [19]. On the other hand, Turan and Mammadov [33] reported the highest antioxidant activity in acetone extracts of *Cyclamen alpinum* Dammann ex. Springer. Molyneux [34] put forward that phenolic compounds can act as free radical scavengers based on their hydrogen-donating property. The above findings indicate that the antioxidant activity of methanol extract is associated with a high phenolic compound level. On the other hand, it can potentially be used to prevent oxidative stress-related and aging-associated diseases, since this taxon has numerous secondary metabolites and neutralizes free radicals in the body [35]. Aliyazıcıoğlu et al. [11] emphasized *that C. longiflorus* had rich phenolic compositions, antioxidant activities and potential for using as raw materials in the pharmaceutical and food industries in the prevention and treatment of various diseases due to oxidative stress.

In Turkey, antioxidant activities of some plants used in traditional medicine (including *Centranthus longiflorus*) were investigated by Çoban et al. [36]. Plant samples were collected from Ankara-Kızılcahamam. The above-ground extracts of plant samples were prepared using aqueous and ethanol. The above-ground ethanol extract of *C. longiflorus* was found to have the most potential antioxidant activity. Hence, Çoban et al. [36] reported that the above-ground ethanol extract can be considered as the best antioxidant source. Nevertheless, it is plausible to suggest that the ethanol extracts of this species may contain more polyphenolic compounds, because the polyphenolic compounds were determined to exhibit potent antioxidant activities [37, 38]. The above and below-ground parts of *Heliotropium samolifolium* Bunge subsp. *erzurumicum* Dönmez were extracted in the presence of different organic solvents (hexane, chloroform, ethyl acetate, ethanol, ethanol+aqueous and aqueous). Especially above ground ethanol+aqueous, chloroform and below-ground ethanol extracts of the *H. samolifolium* subsp. *erzurumicum* were reported to demonstrate the highest antioxidant activity [39]. In the biological activity studies of the endemic *Iris kirkwoodiae* Chaudhary, especially aqueous and methanol extracts exhibited very high antioxidant activity [40]. The antioxidant findings of Çoban et al. [36], Sağlam and Kandemir [39], Emaduldeen [40] are compatible with the antioxidant findings of our study. This may be due to the use of similar organic solvents and similar antioxidant methods. Similar organic solvents cause to appear of similar secondary metabolites, although plant species are different.

3.3. CUPRAC Analysis

The standard graph of Trolox was shown in Figure 3, the trolox equivalent of plant extracts are given in Figure 4 and in Table 3. The ε_T value of the trolox was calculated as 1.66 x 10^4L.mol^{-1}.cm^{-1}. In antioxidant studies, Cu^{2}+ reduction is used to determine electron donation activity. According to trolox equivalents, the above and below-ground methanol extracts were found to have high antioxidant activity. Aliyazıcıoğlu et al. [11] used ferric reducing/antioxidant power (FRAP) assay in methanol sample of *C. longiflorus*. Trolox

equivalent was found to be high in blossom (114 µmol Trolox/100 g DW) and trunk (156 µmol Trolox/100 g DW). In our study, the highest torolox equivalent was seen in above (126 µmol trolox/mg extract) and below-ground (132 µmol trolox/mg extract) methanol extracts. These data were showed parallelism with the results found in other antioxidant methods in our study. Similar Cu^{2+}reduction (CUPRAC) results were found in gall and leaves ethanol extracts of *Andricus quercustozae* by Azmaz et al. [41].

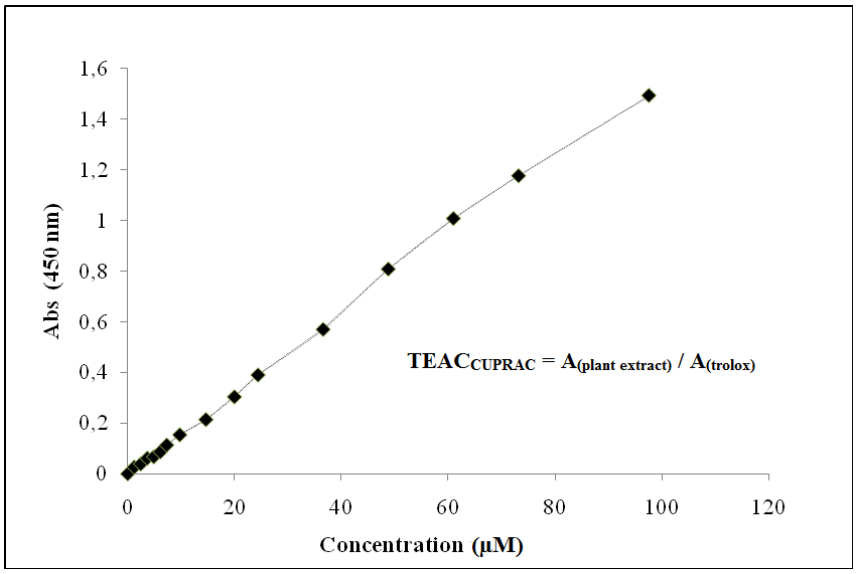

Figure 3. Trolox Cu^{2+} reduction power graph

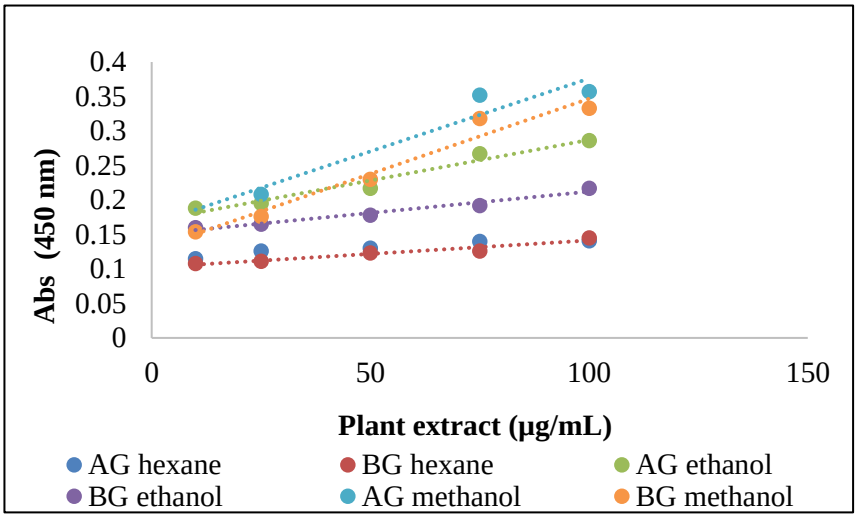

Figure 4. Cu^{2+} reduction power graph of plant extracts

3.4. Antimicrobial Analysis

MIC values of above and below-ground of plant extracts were given in Table 4. The below-ground hexane extract was found to have strong antimicrobial activity on *E. coli* and *P. aeruginosa*. However, the below-ground hexane extract was observed to show intermediate antimicrobial activity on *B. cereus* and *S. aureus*. The above-ground hexane extract had low antimicrobial activity on *E. coli*, *P. aeruginosa* and *C. albicans*. Also, the below-ground ethanol extract of this subspecies demonstrated antimicrobial activity on only *B. cereus* bacterium (Table 4).

Table 3. Trolox equivalent antioxidant capacity ($TEAC_{CUPRAC}$) of plant extracts

Plant Extracts	$TEAC_{CUPRAC}$ µmol trolox/mg extract
Above-ground hexane	18
Above-ground ethanol	72
Above-ground methanol	126
Below-ground hexane	24
Below-ground ethanol	36
Below-ground methanol	132

Table 4. MIC values on different microorganisms of plant extracts (µg/mL)

Plant extracts	*Escherichia coli* (W3110)	*Pseudomonas aeruginosa* (ATCC 27853)	*Bacillus cereus* (ATCC 7064)	*Staphylococcus aureus* (ATCC 6538P)	*Candida albicans* (ATCC 10231)
Above-ground hexane	1500	1500	3000	3000	1500
Below-ground hexane	375	375	750	750	1500
Above-ground ethanol	3000	3000	3000	3000	3000
Below-ground ethanol	3000	3000	1500	3000	3000
Above-ground methanol	3000	3000	3000	3000	3000
Below-ground methanol	3000	3000	3000	3000	3000

Aliyazıoğlu et al. [11] reported that aqueous and methanol extracts of *C. longiflorus* showed strong antimicrobial effect against *Mycobacterium smegmatis* ATCC607 bacterium. In our study, the strongest antimicrobial activity was seen in below-ground hexane extracts. Especially, below-ground hexane extracts have an activity on the Gram-negative, positive bacteria and yeast used in this study. This effect was seen higher in Gram negative bacteria than Gram positive bacteria. Therefore, it would be more appropriate to prepare an antimicrobial drug on Gram negative bacteria rather than Gram positive bacteria from extracts of this taxon. On the other hand, there are no antimicrobial effects on bacteria of above and below-ground ethanol and methanol extracts except below-ground ethanol. Our antimicrobial results do not match the antimicrobial results of Aliyazıoğlu et al. [11]. Such a case can be attributed to the collection of plant samples from different localities at different times and the use of different antimicrobial methods and different microorganism. Also, Aliyazıcıoğlu et al. [11] have made extraction process without separating above and below ground the plant materials. The aqueous and ethanol extracts of *Eryngium creticum* Lam. and *C. longiflorus* distributing in Lebanon were tested with five different bacteria (*Staphylococcus epidermidis* CIP 444, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853) by Makki et al. [10]. Aqueous extracts were reported to exhibit strong antibacterial activity in both plants. The reason why Makki et al. [10] and our antimicrobial results are incompatible may be due to the use of different bacterial strains and different organic solvents in the study. Also, this case may be due to the different cell wall structures of Gram-positive and Gram-negative bacteria.

In another antimicrobial research [39], the above and below-ground extracts of *H. samolifolium* subsp. *erzurumicum*, which distribute around Erzurum, were applied to Gram positive (*Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* NRLLB 1018), Gram negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) standard bacterial strains and a yeast (*Candida albicans* ATCC 10231). All above and below-ground

extracts of the subsp. *erzurumicum* except above-ground hexane and above-ground ethanol+aqueous were obtained to show intermediate antifungal activity on *C. albicans*. The below-ground ethanol extract was found to exhibit moderate activity on *S. aureus*, while the below-ground ethanol extract to exhibit stronger antimicrobial activity on *M. luteus* and *P. aeruginosa*. The below-ground ethanol extract of the *H. samolifolium* subsp. *erzurumicum* did not have any antimicrobial activity on only *E. coli*. Our antimicrobial findings do not match with the antimicrobial findings of Sağlam and Kandemir [39]. It is thought that this is due to the use of different plant extracts in the studies. Since different plant extracts have different secondary metabolites, the effects of these metabolites on microorganisms are different.

Anti-proliferative properties of *C. longiflorus* extracts collected from Lebanon were also examined. It was suggested that the leaf and stem parts of this plant had significant antioxidant and anti-proliferative activities, due to the presence of some secondary metabolites (alkaloids, coumarins, saponin, polyphenols, volatile oils, flavonoids) in the leaves and stem of *C. longiflorus* [42]. In a study, sedative, anticonvulsant and behavior modification activities of subsp. *longiflorus* extracts were investigated. In the effects of aqueous extract (100 mg/kg) compared with diazepam, aqueous was found to have sedative and anticonvulsant effects similar to those produced by diazepam (5 mg/kg) [43].

3.5. DNA Interaction

The DNA interaction results were shown in Figure 5. 1 and 2 lanes belong to pBR322 DNA+H_2O and pBR322 DNA+DMSO control groups, respectively. The extracts of above-ground hexane (Lane 3) and above-ground methanol (Lane 7) had no effect on pBR322 plasmid DNA, while the extracts of the above and below-ground ethanol (Lanes 5 and 6) had the effect of completely eliminating the open ring form. The below-ground methanol extract (Lane 8) had a disintegrating effect on the open ring structure. However, the below-ground hexane extract (Lane 4) was found to have an effect on increasing the concentration of open ring form.

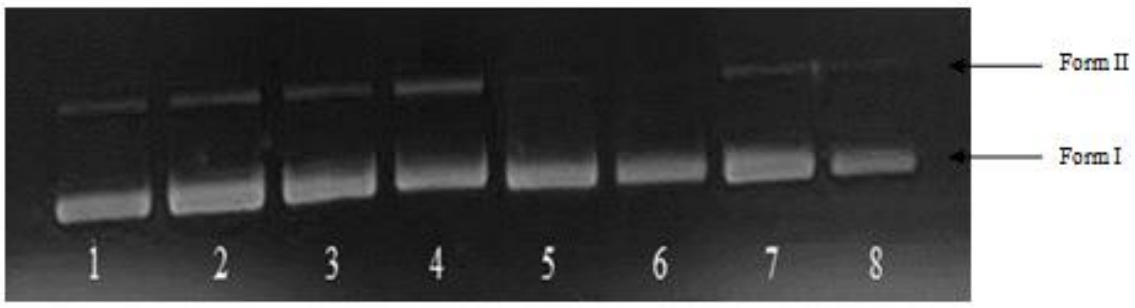

Figure 5. Agarose gel electrophoresis diagram. (Lane 1, 2) pBR322 DNA+H_2O, pBR322 DNA+DMSO control; (Lane 3, 4) pBR322 DNA+AG, pBR322 DNA+BG hexane extract; (Lane 5, 6) pBR322 DNA+AG, pBR322 DNA+BG ethanol extract; (Lane 7, 8) pBR322 DNA+AG, pBR322 DNA+BG methanol extract.

In a DNA interaction study, the below and above-ground chloroform and aqueous, above-ground hexane, below-ground ethyl acetate, ethanol extracts of endemic *H. samolifolium* subsp. *erzurumicum* showed a destructive effect to the structure of pBR322 plasmid DNA [39]. In the biological activity studies of the endemic *Iris kirkwoodiae*, all extracts except root dichloromethane and aqueous extracts showed protection for plasmid DNA against UV and H_2O_2 [40]. In another DNA interaction study, samples of endemic *Iris galatica* Siehe. were extracted with hexane, methanol dichloromethane and aqueous, all of the plant extracts were found to protect DNA against the harmful effects of UV and H_2O_2 [44]. Also, below-ground

ethanol extracts of *Leucojum aestivum* L. were displayed highly effect on pBR322 plasmid DNA [45]. In a similar study with endemic *Linaria corifolia* Desf., the above and below-ground ethanol, ethyl acetate and dichloromethane extracts were determined to show protective activity on plasmid DNA [46]. The plasmid DNA results of the mentioned-above studies are compatible with plasmid DNA results in our study.

4. CONCLUSION

Some biological and antioxidant activities of extracts of *C. longiflorus* subsp. *longiflorus* collected from the vicinity of Tortum (Erzurum) were determined. According to our antioxidant results, both above and below-ground methanol extracts can be used as antioxidant source in many areas. Our antimicrobial data showed that the below-ground hexane extracts of this plant may be added in the content of drugs used in the treatment of diseases Gram-positive and negative bacteria, yeast borne. In addition, above-ground hexane extracts can be included into the structure of drugs for the treatment of Gram negative and yeast diseases. Especially, the above and below-ground ethanol extracts of investigated subspecies were seen to have significant effect on pBR322 plasmid DNA. Briefly, above and below-ground hexane, methanol and ethanol extracts of this plant may be used in many areas of the pharmaceutical industry. Although there are some studies related to this subspecies, this study will contribute to other studies in this field as a new literature source. The study on DNA damage was done by us for the first time.

Acknowledgements

This study was produced within the scope of the project of FMB-BAP 14-073 supported by Amasya University. This study is derived from master thesis Elif Ayar's. “Comparison of antioxidant and antibacterial effects of *Centranthus longiflorus* ssp. *longiflorus* plant extracts prepared in different solvents, XII. National Ecology Congress, 14-17 September 20150”. The mentioned article was presented at this congress and only the abstract section was published in the abstract booklet of this congress**.**

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Orcid

Elif Ayar https://orcid.org/0000-0001-9281-1545
Nezahat Kandemir https://orcid.org/0000-0002-5428-4139
Sevket Kandemir https://orcid.org/0000-0001-6781-0057
Umut Celikoglu https://orcid.org/0000-0003-0995-8154
Onder Idil https://orcid.org/0000-0003-1744-4006

5. REFERENCES

[1]. Güner, A. (2012). *Centranthus* DC. In List of Turkey Plants (Vascular Pants), ed.; Aslan, S., Ekim, T., Vural, M., Babaç, M.T.; Publication of Nezahat Gökyiğit Botanic Garden and Floristic Research Society: İstanbul, Turkey, pp. 314. ISSN:978-605-60425-7-7.

[2]. Baytop, T. (1999). Used Medicine Plants in Turkey. In Therapy with Plant in Turkey (Past and Present). Ed.; Baytop, T.; Nobel Medicine Bookstores: İstanbul, Turkey, 975-420-021-1.

[3]. Rice-Evans, C., Miller, A., Paganga, G. (1997). Antioxidant Properties of Phenolic Compounds. *Trends Plant Sci.*, *2*, 152-59. https://doi.org/10.1016/SI3601385(97)01018-2

[4]. Rammal, H., Farhan, H., Hijazi, A., Bassal, A., Kobeissy, A., Badrtan, B. (2013). Phytochemical Screening and Antioxidant Activity of *Centranthus longiflorus* L. *J. Nat Prod Plant Resour.*, *3*, 29-36.

[5]. Wong, C.C., Li, H.B., Cheng, K.W., Chen, F. (2006). A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chem.*, *97*, 705-711. https://doi.org/10.1016/j.foodchem.2005.05.049

[6]. Sener, B., Mutlugil, A., Bingöl, F. (2008). Analysis of valepotriates in *Centranthus longiflorus* Stev. by reversed-phase high pressure liquid chromatography. *Pharm Biol*., *25*, 133-136. https://doi.org/10.3109/13880208709060916

[7]. Demirezer, L.Ö., Güvenalp, Z., Schiewe, H.J., Strietzel, I., Harmandar, M., Zeeck, A. (1999). Iridoids from *Centranthus longiflorus* ssp. *longiflorus. Phytochemistry*, *51*, 909-912. https://doi.org/10.1016/s0031-9422(99)00152-1

[8]. Kuruüzüm, A-Uz., Güvenalp, Z., Demirezer, L.Ö., Bergere, I., Stroch, K., Zeeck, A. (2002). 4-deoxy iridoid glycoides from *Centranthus longiflorus. Phytochemistry*, *61*, 937-941. https://doi.org/10.1016/S0031-9422(02)00476-4

[9]. Hassan, E., Tayebeh, R., Samaneh, E.T. (2008). Quantification of Valerenic Acid and its derivatives in some species of *Valeriana* L. and *Centranthus longiflorus* Stev. *Asian J. Plant Sci.*, *7*, 195-200. https://doi.org/10.3923/ajps.2008.195.200

[10]. Makki, R., Dirani, Z. E., Rammal, H., Sweidan, A., Al Bazzal, A., Chokr, A. (2015). Antibacterial Activity of Two Lebanese Plants: *Eryngium creticum* and *Centranthus longiflorus*. *J. Nanomed. Nanotechnol.*, *6*, 315-320. https://doi.org/10.4172/2157-7439.1000315

[11]. Aliyazıcıoğlu, R., Korkmaz, N., Akkaya, S., Sener, S.Ö., Badem, M., Karaoğlu, S.A., Eyüpoğlu, O.E. (2016). Phenolic components, antioxidant and antimicrobial activities of *Centranthus longiflorus* L. *Int. J. Adv. Res. Biol. Sci*., *3*, 80-87. https://doi.org/10.22192/ijarbs.2016.03.10.012

[12]. Cowan, M.M. (1999). Plant Products as Antimicrobial Agent. *Clin microbiol Rev.*, *12*, 564-582. https://doi.org/10.1128/CMR.12.4.564

[13]. Okunade, A.L., Elvin-Lewis, M.P.F. (2004). Natural Antimycobacterial Metabolites: Current Status. *Phytochemistry*, *65*, 1017-1032. https://doi.org/10.1016/j.phytochem.2004.02.013

[14]. Taguri, T., Tanaka, T., Kouno, I. (2004). Antimicrobial Activity of 10 Different Plant Polyphenol Against Bacteria Causing Food-Borne Disease. *Biol Pharm Bull.*, *27*, 1965-1969. https://doi.org/10.1248/bpb.27.1965

[15]. Cheesman, L., Nair, J.J., Van Staden, J. (2012). Antibacterial Activity of Crinane Alkaloids From *Boophone disticha* (Amaryllidaceae). *J Ethnopharmacol.*, *140*, 405-408. https://doi.org/10.1016/j.jep.2012.01.037

[16]. Topal, Y. (2013). Investigation of Antioxidant and Antimicrobial Effects of Phenolic Compounds of Some Species of *Alchemilla* L. (Rosaceae) genus. Master thesis, Institute of Science and Technology, Bingöl University, Bingöl, Turkey.

[17]. Kawanishi, S., Hiraku, Y. (2004). Amplification of anticancer drug-induced DNA damage and apoptosis by DNA-binding compounds. *Curr Med Chem-Anticancer*

Agents, *4*(5), 415-419.
https://doi.org/10.2174/1568011043352867

[18]. Richardson, I.B.K. (1972). *Centranthus* DC. In Flora of Turkey and The East Aegean Islands, 4th ed.; Davis, H.P., Chamberlain, D.F., Phil, D., Victoria, A., Matthews, B.; Edinburgh University Press: Edinburg, England, Volume 4, pp. 558-559; ISSN:0852242085

[19]. Mammadov, R., Ili, P., Vaizoğullar, H.E., Makasçı, AA. (2011). Antioxidant Activity and Total Phenolic Content of *Gagea fibrosa* and *Romulea ramiflora*. *Iran J. Chem. Chem. Eng.*, *30*, 57-62.

[20]. Singleton, V.L., Rossi, J.A. (1965). Colimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic*., *16*, 144-158.

[21]. Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci Technol*., *28*, 25-30.
https://doi.org/10.1016/S0023-6438(95)80008-5

[22]. Apak, R., Güçlü, K., Özyürek, M., Karademir, S.E. (2004). A novel total antioxidant capacity index for dietary polyphenols, Vitamin C and E, using their cupric ion reducing capability in the presence of neocuroine: CUPRAC method. *J. Agric. Food Chem*., *52*, 7970- 7981. https://doi.org/10.1021/jf048741x

[23]. Andrews, J.M. (2001). Determination of minimum inhibitory concentrations. *J Antimicrob Chemother*., *48*, 5-16. https://doi.org/10.1093/jac/48.suppl._1.5

[24]. Babu, J., Pramod, W.R., George, T., Nitisha, S. (2007). Standard Review Cold-active microbial Lipases: a versatile tool for industrial applications. *Biotechnol. Mol. Biol. Rev.*, *2*, 39-48.

[25]. Wink, M., Ashour, M.L., El-Readi, M.Z. (2012). Secondary Metabolites from Plants Inhibiting ABC Transporters and Reversing Resistance of Cancer Cells and Microbes to Cytotoxicand Antimicrobial Agents. *Front Microbiol.*, *3*, 130.
https://doi.org/10.3389/fmicb.2012.00130

[26]. Law, P.C., Auyeung, K.K., Chan, L.Y., Ko, J.K. (2012). *Astragalus* Saponins Downregulate Vascular Endothelial Growth Factor Under Cobalt Chloride-Stimulated Hypoxia in Colon Cancer Cells. *BMC Complement Altern Med.*, *12*, 160. https://doi.org/10.1186/1472-6882-12-160

[27]. Zamora-Ros, R., Agudo, A., Luján-Barroso, L., Romieu, I., Ferrari, P., Knaze, V., Sánchez-Cantalejo, E., et al. (2012). Dietary Flavonoid and Lignan Intake and Gastric Adenocarcinoma Risk in The European Prospective Investigation into Cancer and Nutrition (EPIC) study. *Am J Clin Nutr.*, *96*, 1398-1408.
https://doi.org/10.3945/ajcn.112.037358

[28]. Süleyman, H., Güvenalp, Z., Kızılkaya, M., Demirezer, Ö.L. (2007). Sedative effect of *Centranthus longiflorus* ssp. *longiflorus* in rats and the influence of adrenalectomy on its effect. *Yakugaku Zasshi.*, *127*, 1263-1265. https://doi.org/10.1248/yakushi.127.1263.

[29]. Hassan, R., Hussein, F., Akram, H., Ali, B., Ahmed, K., Bassam, B. (2013). Phytochemical Screening and Antioxidant Activity of *Centranthus longiflorus.* L. *J. Nat. Prod. Plant Resour.*, *3*, 29-36.

[30]. Yen, G.C., Wu, S.C., Duh, P.D. (1996). Extraction and Identification of Antioxidant Components from The Leaves of Mulberry (*Morus alba* L.). *J. Agric. Food Chem.*, *44*, 1687-1690.
https://doi.org/10.1021/jf9503725

[31]. Yılmaz, A., Yıldız, S., Tabbouche, S., Kılıç, A.O., Can, Z. (2016) Total Phenolic Content, Antioxidant and Antimicrobial Properties of *Pleurotos ostreatus* Grown on Lime (*Tilia tomentosa*) Leaves. *Hacettepe J. Bio and Chem*, *44*, 119-124. https://doi.org/10.15671/HJBC.20184417585

[32]. Zengin, G., Nithiyanantham, S., Locatelli, M., Ceylan, R., Uysal, S., Aktumsek, A., Palaniswamy, K., Maskovic, P. (2016). Screening of In vitro Antioxidant and Enzyme Inhibitory Activities of Different Extracts from Two Uninvestigated Wild Plants: *Centranthus longiflorus* subsp. *longiflorus* and *Cerinthe minor* subsp. *auriculata*. *Eur. J. Integr.*, *8*, 286-292. https://doi.org/10.1016/j.eujim.2015.12.004

[33]. Turan, M., Mammadov, R. (2018). Antioxidant, Antimicrobial, Cytotoxic, Larvicidal and Anthelmintic Activities and Phenolic Contents of *Cyclamen alpinum*. *Pharmacol Pharm.*, *9*, 100-116. https://doi.org/10.4236/pp.2018.94008

[34]. Molyneux, P. (2004). The use of the stable free radical diphenylpicryllhydrazyl (dpph) for estimating antioxidant activity. *Songklanakarin J. Sci Technol.*, *26*, 211-219.

[35]. Balunas, M., Douglaskinghorn, AD. (2005). Drug discovery from medicinal plants. *Life Sci.*, *78*, 431-441. https://doi.org/10.1016/j.lfs.2005.09.012

[36]. Çoban, T., Saltan, ÇG., Sever, B., İşcan, M. (2003). Antioxidant activities of plants used inTraditional Medicine in Turkey. *Pharm. Biol. 41*, 608-613. https://doi.org/10.1080/13880200390501974

[37]. Hasanaki, Y., Ogawar, S., Fukui, S. (1994). The correlation between active oxygen species and antioxidative effects of flavonoids. *Free Radic Biol Med.*, *16*, 845-850. https://doi.org/10.1016/0891-5849(94)90202-x

[38]. Sanz, MJ., Ferrandiz, M., Cejudo, M., Terencio, MC., Gil, B., Bustos, G., Ubeda, A., Gunasegara, R., Alcanaz, MJ. (1994). Influence of a series of natural flavonoids on free-radical generating systems and oxidative stress. *Xenobiotica*, *24*, 689-699. https://doi.org/10.3109/00498259409043270

[39]. Sağlam, G., Kandemir, N. (2020). Comparison of Biological and Antioxidant Activities of Above and Below-Ground Extracts of Endemic *Heliotropium samolifolium* subsp. *erzurumicum*. *KSU J. Agric. Nat.*, *23*, 1054-1063. https://doi.org/10.18016/ksutarimdoğa.vi.672571

[40]. Emaduldeen, A. (2014). Investigation of Biological Activities of *Iris kirkwoodii*. Master Thesis, Gaziantep University, Institute of Science and Technology, Gaziantep, 2014.

[41]. Azmaz, M., Aksoy, Ö.K., Katılmış, Y., Mammadov, R. (2020). Investigation of the Antioxidant Activity and Phenolic Compounds of *Andricus quercustozae* Gall and Host Plant (*Quercus infectoria*). *Int. J. Second. Metab.*, *7*, 77-87. https://doi.org/10.21448/ijsm.674930

[42]. Makki, R., Rammal, H., Farhan, H., Nasser, M., Dirani, El Z., Hijazi, A. (2015). The antioxidant and anti-tumor activity of the Lebanese *Centranthus longiflorus* L. *World J. Pharm. Sci.*, *3*, 347-354.

[43]. Büyükokuroğlu, M.E., Demirezer, L.O., Güvenalp, Z. (2002). Sedative, Anticonvulsant and Behaviour Modifying Effects of *Centranthus longiflorus* ssp. *longiflorus*: A Study of Comparison to Diazepam. *Pharmazie*, *57*, 559-561.

[44]. Orhan, E. (2015). Investigation of Biological Activities of Endemic *Iris galatica*. Master Thesis, Gaziantep University, Institute of Science and Technology, Gaziantep, 2015.

[45]. Hundur, Ö.D., İdil, Ö., Kandemir, N., Gül, M., Konar, V. (2018). Phytochemical Screening and In vitro Antioxidant, Antimicrobial Activity and DNA Interaction of *Leucojum aestivum*. *Fresen Environ Bull.*, *27*, 6704-6710.

[46]. Gül, M., Öztürk, Çalı, I., Cansaran, A., Idil, O., Kulu, I., Çelikoglu, U. (2017). Evaluation of Phytochemical Content, Antioxidant, Antimicrobial Activity and DNA Cleavage Effect of Endemic *Linaria corifolia* Desf. (Plantaginaceae). *Cogent Chem.*, *3*, 1-14. https://doi.org/10.1080/23312009.2017.1337293

International Journal of Secondary Metabolite
2020, Vol. 7, No. 4, 266–274

https://doi.org/10.21448/ijsm.685864

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Characterization of Native Alginates of Common Alginophytes from the Red Sea Coast of Sudan

Nahid Abdel Rahim Osman[1,*], **Tasneem Omer Suliman**[2], **Khadega Ahmed Osman**[1]

[1]Faculty of Marine Science and Fisheries, Red Sea University, Port Sudan, Sudan
[2]Marine Fisheries Administration, Ministry of Agriculture, Red Sea State, Port Sudan, Sudan

Abstract: The objective of this investigation was to investigate the content, viscosity, and major functional groups of the native alginates extracted from 4 alginophytes collected from the Red Sea coast of Sudan in order to evaluate the quality of the polymer for potential applications. The content of the polymer ranged from 7.99±2.60% to 19.1±1.62% based on dry weight in the 4 seaweeds. The peak characteristics of the major functional groups of alginates were identified between 3469.70 cm^{-1} to 451.31 cm^{-1}. These peaks specify the presence of hydroxyl (OH), carbonyl (C=O), and carboxyl (COOH) groups, respectively. The M/G ratios of the alginate extracted from the Sudanese brown macroalgae are ≤ 1 indicating that the proportion of guluronic acid is slightly greater than the mannuronic acid. The alginate with highest viscosity was that of *Padina pavonica* (Linnaeus) Thivy (190.7±1.14 mPa.s) followed by *Dictyota dichotoma* (Hudson) J.V.Lamouroux (146.96±1.2 mPa.s), *Turbinaria ornata* (Turner) J.Agardh (140.1±1.14 mPa.s), and *Hormophysa cuneiformis* (J.F.Gmelin) P.C.Silva (109±0.6 mPa.s). The values of the parameters of the native alginates extracted from the Sudanese macroalgae are comparatively in conformity with values reported for some species from similar geographical regions. These values indicated that the present biopolymer is of a potential with regard to pharmaceutical and industrial applications.

ARTICLE HISTORY

Received: February 09, 2020
Revised: March 14, 2020
Accepted: December 18, 2020

KEYWORDS

Alginates,
M/G ratio,
Viscosity,
Red Sea,
Sudan

1. INTRODUCTION

Brown macroalgae or Phaeophyta are almost entirely marine macroalgae. While they are the predominant macroalgae in the intertidal and subtidal zones of temperate to Polar Regions, they comparatively become less prominent at lower latitudes of the tropics. In the Red Sea coast of Sudan brown macroalgae are commonly conspicuous on the subtidal zone towards the reef flats in patchy distribution pattern.

Brown macroalgae are rich in bioactive metabolites such as polysaccharides, polyphenols, and terpenes. Polysaccharides or hydrocolloid polysaccharides are a class of macromolecules present primarily in the cell walls of marine macroalgae with varying composition according to the season, age, species, and geographic location [1]. Brown seaweeds are known to produce different polysaccharides, like the polymer group of alginates,

CONTACT: Nahid Abdel Rahim Osman ✉ nahidcoast@yahoo.com Faculty of Marine Science and Fisheries, Red Sea University, Port Sudan, Sudan

ISSN-e: 2148-6905 /© IJSM 2020

fucoidans, and laminarans. Laminarans and fucoidans are the main water-soluble polysaccharides of brown algae whereas the high-molecular mass alginates group is alkali soluble polysaccharides. Alginates perform vital functions pertinent to brown seaweeds biological integrity. They act as a food reserve and provide strength and flexibility to the plant to withstand wave action and maintain ionic equilibrium in the cell. The homogeneity of their structures promotes interaction with external ions and inter-chain hydrogen bonding (e.g. gelation). Of the polysaccharides of the brown macroalgae, the gel forming biopolymers alginates are very important economically and industrially [2]. The most common applications of alginates are in food, cosmetics, medical, and pharmaceutical industries where it is used as stabilizer and thickener, as wound dressings, and as matrices to encapsulate and/or release cells and medicine [3]. Emerging studies have reported that alginates have some biological activities [4,5] that may include immune-stimulation, prevention of chemically induced carcinogenesis and ulcerogenes, obesity control, and lowering of blood sugar and cholesterol level. Further, alginate has recently utilized in the field of tissue engineering [6-8].

Alginates have also been utilized in heavy-metal and toxic materials adsorption, in paper, textile, and food packages industries [9]. Alginates are groups of more than 200 compounds with common yet subtle variations in their physicochemical properties. Alginate is a linear copolymer made up of sequences or blocks of mannuronic acid (M-block), guluronic acid (G-block), and M and G blocks that coexist in different proportions and patterns in the alginate of the different species of brown macroalgae. The composition and distribution pattern of M-block and G-block are the factors responsible for the physicochemical properties of alginates' such as viscosity, sol/gel transition, and water-uptake ability [5]. Alginate with more G-block composition produces stronger, mechanically stable, and brittle gels, while that with more M-block forms soft and elastic gels.

The utilization of alginates in a particular use is dependent on the physicochemical properties of the polymer such as viscosity of the polymer in water solution and gelation. Alginate with high viscosity is considered of high quality compared to those of low viscosity. It has been proven that alginate with more proportion of G units has physicochemical properties suitable for industrial applications, while the alginate with more mannuronic acid units is suitable for pharmaceutical and environmental applications. The ratio of mannuronic acid to guluronic acid (M/G ratio) is used to infer the proportions of M and G in the alginate. This ratio is estimated from the FT-IR spectrum of the alginate with the wavelengths that characterize these acids. The M/G ratio, although the number and size of blocks are not provided, provides a practical estimate to evaluate the suitability of alginate for a particular use [10].

The objective of this investigation was to characterize the native alginates of 4 common brown macroalgae present along the Sudanese Red Sea coast with regard to yield, viscosity, and M/G ratio in order to evaluate its applicability.

2. MATERIAL and METHODS

2.1. Seaweeds Materials

The healthy and fully grown thalli of the brown seaweeds *Dictyota dichotoma*, *Hormophysa cuneiformis*, *Padina pavonica*, and *Turbinaria ornata* were collected from the Sudanese Red Sea coast north of Port Sudan city. The samples were thoroughly washed *in situ* with seawater to remove siltation and epiphytes. In the laboratory the samples were cleaned and washed with fresh water and distilled water to further remove salt and extraneous materials. Subsequently, the materials were spread in the shade to be air-dried. Then, the dry mass of macroalgae mass was ground to a homogenous powder and kept in airtight containers at 4º C until further use.

2.2. Determination of Moisture Content

The moisture content of the seaweed materials was determined gravimetrically according to AOAC [11].

2.3. Determination of Alginate Intrinsic Viscosity

The viscosity of 1% w/v alginate aqueous solution of pH around 3 to 3.5 was measured at room temperature with digital rotary viscometer (Zhengji, DNJ-5S) with due consideration to the manufacturer's guidelines.

2.4. Characterization of Sodium Alginate by Fourier Transform Infra Red

The sodium alginates of the 4 brown macroalgae were examined with Fourier Transform Infra Red (FT-IR) spectroscopy (Shimadzu, FT-IR 8400S). Small portion of the alginate was mixed with potassium bromide and compressed into a firm disc for the FT-IR examination. The spectra were obtained in transmission mode in the range of 400 to 4000 cm^1 and were the average of 2 measurements undertaken at a resolution of 2 cm^{-1} and 40 scans. The ratio of the mannuronic acid to guluronic acid (M/G) in the native sodium alginate extracted in this investigation was determined based on the infrared spectra. The absorbance at the wave number 1100 cm^{-1} corresponding to the mannuronic acid and at the wave number 1125 cm^{-1} corresponding to the guluronic acid was determined with the most probable baseline method [13] and the equation:

A= 2-logT where A is the absorbance and T is the transmission.

3. RESULTS and DISCUSSION

The content and viscosity of sodium alginate in *D. dichotoma*, *H. cuneiformis*, *P. pavonica*, and *T. ornata* are given in Table 1. The highest content of alginate was obtained from *H. cuneiformis* (19.1±1.62%) and *T. ornata* (14.3±1.2%) respectively. These two algae belong to the same order Fucales. Comparatively, lower content of alginate was obtained from *D. dichotoma* (8.8±2.12%) and *P. pavonica* (7.99±2.60%) that belong to the order Dictyotales.

Table 1. Content and viscosity of sodium alginate in *D. dichotoma*, *H. cuneiformis*, *P. pavonica*, and *T. ornata* from Sudan.

No	Species name	Content of alginate (%)	Viscosity (mPa-s)
1	*Dictyota dichotoma*	8.8±2.12	146.96±1.2
2	*Hormophysa cuneiformis*	19.1±1.62	109±0.6
3	*Padina pavonica*	7.99±2.60	190.73±1.14
4	*Turbinaria ornata*	14.3±1.2	140±1.14

The value of the alginate content of the present *D. dichotoma* is different from that obtained for the same species from the Egyptian coast (22.9% to 20.9%) as recorded in Deyab et al. [14]. The difference in the yield could be attributed to the longer extraction time (3 hrs) and the high temperature (100º C) employed during the extraction of the Egyptian species or to the acidic treatment of the algal mass before extraction. It is worth to mention that the alginate content of the Dictyotales from the Sudanese Red Sea coast (*D. dichotoma* and *P. pavonica*) is comparable to its content in Dictyotales from the Mexico coast as reported in García-Ríos et al. [15] where it was 7.4±1.1% for *Dictyota caribaea* Hörnig & Schnette and 5.4±0.2% for *Padina perindusiata* Thivy.

On the other hand, some of the values of alginate content of the 4 brown macroalgae could relatively be considered in conformity with those reported for some tropical brown macroalgae. For instance, the alginate content of a *Sargassum* species, ranged from 12% to 16.5% when the

alga was treated with an acidic solution prior to the extraction [16]. However, the yield of alginate of the same alga increased remarkably to 25-30% when the alga was treated with alkali solution before the extraction was performed.

The content of alginate of the present *T. ornata* (14.3%) is congruent with that of the same species from Indonesia (13.33%) recorded in Widyastuti [17]. Also, the content of alginate in *Turbinaria decurrens* Bory was 14.2% [18] similar to the value obtained for the *T. ornata* in this investigation. Nevertheless, the alginate content of the former alga fluctuates from 9.6% to 16.9% when the algal mass was treated with different concentrations of formalin (2% to 40%). It is worth to mention that the lower concentrations of the formalin (2% and 10%) as well as the highest concentration (30% to 40%) produced lower amount of alginate compare to the control sample (14.2%). The alginate content in *Hormophysa triquetra* (C.Agardh) Kützing extracted for 1 hour time was 19.20% relatively similar to the present value of alginate content in *H. cuneiformis*. Relatively higher amount of alginate (12.4%) were reported for *Padina tetrastromatica* Hauck than that reported here for *P. pavonica* (7.99%).

It was reported that the yield of alginate increases with the increase in alkali concentration, the time of extraction, and the pretreatment of the algal materials with acids [16,18]. In line with this, the yield of sodium alginate extracted from *Sargassum* species (Fucales) ranged from a minimum of 12.13% to 30.1% due to application of different extraction methods [16]. However, acid pre-treatment of the algal mass before the alkali extraction of the alginate has been reported to reduce the viscosity of the alginate [19]. Therefore, acid treatment step of the algal mass was not performed in this investigation. The alginate yield of the present brown macroalgae is anticipated to increase with the increase of the time and temperature of the extraction.

The results obtained in this investigation and those of Garcia-Rios et al. [15] may indicate that members of the Fucales comparatively contain more alginates than Dictyotales. The viscosity of the sodium alginate extracted in this study ranged from a lowest value of 109±0.6 mPa-s for *H. cuneiformis* to a highest value of 190.7.2±1.14 mPa-s for *P. pavonica* (Table 1). It's noteworthy to mention that the alginate of *H. cuneiformis,* which has produced the highest yield of alginate in this study, has the lowest viscosity. It has been reported that alginate has a wide range of viscosity. The viscosities of 1% w/v aqueous solutions of different types of sodium alginate have dynamic viscosities starting from 20 to 400 mPa·s at 20º C [5,20]. The values of the viscosity of the alginates extracted in this investigation are normal and within the known rage of the biopolymer viscosity.

The viscosity of alginates is dependent on the molecular weight of the polymer, the content of uronic acids, and the pH during the extraction process [10]. Alginates with high molecular weight and high guluronic acid content are more viscose. Therefore, it might be possible to infer that alginates from the tested species have low to intermediate molecular weight. Nevertheless, alginates with high viscosities were obtained under controlled extraction condition particularly on commercial scale. For instance, manipulation of the temperature, pH, and time of extraction produces alginates with high or low viscosity. Further, alginates with low viscosity are preferred for use in pharmaceutical industry in controlled-release drug delivery system [21] among other applications as they form softer hydrogels. Accordingly, based on the present values of the viscosity, the alginate of the investigated species might be suitable for pharmaceutical application taking into consideration that other prerequisites are also met. Typical FT-IR spectrum of sodium alginate shows absorption bands at 3390, 2930, 1615, 1410, 1320, 1300, 1170, 1150, 1125, 1090, 1035, 950, 900, 890, 815 and 780 cm−1. The FT-IR spectra of the alginate obtained from the 4 Sudanese brown macroalgae are shown in Figures 1, 2, 3 and 4. The FT-IR spectra of *D. dichotoma* alginate (Figure 1) showed strong to medium bands at 3419.56 cm^{-1}, 2921.96 cm^{-1}, 1616.24 cm^{-1}, 1415.65 cm^{-1}, 1126.35 cm^{-1},

1091.63 cm^{-1}, 1031.85 cm^{-1}, 946.88 cm^{-1}, 877.56 cm^{-1}, 838.98 cm^{-1}, 790.76 cm^{-1}, and weak bands at and 821.62 cm^{-1} and 1265.22 cm^{-1}.

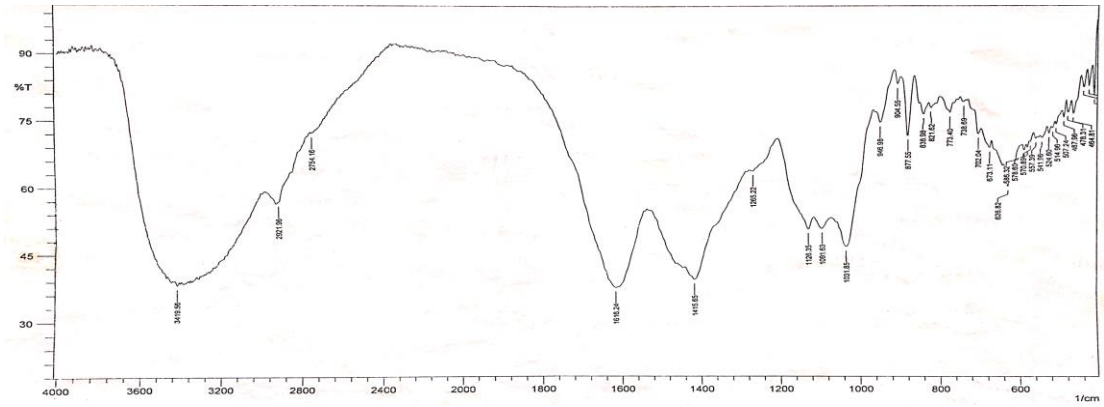

Figure 1. The FT-IR spectrum of the sodium alginate of *D. dichotoma* (Sudan, Red Sea coast)

The strong and medium bands of *H. cuneiformis* alginate FT-IR spectrum (Figure 2) occured at 3469.70 cm^{-1}, 2921.96 cm^{-1}, 1728.10 cm^{-1}, 1639.38 cm^{-1}, 1458.08 cm^{-1}, 1377.08 cm^{-1}, 1141.78 cm^{-1}, 1027.99 cm^{-1}, 948.91 cm^{-1}, 840.91 cm^{-1}, 790.76 cm^{-1}, and 610.11 cm^{-1}.

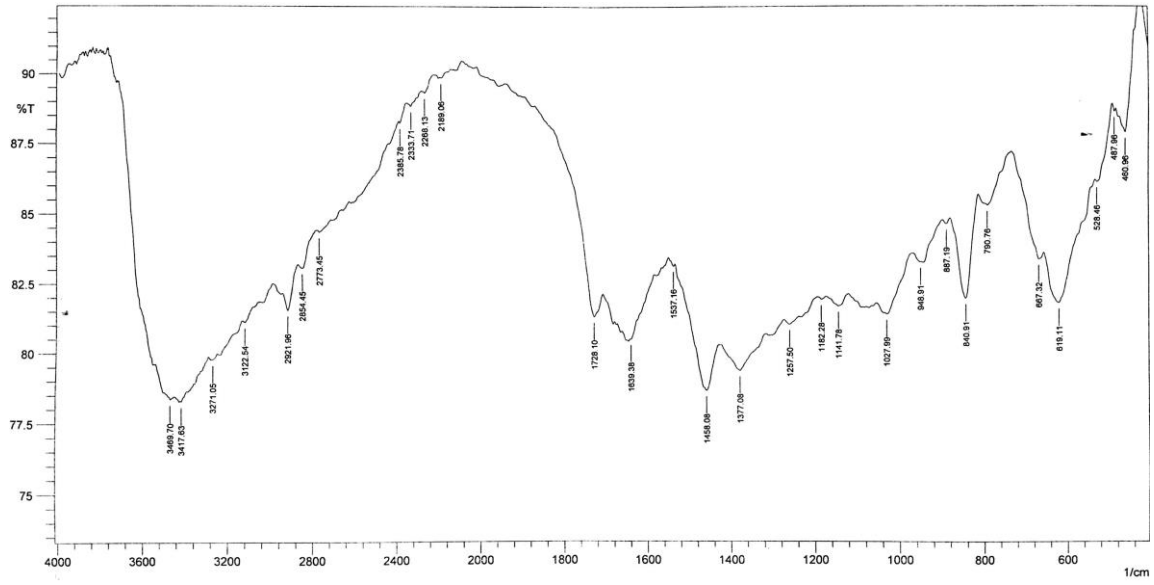

Figure 2. The FT-IR spectrum of the sodium alginate of *H. cuneiformis* (Sudan, Red Sea coast)

Almost all the bands of *P. pavonica* alginate FT-IR spectrum were strong and broad (Figure 3). They were recorded at 3382.91 cm^{-1}, 2927.74 cm^{-1}, 1728.17 cm^{-1}, 1616.17 cm^{-1}, 1458.08 cm^{-1}, 1407.94 cm^{-1}, 1124.42 cm^{-1}, 1093.56 cm^{-1}, 1029.92 cm^{-1}, 946.98 cm^{-1}, and 636.27 cm^{-1}. In additions, bands at 904.55 cm^{-1}, 875.62 cm^{-1}, 850.55 cm^{-1}, 840.91 cm^{-1}, 821.62 cm^{-1} were also detected.

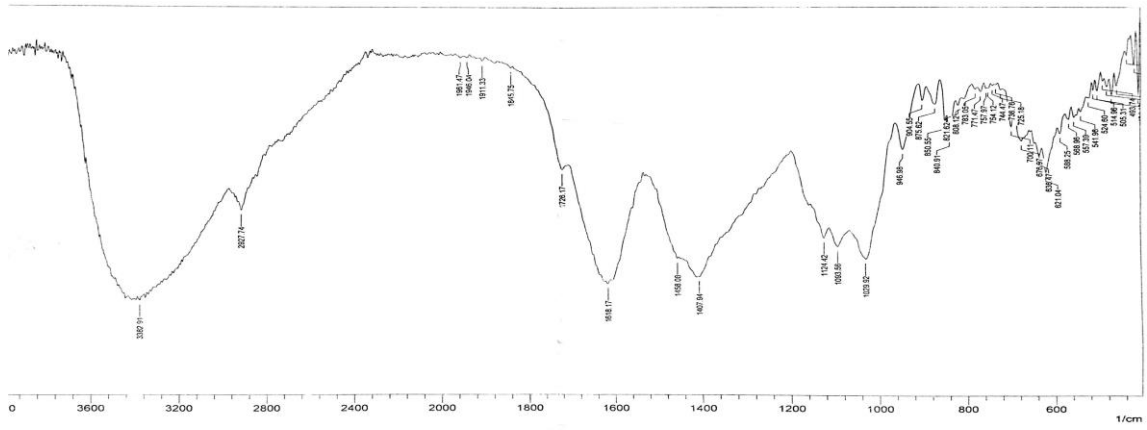

Figure 3. The FT-IR spectrum of the sodium alginate of *P. pavonica* (Sudan, Red Sea coast)

In *T. ornata* alginate FT-IR spectrum the following bands were recorded: 3423.41 cm^{-1}, 2931.60 cm^{-1}, 1623.95 cm^{-1}, 1512.09 cm^{-1}, 1417.58 cm^{-1}, 1359.72 cm^{-1}, 1122.49 cm^{-1}, 1087.78 cm^{-1}, 1033.77 cm^{-1}, 1026.0 cm^{-1}, 964.39 cm^{-1}, 875.62 cm^{-1}, and 838.98 cm^{-1} (Figure 4).

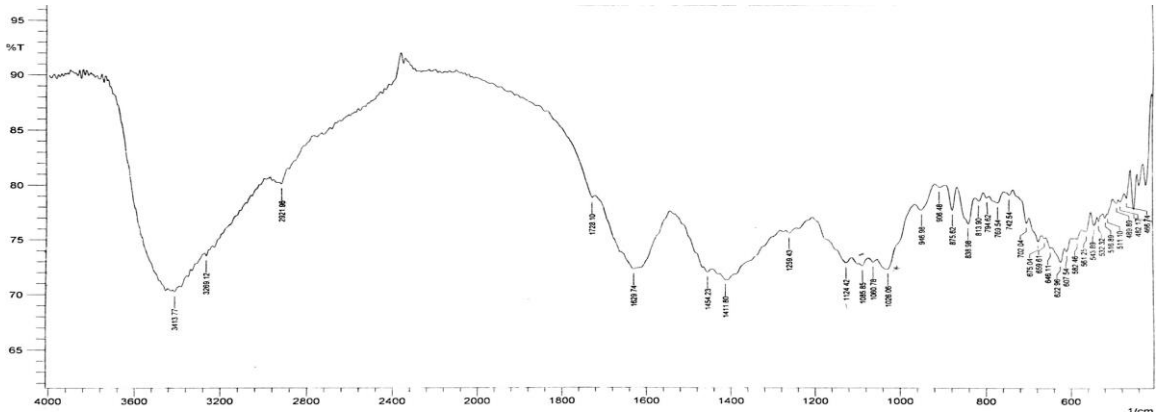

Figure 4. The FT-IR spectrum of the sodium alginate of *T. ornata* (Sudan, Red Sea coast)

Though, the alginate FT-IR spectra of the 4 seaweed species exhibited similar wavelength pattern, evident differences are perceptible between the fingerprint regions of the spectra (1400 cm^{-1} to 400 cm^{-1}). All spectra exhibited the characteristics bands of alginate [16,18, 22]. For instance the bands associated with stretching vibrations of O-H bonds of alginate that appears in the range of 3000-3600 cm^{-1} have been reported in all the present alginate spectra. Similarly, stretching vibrations of aliphatic C–H that are observed at 2900–2930 cm^{-1} were also detected here together with the bands around 1600 cm^{-1} and 1400 cm^{-1} which correspond to the carbonyl and carboxyl groups respectively. All the spectra showed a peak at 946.88 to 946.98 cm^{-1} which may belong to the mannuronic acid. Broadly, the alginates IR spectra of the tested species are in conformity with those reported for Sargassum species from the Indian coast [23] in particular with regard to the presence of peaks around 1728 to 1735 cm^{-1}.

In this investigation, the bands appeared around 1025 and 1100 cm^{-1} in the fingerprint region of the alginate FT-IR spectrum were assumed to refer to the absorbance of mannuronic and guluronic acids respectively[24, 25]. The M/G ratios of *D. dichotoma* (0.92), *H. cuneiformis* (0.97)**,** and *P. pavonica* (0.93) were all slightly ≤ 1 (Table 2) indicating that the content of mannuronic acid is slightly less than that of the of guluronic acid in the alginates of these species. This result corroborates with García-Ríos et al. [15] who reported that the alginates from the seaweed of the Mexico contained more mannuronic acid. For *T. ornata* the M/G ratio was slightly greater than one (1.01) suggesting that the alginate of this alga may

contain equal proportion of mannuronic and guluronic acids or the less mannuronic acid units are slightly greater than the guluronic acid ones.

Table 2. The M/G ratios of sodium alginate in *D. dichotoma*, *H. cuneiformis*, *P. pavonica*, and *T. ornata* from Sudan.

No	Species name	Alginate M/G ratio
1	*Dictyota dichotoma*	0.92
2	*Hormophysa cuneiformis*	0.97
3	*Padina pavonica*	0.93
4	*Turbinaria ornata*	1.01

On a broad basis these results are in accord with the information on the alginate of tropical brown macroalgae [15] that has been reported to have low proportion of mannuronic acid than guluronic acid and consequently low M/G ratio and viscosity. The M/G ratio is an index of the nature of gels produced, therefore alginates with low M/G ratio (<1) correspond to high values of guluronic than mannuronic acid form brittle gels [12], whereas alginates with high M/G ratio (>1) due to low content of guluronic acid produce soft and elastic gels. The M/G ratio of Padina spp was found to be 0.85 [26] slightly lower than that of the present *P. pavonica*. The dissimilarity in M/G ratio within species was attributed to variation in species age, extraction method, algal tissue, and geographical location [2, 27, 28].

4. CONCLUSION

The values of the parameters of the native alginates extracted from the Sudanese macroalgae are comparatively in conformity with values reported for some species from similar geographical region. These values indicated that the present biopolymer is of a potential with regard to pharmaceutical and industrial applications. It is worth to mention that some treatments applied during alginate extraction process have a significant bearing on enhancing the yield and the quality of the polymer. Further research should be carried out to test the effect of these treatments on the yield and characteristic of alginates from Sudanese materials.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Orcid

Nahid Abdel Rahim Osman https://orcid.org/0000-0001-6069-3396

5. REFERENCES

[1]. Gupta, S., and Abu-Ghannam, N. (2011). Bioactive potential and possible health effects of edible brown seaweeds. *Trends in Food Science & Technology*, *22*, 315-326.

[2]. Masuelli, M.A., Illanes, C.O. (2014). Review of the characterization of sodium alginate by intrinsic viscosity measurements. Comparative analysis between conventional and single point methods. *International Journal of BioMaterials Science and Engineering*, *1*(1), 1-11.

[3]. Rhein-Knudsen, N., Ale, M.T., Meyer, A.S. (2015). Seaweed Hydrocolloid Production: An Update on Enzyme Assisted Extraction and Modification Technologies. *Mar. Drugs*, *13*, 3340-3359. https://doi.org/10.3390/md13063340

[4]. Hernande-Carmona, G. (2013). Conventional and alternative technologies for the extraction of algal polysaccharides. In: Functional Ingredients from Algae for Food and Naturaceuticals. Woodhead Publishing Limited.

[5]. Szekalska, M., Pucilowska, A., Szymanska, E., Ciosek, P., Winnicka, K. (2016). Alginate: current use and future perspectives in pharmaceutical and biomedical applications. International *Journal of Polymer Science*, 2016, ID: 7697031. https://doi.org/10.1155/ 2016/7697031

[6]. Lee, K.Y., and Mooney, D.J. (2012). Alginate: properties and biomedical applications. *Prog Polym Sci*., *37* (1), 106–126. https://doi.org/10.1016/j.progpolymsci.2011.06.003

[7]. Nalamothu, N., Potluri, A., Muppalla, M. B. (2014). Review on marine alginates and its applications. *Indo American Journal of Pharm Research*, *4* (10), 4006-4015.

[8]. Guarino, V., Caputo, T., Altobelli, R., and Luigi Ambrosio, L. (2015). Degradation properties and metabolic activity of alginate and chitosan polyelectrolytes for drug delivery and tissue engineering applications. *AIMS Materials Science*, *2*(4), 497-502, https://doi.org/10.3934/matersci.2015.4.497

[9]. Parreidt, T. S., Müller, K., Schmid, M. (2018). Alginate-Based Edible Films and Coatings for Food Packaging Applications. *Foods*, *7*(170). https://doi.org/10.3390/foods71001 70

[10]. Silva, T.L., Vidart, J.M., Silva, M.G., Gimenes, M.L., Vieira, M.G. (2018). Chapter 4 Alginate and Sericin : Environmental and Pharmaceutical Applications. In: Shalaby, E. (edi). Biological Activities and Application of Marine Polysaccharides.

[11]. AOAC. (1990). Official Methods of Analysis, 16th ed. Association of official Analytical Chemists, Washington, DC.

[12]. Torres, M.R., Sousa, A.P.A., Filho, E.A.T.S., Melo, D.F., Feitosa, J.P.A., Paulab, R.C.M., Limaa, M.G.S. (2007). Extraction and physicochemical characterization of Sargassum vulgare alginate from Brazil. *Carbohydr Res.*, *342*, 2067-2074.

[13]. Rochas, C., Lahaye, M., Yaphye, W. (1986). Sulfate content of carrageenan and agar determined by infrared spectroscopy. *Botanica Marina*., *29*, 335-340.

[14]. Deyab, M.A., El-Katony, T.M., El-Adl M.F., Ward, F.M. (2017). Temporal variation in chemical composition of Dictyota dichotoma (Hudson) J.V. Lamouroux (Dictyotales, Phaeophyceae) from Red Sea Coast, Egypt. *Journal of Coastal Life Medicine*, *5*(4), 149-155.

[15]. García-Ríos, V., Ríos-Leal, E., Robledo, D., Freile-Pelegrin1, Y. (2012). Polysaccharides composition from tropical brown seaweeds. *Phycological Research*, *60*, 305–315.

[16]. Latifi, A.M., Nejad, E.S., Babavalian, H. (2015). Comparison of Extraction Different Methods of Sodium Alginate from Brown Alga *Sargassum* sp. Localized in the Southern of Iran. *Journal of Applied Biotechnology Reports*, *2*(2), 251-255.

[17]. Widyastuti, S. (2009). Alginate content of the seaweeds grown in coastal zone of Lombok extracted by two extraction methods. *Jurnal Teknologi Pertanian*, *10*(3), 144-152.

[18]. Subramanian, V., Ganapathy, K., and Dakshinamoorthy, B. (2015). FT-IR, ^{1}H- NMR and ^{13}C- NMR spectroscopy of alginate extracted from Turbinaria decurrens (Phaeophyta). *World Journal of Pharmacy and Pharmaceutical Sciences*, *4*(12), 761-771.

[19]. Hernandez-Carmona, G., McHugh, D.J., Lopez-Gutierrez, F. (1999). Pilot plant scale extraction of alginate from Macrocystis pyrifera. 2. Studies on extraction conditions and methods of separating the alkaline-insoluble residue. *J. Appl. Phycol.*, *11*, 493–502.

[20]. Sachan, N.K., Pushkar, S., Jha, A., Bhattcharya, A. (2009). Sodium alginate: the wonder polymer for controlled drug delivery. *Journal of Pharmacy Research*, *2*(8),1191-1199.

[21]. Nahar, K., , Hossain, M. K., Khan, T. A. (2017). Alginate and its versatile application in drug delivery. J. *Pharm. Sci. & Res*., *9*(5), 606-617.

[22]. Torres, M.L., Cortizo, A.M., Oberti, T.G., Fernández, J.M. (2016). Characterization of commercial and algae (*Undaria pinnatifida*) extracted sodium alginate for future application in bone tissue engineering. Colaob, 24 to 27 de Agosto de 2016 Foz do Iguacu. PR.

[23]. Ganapathi, K., Subramanian, V., Mathan, S. (2013). Bioactive potentials of brown seaweeds, *Sargassum myriocystum* J. Agardh, *S. plagiophyllum* C.Agardh and *S. ilicifolium* (Turner) J. Agardh. *Int. Res J Pharm. App Sci.*, *3*(5), 105-111.

[24]. Filippov, M.P., and Kohn, B.R. (1974). Determination of composition of alginates by infrared spectroscopic method. *Chem. Zvesti*, *28*(6), 817-819.

[25]. Pereira, L., Sousa, A., Coelho, H., Amado, A.M., Ribeiro-Claro, P.J.A. (2003). Use of FT-IR, FT-Raman and ^{13}C-NMR spectroscopy for identification of some seaweed phycocolloids. *Biomolecular Engineering*, *20*, 223-228.

[26]. Omar, S., Ahmad, N., Ahmad, F. (1988). Composition of Alginates from Brown Seaweeds, *Sargassum* and *Padina* spp. *Pertanika*, *11*(1), 79-85.

[27]. Murillo, Á., and Hernández, C. (2007). Monomer composition and sequence of sodium alginate extracted at pilot plant scale from three commercially important seaweeds from Mexico. *J. Appl Phycol*, *19*, 545–548. https://doi.org/10.1007/s10811-007-9168-5

[28]. Fertah, M., Belfkira, A., Dahmane, E., Taourirte, M., Brouillette, F. (2017). Extraction and characterization of sodium alginate from Moroccan *Laminaria digitata* brown seaweed. *Arabian Journal of Chemistry*, *10*, 3707-3714.

International Journal of Secondary Metabolite
2020, Vol. 7, No. 4, 275–285

https://doi.org/10.21448/ijsm.715530

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Examination of Substrate Specificity of the First Adenylation Domain in *mcyA* Module Involved in Microcystin Biosynthesis

Gozde Yaman[1], **Mete Yilmaz**[1,2,*]

[1]Program in Biotechnology, Graduate School of Natural and Applied Sciences, Bursa Technical University, Bursa, Turkey
[2]Department of Bioengineering, Faculty of Engineering and Natural Sciences, Bursa Technical University, Bursa, Turkey

Abstract: The cyanotoxin microcystin (MC) is a secondary metabolite, synthesized by nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) enzymes. It has many isoforms and the mechanism of its diversity is not well understood. One of the MC synthetase genes, *mcyA*, codes for the McyA module containing two adenylation (A) domains. The first domain, McyA-A1, generally binds to L-serine (L-ser). Then the *N*-methyl transferase (NMT) domain converts L-Ser into *N*-methyldehydroalanine (Mdha), which usually occupies position 7 on the MC molecule. However, various other amino acids (AAs) might also be present at this position. In this study, bioinformatic analyses of selected cyanobacteria were performed to understand whether genetic information in the first adenylation domain of *mcyA* could explain incorporation of different AAs at position 7 of the MC molecule. Binding pocket signatures of McyA-A1 and putative activated AAs were determined via various bioinformatics tools. Maximum likelihood phylogenetic trees of full length *mcyA*, *mcyA*-A1 and 16S rRNA genes were prepared in Mega 6. Phylogenetic analysis of *mcyA*-A1 nucleotide sequences was in agreement with the predictions of activated AAs by McyA-A1. In comparison with the 16S rRNA and full length *mcyA* gene trees, *mcyA*-A1 phylogenetic trees suggested horizontal transfer of the A domain in either *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek or *Planktothrix rubescens* (De Candolle ex Gomont) Anagnostidis & Komárek strains. Predictions of activated AAs were generally in agreement with the chemically determined position 7 AAs. However, there were exceptions suggesting the multispecificity of the first A domain of McyA in some cyanobacteria.

ARTICLE HISTORY

Received: April 06, 2020
Revised: September 17, 2020
Accepted: November 21, 2020

KEYWORDS

Microcystin,
Nonribosomal Peptides,
Polyketide Synthase,
Adenylation Domain,
Bioinformatics

1. INTRODUCTION

There are approximately 500 natural amino acids [1] and they can be classified based on their R groups, stereoisomer structures (D- and L-form), being proteinogenic or nonproteinogenic. Proteinogenic amino acids are all 20 kinds of L-form amino acids, where mRNA, tRNA and ribosomes are involved in the protein synthesis pathway. Nonproteinogenic amino acids are D-form or β-amino acids and synthesized by either non-ribosomal peptide

CONTACT: Mete Yilmaz ✉ mete.yilmaz@btu.edu.tr Department of Bioengineering, Faculty of Engineering and Natural Sciences, Bursa Technical University, Bursa, Turkey

ISSN-e: 2148-6905 /© IJSM 2020

synthetases (NRPSs), polyketide synthases (PKSs) or hybrid NRPS/PKS modular enzyme systems.

Cyanobacteria can produce a variety of toxic secondary metabolites (cyanotoxins) which are generally subdivided into neurotoxins, hepatotoxins, cytotoxins and dermatotoxins. Cyanotoxins are usually synthesized nonribosomally [2,3]. Microcystin (MC) is also a nonribosomally synthesized cyanotoxin, which shows hepatotoxic and neurotoxic effects, inhibiting eukaryotic serine/threonine protein phosphatases PP1 and 2 [4,5]. Its general structure is cyclo(-D-Ala[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-Glu[6]-Mdha[7]), where X and Z are various L-amino acids (Figure 1) and D-Glu is D-iso-glutamic acid, Mdha is *N*-methyl-dehydroalanine, D-Ala is D-Alanine, D-MeAsp is D-*erythro*-β-methyl aspartic acid and Adda is (2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Microcystin producing species are found in various genera including *Microcystis*, *Anabaena*, *Nostoc*, *Planktothrix (Oscillatoria)*, *Phormidium*, *Fischerella* and *Hapalosiphon* [6,7].

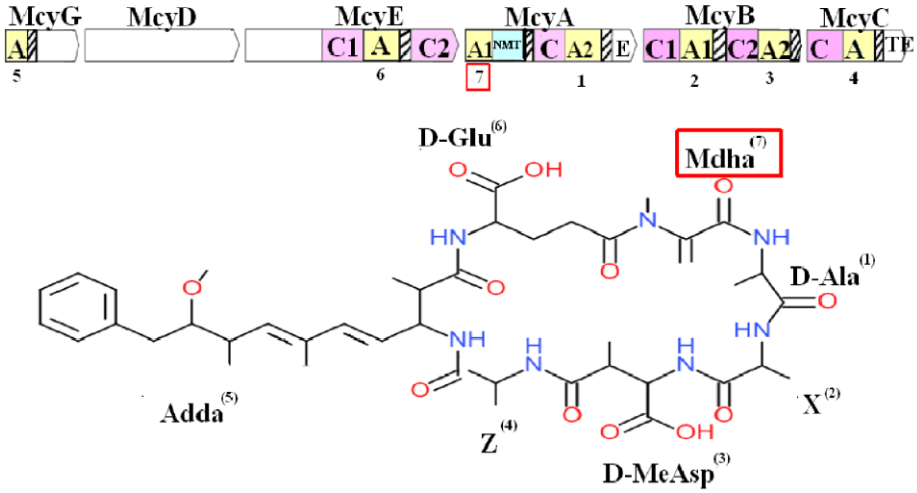

Figure 1. Schematic representation of MC synthetase operon (top) and MC structure (below). Position 7, which is investigated in this study, is marked. A, adenylation domain; C, condensation domain; NMT *N*-methyl transferase domain; TE, thioesterase domain. Peptidyl carrier protein is indicated in rectangular shape with lines.

Sequencing of MC biosynthesis genes was initially completed for *Microcystis aeruginosa* PCC 7806 [4], followed by *Planktothrix agardhii* NIVA CYA 126/8 [8] and *Anabaena* sp. 90 [9]. Within the MC synthetase operon, NRPS modules are expressed by *mcyA*, *-B* and *-C*; PKS modules are expressed by *mcyD*; hybrid NRPS / PKS modules are expressed by *mcyG* and *mcyE* genes. The *mcyJ*, *-F*, *-H*, *-I*, *-L* and *-T* genes do not have a modular structure and generally only contain a domain (Table 1). A module is a functional unit in NRPS or PKS and each module contains active units called domains. Adenylation (A), condensation (C) and peptidyl carrier protein (PCP) domains are the core domains in NRPS modules. The A domain recognizes the corresponding AA and activates it by using ATP. The PCP domain transfers the -SH (thiol) group to the activated residue. The C domain synthesizes the peptide bond between the -SH groups of the two activated residues. In addition, some modules may have modified domains called tailoring enzymes such as epimerization (E) and *N*-methyl transferase (NMT) domains [10].

The microcystin synthesis assembly line is started by McyG, which activates phenylacetate. Polyketide synthase modules of McyD, McyE and McyG complete the Adda structure at position 5. NRPS modules of McyA, McyB, McyC and McyG incorporate the

remaining AAs [4,5]. There are two A domains in McyA and the order of the domains is A1-NMT-PCP-C-A2-E in most MC producing species; however the NMT domain may not always be present (Table 1). Within these domains, the A1 domain usually activates L-Serine. Then, NMT domain transfers the methyl group from *S*-Adenosyl-L-methionine (SAM) to L-Serine followed or preceded by a dehydration reaction for the formation of Mdha at position 7 of the MC molecule. Various AAs can be incorporated in all 7 positions [11,12].

Table 1. Microcystin synthetase (*mcy*) genes and McyA domain order in cyanobacteria strains investigated in this study. Extra genes are marked in bold.

Cyanobacteria	mcy genes present	Domain order of McyA	Ref.
Anabaena sp. 90	A-B-C-D-E-F-G-H-I-J	A1-NMT-PCP-C-A2-T-E	[9]
Microcystis aeruginosa K-139	A-B-C-D-E-F-G-H-I-J	A1-NMT-PCP-C-A2-T-E	[9]
M. aeruginosa NIES-843	A-B-C-D-E-F-G-H-I-J	A1-NMT-PCP-C-A2-T-E	[3]
M. aeruginosa PCC7806	A-B-C-D-E-F-G-H-I-J	A1-NMT-PCP-C-A2-T-E	[4]
M. viridis NIES-102	A-B-C-D-E-F-G-H-I-J*	A1-NMT-PCP-C-A2-T-E*	
Fischerella sp. CENA161	A-B-C-D-E-F-G-H-I-J	A1-NMT-PCP-C-A2-T-E	[6]
Planktothrix agardhii NIVA-CYA 126/8	A-B-C-D-E-G-H-J-T	A1-NMT-PCP-C-A2-T-E	[8]
P. rubescens PCC7821	A-B-C-D-E-G-H-J**	A1-PCP-C-A2-T-E	[11]
P. rubescens NIVA-CYA 98	A-B-C-D-E-G-H	A1-PCP-C-A2-T-E	
Nostoc sp.152	A-B-C-D-E-F-G-H-I-L	A1-NMT-PCP-C-A2-T-E	

*This study, Genbank: AP019314.1; **Genbank: CZCZ01000002. Abbreviations: A, adenylation domain; NMT, *N*-methyl-transferase domain; PCP, peptidyl carrier protein domain; C, condensation domain; E, epimerization domain.

The substrate specificity of A domains is important to understand the structure of secondary metabolites, including MC. The substrate specificity determination of A domains is usually carried out by determining of binding pocket residues of A domains via bioinformatics analyses. Ten residues constitute the binding pocket of an A domain and are numbered as "Positions-235, 236, 239, 278, 299, 301, 322, 330, 331 and 517" depending on their location in the GrsA module in *Bacillus brevis* (GenBank: CAA33603.1) [13,14]. Position-235 is generally occupied by Asp (Asp_{235}) and position-517 generally by a Lys residue (Lys_{517}). These residues are highly conserved, where Asp_{235} binds to -NH_2 group and Lys_{517} binds to -COOH group of activated AAs. Rest of the eight residues in the binding pocket can be variable and this variability is thought to be an important factor for A domain substrate specificity. It is suggested that A domain substrate specificity is associated with AA interactions between R groups of the activated AAs and R groups of the eight residues in the binding pocket [15,16].

There are nearly 280 variants of MCs [17] and the mechanisms that cause this diversity are not well understood. As more sophisticated techniques are used, both environmental samples and cyanobacteria cultures prove to produce more diverse and novel MC analogues [18]. Current data suggest that this diversity is based on either genetic diversity coded in the MC synthetase genes (e.g. insertions or deletions within various *mcy* genes) or biochemical status of MC producing cells [12]. For instance, point mutations for a few critical AAs in the A domain of GrsA were reported to change the substrate specificity of A domain in GrsA [13]. Fewer *et al*. [19] demonstrated recombination events between A domains of McyB1 and McyC. They reported that A domains of McyB1 and McyC were recombination hotspots for MC variants.

To our knowledge, there are no detailed bioinformatics studies regarding the first A domain of *mcyA*. Therefore, the main purpose of this study was to investigate whether genetic information in this domain could explain incorporation of different AAs at position 7 of the MC molecule. This is important since the toxicity of MCs might change with incorporation of

various AAs. Additionally, mechanisms explaining A domain AA selectivity will help in combinatorial biosynthesis of new NRPS and PKS enzymes [5]. For this purpose, ten MC producing cyanobacteria with sequenced MC synthetase genes were selected for bioinformatics analyses (i) to predict the binding pocket residues of the A1 domain in McyA (McyA-A1); (ii) to determine activated AAs by McyA-A1 based on the binding pocket residues; (iii) to investigate concordance between activated AAs and chemically determined position-7 AAs of MCs reported in the literature for the studied strains; (iv) to investigate concordance between activated AAs and phylogenetic clustering of *mcyA*-A1 sequences.

2. MATERIAL and METHODS

A detailed literature search was conducted to determine the best investigated cyanobacteria strains in terms of MC variant production to report the variation at position 7 of the MC molecule (Table 2). Ten cyanobacteria with sequenced MC synthetase genes and detailed MC characterizations were selected (Table 2) based on literature and GenBank searches. Full length *mcyA* and 16S rRNA gene nucleotide sequences of these cyanobacteria were downloaded from NCBI (National Center for Biotechnology Information) (https://www.ncbi.nlm.nih.gov) using Mega 6 [20]. Alignment was performed using Muscle [21] as implemented in Mega 6 [20] using codons for *mcyA* and nucleotides for 16S rRNA genes. Since *mcyA* is composed of domains, the borders of the first adenylation domain (mcyA-A1) in *mcyA* were determined using the NCBI conserved domain program [9]. Then the determined McyA-A1 domains were extracted from the full length *mcyA* alignment and a new codon-based alignment was performed after addition of the *B. brevis* GrsA domain nucleotide sequence. The nucleotide sequence alignment was converted to an AA alignment and binding pocket residues of McyA-A1 were predicted by comparison to the *B. brevis* GrsA domain AA sequences and by using an online tool [14]. Predictions of the activated AAs by the A domains were also performed using the same online tool.

In order to construct phylogenetic trees, best-fitting models among 24 different nucleotide substitution models were estimated in Mega 6 [20] using an automatic neighbor-joining tree. Models with the lowest Bayesian Information Criterion scores were assumed to describe the substitution patterns best. These models were used to construct phylogenetic trees of the full length *mcyA*, *mcyA* adenylation domain (*mcyA*-A1) and 16S rRNA gene nucleotide sequences of investigated cyanobacteria using Mega 6 [20]. Phylogenetic tree of the 16S rRNA genes of selected cyanobacteria (10 nucleotide sequences and a total of 1350 nucleotide positions) was constructed with the maximum likelihood (ML) method using the Hasegawa, Kishino, and Yano (HKY) model [22]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3331)). Unrooted phylogenetic tree for the full length *mcyA* gene (10 nucleotide sequences and 7077 nucleotide positions) was constructed with the ML method using the general time reversible (GTR) model [23]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8351)). Phylogenetic tree for the *mcyA*-A1 domain nucleotide sequences (11 sequences and 1152 nucleotide positions) was constructed with the ML method based on the GTR model [23]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.1089)). For all phylogenetic trees, 1000 bootstrap replicates were used to test the stability of monophyletic groups. *GrsA* [24] and 16S rRNA gene nucleotide sequences of *Bacillus brevis* (Genbank: X15577.1) were chosen as the outgroups for *mcyA*-A1 and 16S rRNA gene-based phylogenetic trees, respectively.

3. RESULTS and DISCUSSION

Binding pocket analyses for McyA-A1 sequences were performed to predict which AA was incorporated at position 7 of the MC molecule. The predicted binding pocket residues and the predicted AAs potentially activated by McyA-A1 are summarized in Table 2. Of the ten cyanobacteria species, eight were predicted to activate L-Ser and two *Planktothrix rubescens* strains were predicted to activate L-Thr. Binding pocket signatures for all *Microcystis* strains

Table 2. Microcystin (MC) variants produced, predicted binding pocket residues in McyA-A1, and predicted activated amino acids (AAs) by McyA-A1 in cyanobacteria strains used in this study.

Cyanobacteria	MCs produced	Predicted binding pocket residues	Predicted AAs	Ref.
Anabaena sp. 90[1]	[D-Asp[3], MeSer[7]]MC-LR; [MeSer[7]]MC-LR**;** MC-RR [Dha7]MC-LR*; [Dha7]MC-RR; [D-Asp[3]]MC-HillR; [D-Asp[3]] MC-LR; [D-Asp[3]]MC-RR; [DMAdda[5]] MC-LR; MC-LR; MC-HillR; MC-LR	DVWHLSLID	L-Ser	[9,12]
Planktothrix agardhii NIVA-CYA 126/8[2]	[D-Asp[3]]MC-RR; [D-Asp[3]]MC-LR	DVWHISLID	L-Ser	[7,12]
Planktothrix rubescens NIVA-CYA 98[3]	[Dha[7]]MC-LR; [Dha[7]]MC-RR	DFWNIGMVH	L-Thr	[25]
Planktothrix rubescens sp. PCC7821[4]	[Asp[3], Dhb[7]]MC-RR; [Asp[3]]MC-LR*	DFWNIGMVH	L- Thr	[7,26]
Microcystis aeruginosa PCC7806[5]	[D-Asp[3], MeSer[7]]MC-LR; [MeSer[7]]MC-LR; [Dha[7]]MC-LR; [D-Asp[3], Dha[7]]MC-LR; [D-Asp[3]]MC-LR; MC-LR	DVWHFSLID	L-Ser	[12]
Microcystis aeruginosa NIES-843[6]	MC-LR; MC-RR; MC-YR	DVWHFSLID	L-Ser	[3]
Microcystis aeruginosa K-139[7]	[Dha[7]]MC-LR; [Asp[3], Dha[7]]MC-LR	DVWHFSLID	L-Ser	[12]
Microcystis viridis NIES-102[8]	[Ser[1], D-Asp[3],Dha[7]]MC-LR; [X,MeSer[7]] MC-(Hil)R**;** [Dha7]MC-LR; MC-RR; [D-Asp[3]]MC-YR; MC-FR [D-Asp[3]]MC-RR; MC-WR; [D-Asp[3]]MC-LR; MC-HillR;	DVWHFSLID	L-Ser	[12]
Nostoc sp.152[9]	[D-Asp[3], ADMAdda[5], Dha[7]] MC-(Hil)R; [DMAdda[5]] MC-LR; [Mdhb[7]] MC-LR; MC-YR; [ADMAdda[5], Dha[7]]MC-LR; [D-Asp[3],ADMAdda[5], Dha[7]]MC-LR; [ADMAdda[5]]MC-LR [ADMAdda[5], MeSer[7]]MC-LR;	DVWHISLID	L-Ser	[25,27]

	[D-Ser[1], ADMAdda[5]] MC-LR; [ADMAdda[5]]MC-HillR**;** [D-Asp[3],DMAdda[5]] MC-LR; [D-Asp[3],DMAdda[5]] MC-VR; [D-Asp[3],ADMAdda[5]] MC-LHar; [D-Asp[3], ADMAdda[5]] MC-HillR			
Fischerella sp. CENA 161[10]	MC-LR	DVWHISLID	L-Ser	[6]

Genbank numbers: [1]AJ536156; [2]AJ441056.1; [3]AM990462.1; [4]CZCZ01000002; [5]AF183408; [6]AP009552; [7] AB019578; [8] AP019314.1; [9] KC699835.1; [10] KX891213. Abbreviations: Dhb, dehydrobutyrine; Dha, dehydroalanine; ADMAdda, *O*-acetyl-*O*-demethylAdda; DMAdda, *O*-demethylAdda; Hil, homoisoleucine; MeSer, *N*-Methylserine; D-Asp, desmethyl aspartic acid. * trace amount

were DVWHFSLID. While binding pocket signatures for the nostocalean genera *Nostoc* sp. 152, *Fischerella* sp. CENA161 and the oscillatorian *Planktothrix agardhii* NIVACYA 126/8 were DVWHISLID; it was DVWHLSLID for *Anabaena* sp. 90, both with one AA change from the *Microcystis* binding pocket signature. Binding pocket signature for *P. rubescens* strains was DFWNIGMVH.

Among the potentially L-Thr activating *P. rubescens* strains, NIVACYA 98 was reported to produce Dha^7 containing MCs [25] and PCC 7821 was reported to produce both Dhb^7 and Mdha^7 containing MCs [11,25] (Table 2). Three different residues, namely MeSer^7, Dha^7 and Mdha^7 were reported for *Anabaena* sp. 90, *M. viridis* NIES 102 and *M. aeruginosa* PCC7806 [12,27]. While production of Mdha^7 were reported for *Planktothrix agardhii* NIVA CYA 126/8 [12], *Microcystis aeruginosa* NIES 843 and *Fischerella* sp. CENA161 [3,6], Dha^7 was the only AA residue reported for *M. aeruginosa* K-139 [12]. Among the investigated species in this study, Mdhb^7 formation was only reported for *Nostoc* sp. 152 [27] that also produced Dha^7, MeSer^7 and Mdha^7 [12, 25] containing MCs (Table 2).

In most MC producing cyanobacteria, McyA-A1 domain usually activates L-Ser. In the case of L-Thr activation by the McyA-A1 domain, Dhb^7 formation may occur in species lacking NMT domain, as expected for the *P. rubescens* strains in this study (Table 1). However, in previous LC/MS analyses of *P. rubescens* NIVACYA 98, only Dha^7 formation was reported [7,25], which indicated L-Ser activation instead of L-Thr and conflicted with the binding pocket analysis results in this study (Table 2). Similarly, Kurmayer *et al*. [11] reported that *P. rubescens* PCC7821 produced MCs containing both Mdha^7 (5%) and Dhb^7 (95%), which indicated activation of both L-Ser and L-Thr. These results contradicted the proposition that polar AA activating A domains such as McyA-A1 had higher selectivity than hydrophobic AA activating A domains [11,28].

Another similar situation was observed for the McyA-A1 domain in *Nostoc* sp. 152, which was predicted to activate L-Ser. MCs containing Mdha^7, MeSer^7, Dha^7 [25,28] and Mdhb^7 [27] were reported for *Nostoc* sp. 152 (Table 2). Mdhb^7 formation (probably a minor variant [29]) indicates L-Thr activation and shows an active NMT, since it is a methylated form of Dhb. Apparently, L-Thr and L-Ser are simultaneously activated by McyA-A1 for *Planktothrix rubescens* PCC7821 and *Nostoc* sp. 152. This multispecificity suggests that there is a flexibility for McyA-A1 domain to activate various AAs during MC production.

The afore-mentioned A domain flexibility has been reported for various secondary metabolites. For example, the A domain involved in the biosynthesis of maremycin from *Streptomyces* sp. was predicted to activate Cys based on the specificity-conferring code, however *in vitro* and *in vivo* studies showed the activation of Cys, Me-Cys and Ser. It should be noted that in vitro catalytic efficiency of A domain towards Cys and Me-Cys were

comparable and 4 times higher than Ser, in agreement with the observation of the minor product maremycin D incorporating Ser *in vivo* [30]. In a comparable situation, L-Phenylalanine activating A domain for the antibiotic Tyrocidine A was shown to be flexible for various AAs *in vitro* [31]. The specificity of this A domain towards L-Phe in vivo suggested that gatekeeping function of the condensation (C) domain might be in place [31]. In fact, Meyer *et al*. [3] demonstrated flexibility of A domains and the gatekeeping and specificity-regulatory role of the C domains of *mcyB* and *mcyC* genes of the microcystin synthetases in *Microcystis* spp. Their *in vitro* experiments showed that the predicted AAs were used by A domains when the C domain was present. On the other hand, minor amounts of unanticipated AAs were incorporated in the MC molecules due probably to the leaky control of the C domain [3], which probably would explain the minor incorporation of un-predicted AAs by *mcyA*-A1 (e.g. Mdha^7 in *P. rubescens* PCC7821). However, it doesn't explain the sole production of Dha^7 containing MCs in *P. rubescens* NIVACYA 98, in which L-Thr activation was predicted (Table 2).

Anabaena sp. 90, *Microcystis* spp., *Planktothrix agardhii* NIVACYA 126/8 and *Fischerella* sp. CENA 161 investigated in this study were predicted to activate L-Ser (Table 2). Previous LC/MS analyses of these species reported MCs containing Mdha^7, MeSer^7 and Dha^7, supporting the activation of L-Ser by McyA-A1 [19]. MeSer^7 was suggested to form through an incomplete dehydration reaction involving the condensation domain of *mcyA* [18]. On the other hand, formation of Dha^7 might have resulted due to transient inactivity of NMT domain or SAM limitation during MC biosynthesis [12].

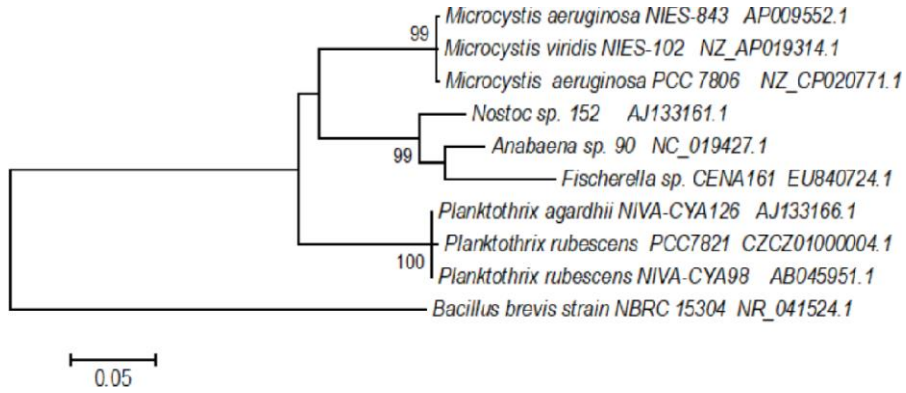

Figure 2. Phylogenetic tree of the 16S rRNA genes of selected cyanobacteria. Bootstrap values above 50 percent from 1000 replicates are at the nodes. Branch lengths represent the number of substitutions per site. All positions with less than 95% site coverage were eliminated. *B. brevis* 16S rRNA gene was used as the outgroup.

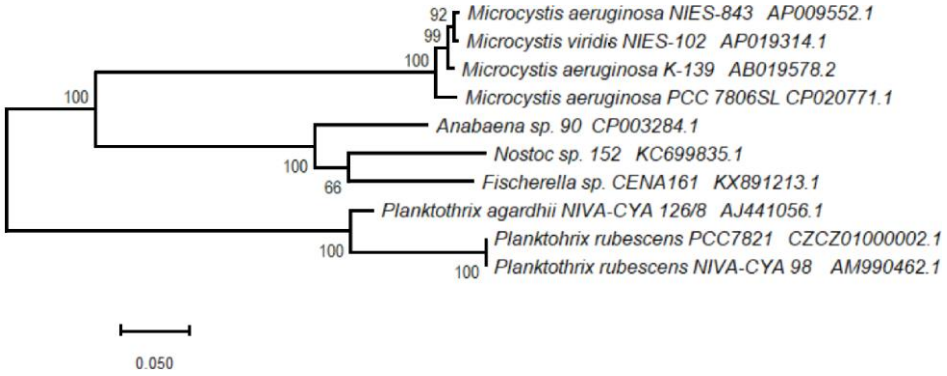

Figure 3. Unrooted phylogenetic tree for the full length *mcyA* gene. Bootstrap values above 50 percent from 1000 replicates are at the nodes. All positions with less than 95% site coverage were eliminated. Nucleotide accession numbers are given at the ends of sequence names. Branch lengths represent the number of substitutions per site.

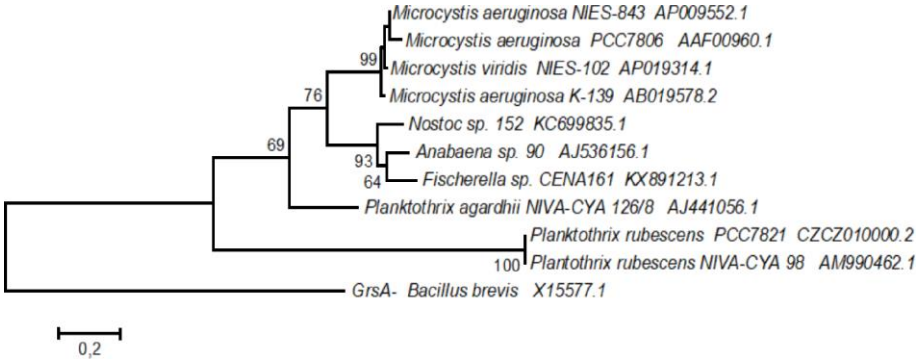

Figure 4. Phylogenetic tree for the *mcyA*-A1 domain nucleotide sequences. Bootstrap values above 50 percent from 1000 replicates are at the nodes. All positions with less than 95% site coverage were eliminated. *GrsA* was used as the outgroup. Nucleotide accession numbers are given at the ends of sequence names. Branch lengths represent the number of substitutions per site.

Phylogenetic analysis of the 16S rRNA gene of the investigated cyanobacteria species clearly separated strains belonging to Nostocales, Oscillatoriales and Chrooccocales with high bootstrap support (Figure 2). A similar tree, with high bootstrap support, was obtained when the full length *mcyA* nucleotide sequences were analyzed (Figure 3). However, *P. agardhii* NIVACYA 126/8 did not cluster with *P. rubescens* strains when *mcyA*-A1 domain was analyzed (Figure 4). Clustering of other strains were in agreement with the clusters obtained in 16S rRNA and *mcyA* gene phylogenetic analyses.

In general, phylogenies of full length *mcyA* gene and *mcyA*-A1 domain sequences followed that of 16S rRNA gene of the analyzed strains. The exception was clustering of *P. agardhii* NIVACYA126/8 *mcyA*-A1 domain sequences with Nostocales and Chrococcales sequences, albeit with low bootstrap support. This suggests that either *P. agardhii* or *P. rubescens* acquired its A1 domain via horizontal gene transfer after the separation of both species [5,11]. Phylogenies of the *mcyA* condensation domains of the investigated strains had similar topology to full length *mcyA* and 16S rRNA gene trees (data not shown), further strengthening the idea that some *Planktothrix* strains might have acquired their A domains via horizontal transfer.

4. CONCLUSION

The phylogenetic tree and clustering of A1 domains are in agreement with the prediction of activated AAs by A1 domains. L-Thr activating *P. rubescens* A1 domains are clearly separated from the L-Ser activating domains. These results suggest that phylogenetic analyses of A domains might potentially give information on the activated AAs by these domains. On the other hand, MC congener analysis of these strains show that binding pocket or phylogenetic analysis alone may not explain the AA flexibility of A domains in some strains. Further work involving heterologous expression of *mcyA*-A1 and -C domains together or separately, followed by protein purification and *in vitro* substrate specificity assays might help to explain variation of position 7 AAs in MCs.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Orcid

Gozde Yaman https://orcid.org/0000-0001-6044-3194
Mete Yilmaz https://orcid.org/0000-0002-0982-727X

5. REFERENCES

[1]. Walsh, C.T., O'Brien, R.V., Khosla, C. (2013). Nonproteinogenic amino acid building blocks for nonribosomal peptide and hybrid polyketide scaffolds. *Angew Chem Int Ed Engl*., *52*(28), 7098–7124. http://dx.doi.org/10.1002/anie.201208344

[2]. Dittmann, E., Gugger, M., Sivonen, K., Fewer, D.P. (2015). Natural product biosynthetic diversity and comparative genomics of the cyanobacteria. *Trends Microbiol*., *23*(10), 642–652. http://dx.doi.org/10.1016/j.tim.2015.07.008

[3]. Meyer, S., Kehr, J.C., Mainz, A., Dehm, D., Petras, D., Süssmuth, R.D., Dittmann, E. (2016). Biochemical dissection of the natural diversification of microcystin provides lessons for synthetic biology of NRPS. *Cell Chem Biol*., *23*(4), 462–471. http://dx.doi.org/10.1016/j.chembiol.2016.03.011

[4]. Tillett, D., Dittmann, E., Erhard, M., von Döhren, H., Börner, T., Neilan, B.A. (2000). Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide–polyketide synthetase system. *Chem Biol*., *7*(10), 753–764. http://dx.doi.org/10.1016/s1074-5521(00)00021-1

[5]. Dittmann, E., Fewer, D.P., Neilan, B.A. (2013). Cyanobacterial toxins: biosynthetic routes and evolutionary roots- Review Article. *FEMS Microbiol Rev*., *37*, 23-43. http://dx.doi.org/10.1111/j.1574-6976.2012.12000.x

[6]. Heck, K., Alvarenga, D.O., Shishido, T.K., Varani, A.M., Dörr, F.A., Pinto, E., Rouhiainen, L., Jokela, J., Sivonen, K., Fiore, M. F. (2018). Biosynthesis of microcystin hepatotoxins in the cyanobacterial genus *Fischerella*. *Toxicon*., *141*, 43–50. http://dx.doi.org/10.1016/j.toxicon.2017.10.021

[7]. Shishido, T.K., Jokela, J., Humisto, A., Suurnäkki, S., Wahlsten, M., Alvarenga, D.O., Sivonen, K., Fewer, D.P. (2019). The biosynthesis of rare Homo-amino acid containing variants of microcystin by a benthic cyanobacterium. *Mar Drugs*, *17*(5), 271. http://dx.doi.org/10.3390/md17050271

[8]. Christiansen, G., Fastner, J., Erhard, M., Borner, T., Dittmann, E. (2003). Microcystin biosynthesis in *Planktothrix*: Genes, evolution, and manipulation. *J. Bacteriol*., *185*(2), 564–572. http://dx.doi.org/10.1128/jb.185.2.564-572.2003

[9]. Rouhiainen, L., Vakkilainen, T., Siemer, B.L., Buikema, W., Haselkorn, R., Sivonen, K. (2004). Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. *Appl Environ Microbiol*., *70*(2), 686–692. http://dx.doi.org/10.1128/aem.70.2.686-692.2004

[10]. Mootz, H.D, Schwarzer, D., Marahiel, M.A. (2002). Ways of assembling complex natural products on modular nonribosomal peptide synthetases. *Chem BioChem*., *3*, 490–504. http://dx.doi.org/10.1002/1439-7633(20020603)3:6<490::AID-CBIC490>3.0.CO;2-N

[11]. Kurmayer, R., Christiansen, G., Gumpenberger, M., Fastner, J. (2005). Genetic identification of microcystin ecotypes in toxic cyanobacteria of the genus *Planktothrix*. *Microbiol*., *151*(5), 1525–1533. http://dx.doi.org/10.1099/mic.0.27779-0

[12]. Fewer, D.P., Tooming-Klunderud, A., Jokela, J., Wahlsten, M., Rouhiainen, L., Kristensen, T., Rohrlack, T., Jakobsen, K., Sivonen, K. (2008). Natural occurrence of microcystin synthetase deletion mutants capable of producing microcystins in strains of the genus *Anabaena* (Cyanobacteria). *Microbiol*., *154*(4), 1007–1014. http://dx.doi.org/10.1099/mic.0.2007/016097-0

[13]. Stachelhaus, T., Mootz, H.D., Marahiel, M.A. (1999). The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. *Chem Biol*., *6*(8), 493–505. http://dx.doi.org/10.1016/s1074-5521(99)80082-9

[14]. Ansari, M.Z., Yadav, G., Gokhale, R.S., Mohanty, D. (2004). NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. *Nucleic Acids Res*., *1*(32), 405-413. http://dx.doi.org/10.1093/nar/gkh359

[15]. Lautru, S., Challis, L.G. (2004). Substrate recognition by nonribosomal peptide synthetase multi-enzymes. *Microbiol.*, *150*(6), 1629–1636. http://dx.doi.org/10.1099/mic.0.26837-0

[16]. Kudo, F., Miyanaga, A., Eguchi, T. (2019). Structural basis of the nonribosomal codes for nonproteinogenic amino acid selective adenylation enzymes in the biosynthesis of natural products. *J. Ind Microbiol Bio.*, *46*, 515-536. http://dx.doi.org/10.1007/s10295-018-2084-7

[17]. Bouaïcha, N., Miles, C.O., Beach, D.G., Labidi, Z., Djabri, A., Benayache, N.Y., Quang, T.N. (2019). Structural Diversity, Characterization and Toxicology of Microcystins. *Toxins*, *11*(12), 714. http://dx.doi.org/10.3390/toxins11120714

[18]. Yilmaz, M., Foss, A.J., Miles, C.O., Özen, M., Demir, N., Balcı, M., Beach, D.G. (2019). Comprehensive multi-technique approach reveals the high diversity of microcystins in field collections and an associated isolate of *Microcystis aeruginosa* from a Turkish lake. *Toxicon*, *167*, 87-100. http://dx.doi.org/10.1016/j.toxicon.2019.06.006

[19]. Fewer, D.P., Rouhiainen, L., Jokela, J., Wahlsten, M., Laakso, K., Wang, H., Sivonen, K. (2007). Recurrent adenylation domain replacement in the microcystin synthetase gene cluster. *BMC Evol Biol.*, *7*(1), 183. http://dx.doi.org/10.1186/1471-2148-7-183

[20]. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*., *30*(12), 2725-2729. http://dx.doi.org/10.1093/molbev/mst197

[21]. Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*., *32*(5), 1792–1797. http://dx.doi.org/10.1093/nar/gkh340

[22]. Hasegawa, M., Kishino, H., Yano, T. (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.*, *22*(2), 160-174. http://dx.doi.org/10.1007/bf02101694

[23]. Nei, M., Kumar, S. (2000). Molecular Evolution and Phylogenetics. Oxford University Press: New York, USA, 332 pp, ISBN-10: 0195135857.

[24]. Mikalsen, B., Boison, G., Skulberg, O.M., Fastner, J., Davies, W., Gabrielsen, T.M., Rudi, K., Jakobsen, K.S. (2003). Natural variation in the microcystin synthetase operon *mcy*ABC and impact on microcystin production in microcystis strains. *J. Bacteriol.*, *185*(9), 2774-2785. http://dx.doi.org/10.1128/jb.185.9.2774-2785.2003

[25]. Gehringer, M.M., Adler, L., Roberts, A.A., Moffitt, M.C., Mihali, T.K., Mills, T.J. T., Fieker, C., Neilan, B.A. (2012). Nodularin, a cyanobacterial toxin, is synthesized in planta by symbiotic *Nostoc* sp. *ISME J*., *6*(10), 1834–1847. http://dx.doi.org/10.1038/ismej.2012.25

[26]. Tooming-Klunderud, A., Fewer, D.P., Rohrlack, T., Jokela, J., Rouhiainen, L., Sivonen, K., Kristensen, T., Jakobsen, K.S. (2008). Evidence for positive selection acting on microcystin synthetase adenylation domains in three cyanobacterial genera. *BMC Evol.*

Biol., *8*(1), 256.
http://dx.doi.org/10.1186/1471-2148-8-256

[27]. Puddick, J., Prinsep, M., Wood, S., Kaufononga, S., Cary, S., Hamilton, D. (2014). High levels of structural diversity observed in microcystins from *Microcystis* CAWBG11 and characterization of six new microcystin congeners. *Mar. Drugs*, *12*(11), 5372-5395. http://dx.doi.org/10.3390/md12115372

[28]. Challis, G.L., Ravel, J., Townsend, C.A. (2000). Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. *Chem Biol*., *7*(3), 211–224.
https://doi.org/10.1016/S1074-5521(00)00091-0

[29]. Sivonen, K., Carmichael, W.W., Namikoshi, M., Rinehart, K.L., Dahlem, A.M., Niemela, S.I. (1990). Isolation and characterization of hepatotoxic microcystin homologs from the filamentous freshwater cyanobacterium *Nostoc* sp. strain 152. *Appl. Environ. Microbiol.*, *56*(9), 2650-2657.
http://dx.doi.org/10.1128/AEM.56.9.2650-2657.1990

[30]. Huang, T., Duan, Y., Zou, Y., Deng, Z., Lin, S.b(2018). NRPS protein MarQ catalyzes flexible adenylation and specific S-methylation. *ACS Chem. Biol.*, *13*(9), 2387-2391. http://dx.doi.org/10.1021/acschembio.8b00364

[31]. Schaffer, M.L., Otten, L.G. (2009). Substrate flexibility of the adenylation reaction in the Tyrocidine non-ribosomal peptide synthetase. *J. Mol. Catal B-Enzym.*, *59*(1-3), 140-144. http://dx.doi.org/10.1016/j.molcatb.2009.02.004

International Journal of Secondary Metabolite
2020, Vol. 7, No. 4, 286–296

https://doi.org/10.21448/ijsm.753053

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Callus Induction and Micropropagation of *Lilium candidum* L. Using Stem Bulbils and Confirmation of Genetic Stability via SSR-PCR

Hilal Busra Tokgoz[1], **Filiz Altan**[1,*]

[1]Department of Molecular Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey

Abstract: Natural populations of *Lilium candidum* L. are remarkably affected by biotic and abiotic factors therefore there is a requirement to develop effective micropropagation protocol to provide mass production, multiplication and conservation of these plants. For this reason, this study was aimed to develop an efficient micropropagation method for multiple shoot production via somatic embryogenesis induced from *L. candidum* stem bulbils and also to determine the genetic stability of *in vitro* grown plants using SSR markers. The obtained results of this study are the first comprehensive reports including an investigation of genetic fidelity on somatic embryogenesis of *L. candidum*. After surface sterilization of bulbils, the calculated regeneration percentage of them was 89.5% and the callus induction was achieved using leaf segments of *in vitro* grown bulbils. The well formed somatic embryos were obtained from smooth whitish-yellow colored calli and these somatic embryos produced well formed healthy *L. candidum* seedlings with adventitious roots. All rooted seedlings were easily adapted to greenhouse conditions and the genetic stability of *in vitro* grown seedlings were determined by using SSR-PCR technique and it was calculated as 100%.

ARTICLE HISTORY

Received: June 15, 2020
Revised: September 22, 2020
Accepted: December 03, 2020

KEYWORDS

Callus Culture,
Genetic Stability,
Lilium candidum L.,
Somatic Embryogenesis,
SSR

1. INTRODUCTION

Lilium candidum being a member of Liliaceae family is a perennial herbaceous medicinal and aromatic plant [1]. Because of its attractive white flowers, aromatic and medicinal components, *L. candidum* has widely been cultivated in many countries such as in USA, Italy, Netherlands, Spain, Germany, France and Turkey [2]. *L. candidum* is a species adapted to the Mediterranean climate. The other *Lilium* species, which are spread in our country are distributed in areas under the influence of the Black Sea climate which is cooler and more rainy climate. Their natural populations are remarkably affected by biotic and abiotic factors such as anthropological pressure, diseases, pathogen attacks, carbon fuel pollution, dramatic climate changes, therefore there is a requirement to develop effective micropropagation protocol to provide mass production, multiplication and conservation of these plants [3].

Plant tissue culture techniques providing very useful approach for rapid propagation of plant species help to preserve especially the economically important and endangered species. *In vitro* propagation supports the proliferation of plant cells, tissues and organs by incubating

CONTACT: Filiz Altan ✉ afiliz@mu.edu.tr Department of Molecular Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey

ISSN-e: 2148-6905 /© IJSM 2020

them in an aseptic condition in liquid or on semi-solid nutrient medium [4,5]. Although the earliest studies on plant tissue culture date back to the early twentieth centuries, the main studies improved completely from 1970s onwards, as technological improvements began to be increased and theoretical limitations started to be overcome by expanding interest in usage of biotechnological techniques [6]. Until today many plant tissue culture techniques have been developed for improvement and breeding of different group plant species such as conifers [7], dicots [4] and monocots [8].

Somatic embryogenesis being one of the most important micropropagation tools has been applied throughout different types of *in vitro* systems for plant mass production. This tool serves also many advantages for *in vitro* propagation of true-to-type clones, rapid regeneration of genetic transformed and somatic hybridized plants and induction and selection of mutant types. Additionally, somatic embryogenesis plays an important role in key studies on totipotency and understanding of principle pathways of morphogenesis. Because of all these possible advantages of somatic embryogenesis, it has been tempting studies on investigation of *in vitro* conditions for somatic embryo induction of different plant species [9] and an excessive number of procedures for effective *in vitro* regeneration based on somatic embryogenesis have been developed for many economically important plant species [10].

The continuity of genetic stability during *in vitro* growing and subculturing periods is very important for clonal propagation of especially medicinal, aromatic, rare and endangered plant species [4]. It is important to maintain genetic stability in micropropagated cultures. Molecular markers are more stable and highly reproducible compared to the various morphological, cytological and protein markers used to detect variation in tissue cultivated plants. A molecular marker such as SSR, ISSR, RAPD, AFLP etc. is beneficially used in tissue culture studies to test the genetic stability of *in vitro* regenerates [11]. In all prokaryotes and eukaryotes, SSRs expressing sequences between 1 and 6 nucleotides on DNA are one of the preferred markers in genetic diversity studies due to their high mutation rate and consequently high polymorphism rate. They are an excellent source of polymorphism for eukaryotic genomes [12].

Although there are many published papers for method development on somatic embryogenesis of *Lilium* spp., [13-20], none of them reported genetic stability investigation after *in vitro* propagated *L. candidum* natural populations using SSR molecular marker techniques. The current study was carried out to improve efficient micropropagation method for multiple shoot propagation via somatic embryogenesis induced from *L. candidum* stem bulbils and also to determine the genetic stability of *in vitro* grown plants using SSR markers. The obtained results of this study are the first comprehensive reports including investigation of genetic fidelity on somatic embryogenesis of *L. candidum*.

2. MATERIAL and METHODS

2.1. Plant Materials

Plant samples belonging to natural populations of *L. candidum* L. were collected from Nif Mountain (İzmir, Turkey). The legal authorization letter for sample collection was obtained from Republic of Turkey Ministry of Agriculture and Forestry, document number 36178555-604.01.01/488551 and all collected samples were taxonomically identified by Dr. Hasan Yildirim and Dr. Ademi Pirhan.

2.2. *In vitro* Culture Establishment

The stem bulbils of *L. candidum* (Figure 1A-B) obtained from natural populations were sterilized according to surface sterilization protocol of Özüdoğru *et al*. [4]. After surface sterilization, the bulbils were transferred to semi solid Murashige and Skoog (MS) [21].

Medium supplemented with 4.44 µM 6-Benzylaminopurine (BAP), 20 g L^{-1} sucrose and 7 g L^{-1} agar. To enhance *in vitro* regeneration of *L. candidum* bulbils, surface sterilized bulbils were secondly transferred to Olive Medium (OM), [22]. Medium supplemented with 4.44 µM BAP, 20 g L^{-1} sucrose, 3.5 g L^{-1} agar and 1.5 g L^{-1} phytagel [23]. We used 29 stem bulbils for each experiment. For each experiment, approximately 30 leaf fragments were used as the explant source for callus induction. For callus induction, ~ 0.5×0.75 cm leaf fragments derived from *in vitro* regenerated bulbils (Figure 1C-D) were transferred to MS medium supplemented with 10.7 µM Naphthaleneacetic acid (NAA), 20 g L^{-1}sucrose and 7 g L^{-1} agar. Somatic embryos obtained from *in vitro* grown calli via OM medium were supplemented with 10.7 µM NAA, 20 g L^{-1} sucrose and 7 g L^{-1} agar (Figure 1E-F). It was determined that OM medium supplemented with 10.7 µM Naphthaleneacetic acid (NAA), 20 g L^{-1} sucrose and 7 g L^{-1} agar was more effective for somatic embryos, experiments were continued with this medium. All *in vitro* cultures were incubated at 25±2 °C, under a 8 hours dark / 16 hours light photoperiod, with light provided by cool daylight fluorescent lamps (50 $\mu mol^{-1}m^{-2}s^{-1}$).

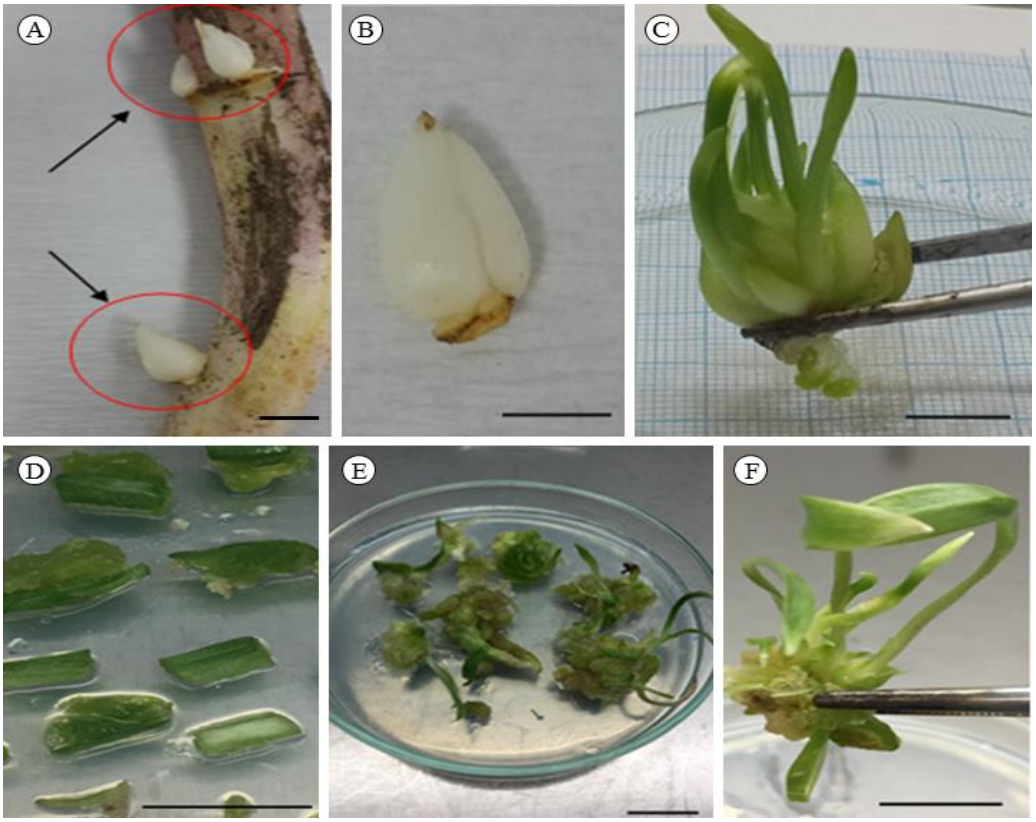

Figure 1. Morphological structure of *L. candidum* derived from native and *in vitro* grown plants (Bars 1cm) (**A**) Bulbils along the plant stem (**B**) One of stem bulbils used for *in vitro* culture initiation (**C**) *in vitro* regenerated bulbils (**D**) ~ 0,5×0,75 cm leaf fragments derived from *in vitro* regenerated bulbils (**E**) *in vitro* callus induction and somatic embryos (**F**) *in vitro* grown seedling obtained from callus.

2.3. Acclimatization to Greenhouse Conditions

Multiple rooted shoot clusters derived from *in vitro* grown somatic embryos of *L. candidum* (Figure 2A) were acclimatized under greenhouse conditions by transferring them into 100 mL plastic pots (Figure 2B) including nitrogen-rich peat and to gradually decrease the relative humidity of peat, the pots were closed with transparent pots and a hole was drilled on top of the transparent pots every day [4]. The plastic pots were irrigated with tap water for seven days, the transparent pots were removed after seven days and the plastic pots were transferred to greenhouse conditions (Figure 2C).

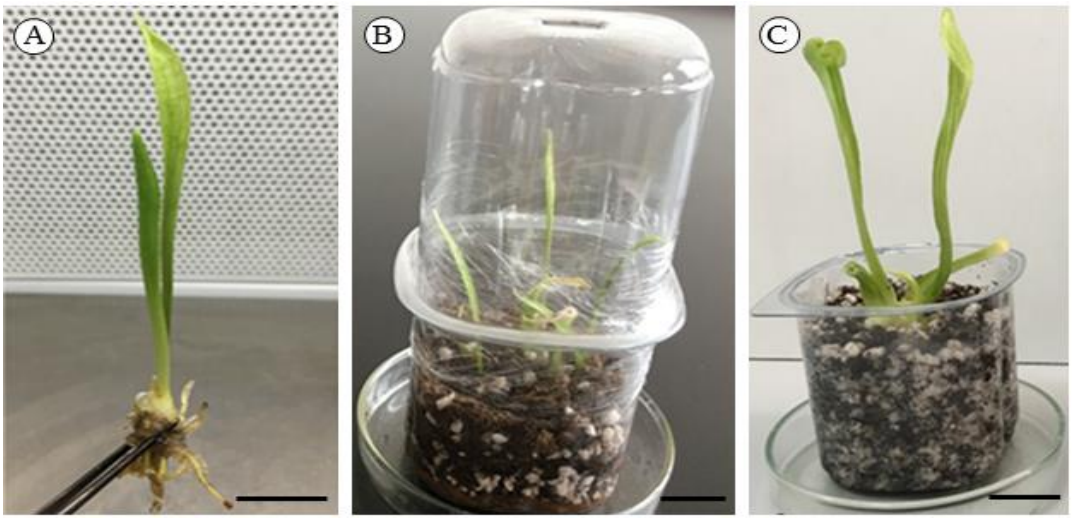

Figure 2. Acclimatization of *L. candidum in vitro* grown shoot clusters to greenhouse conditions (Bars 1 cm long) (**A**) Multiple rooted shoot cluster derived from *in vitro* grown somatic embryos of *L. candidum*, (**B**) Acclimatization under greenhouse conditions by transferring them into 100 mL plastic pots, (**C**) Transferring of acclimatized plants to green house conditions.

2.4. Data Collection and Statistical Analysis

In vitro regeneration of *L. candidum* bulbils, callus induction and somatic embryogenesis data were calculated as percentage values. All data were collected after four weeks incubation at standard culture conditions and all treatments were repeated at least three times. The statistical analysis of the non-parametric data was performed by means of the test for homogeneity rates, and the differences obtained by treatments were chosen using non-parametric statistical test [24]. Separate data were exposed to ANOVA, monitored by the least significant difference test at $P \leq 0.05$ to compare means. Data were analyzed by SPSS package program (IBM, version 21).

2.5. Determination of Genetic Stability

DNA isolation was performed from the mother plant, callus derived from *in vitro* regenerated bulbils, developing somatic embryogenesis, and acclimated seedlings (callus, somatic embryogenesis and acclimated seedlings were obtained after the third subculture) in order to confirm whether there was a variation. All samples were stored at -20 °C. SSR markers we previously determined as polymorphic were used in the naturally grown *L. candidum* genotypes.

2.6. DNA Isolation

DNA isolation was performed in all the above mentioned examples by modifying the method of Lodhi *et al*., [25]. After isolation, DNA samples were run on a 1% agarose gel to determine their purity and were visualized with a gel imaging system (MS, Major Science).

2.7. SSR Analysis

For SSR analysis, SSR primers to be used in the study were determined by referring to Du *et al*. [26]. Of these SSR primers, 12 pairs of them were identified as polymorphic in naturally grown *L. candidum* populations in Turkey (Table 1).

The total volume of PCR amplification reaction used in the study was 20 µL. Each PCR reaction consisted of 1×Taq PCR buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 units Go Taq Polymerase (Promega Go taq- M8295), 100 ng DNA and dH_2O. PCR amplification; The initial denaturation step was carried out at 94 °C for 5 minutes followed by 35 cycles of 1 minutes denaturation at 94 °C, 30 second annealing at (48-

55 °C) and 1 minutes extension at 72 °C with a final extension at 72 °C for 7 minutes. The PCR products obtained were run at a consistent voltage of 4.5 V/cm on a 4 % agarose (1.2% Biomax Basic Agarose and 2.8% Delta Micropor Agarose) gel in 1×TBE buffer for 3 hours by electrophoresis and bands were visualized by EtBr (Sigma-Aldrich®). Fragment size was determined using 100 bp ladder (ABM Cat: G016).

Table 1. SSR primers used for genetic stability determination in micropropagated *L. candidum* [26].

Primer no	Primer name	Repeat motif	Primer sequence (5'-3')
1	LS-ZJU07	$(AG)_6$	F:TGATCTCTGAGCTCCCCACT R:TGAGAATTGGATCAGGCGTT
2	LS-ZJU08	$(ACC)_7$	F:CATCAGCAACAACAAACCCA R:CTATGATTATAAGGCCGCCG
3	LS-ZJU09	$(CAG)_6$	F:AAGTCAGCAGCAACAGCAGA R:CAGGTAAAAATCCGCCAGAA
4	LS-ZJU11	$(GAG)_6$	F:TTCCAAGACCAGGACGACTC R:TTCCTGCCCAAATTGAACTC
5	LS-ZJU12	$(TC)_6$	F:CCATAGCTTCGTAGCTGCCT R:AAGTTGCCTAGAATGCCGAA
6	LS-ZJU13	$(GAC)_6$	F:GCTGTATAGCAGGACGGAGG R:TCGATTGTCTGCTTGACGAG
7	LS-ZJU16	$(CCT)_5$	F:GGCTCGCTCCTCTTCTCTCT R:GTCGTCCTAGCGGCATTAAG
8	LS-ZJU32	$(TTG)_5$	F:GTTTCCAACTGCGGATGTTT R:TGTTCAACTCCGTGCCACTA
9	LS-ZJU35	$(TGC)_5$	F:AAAAGCTCCAGCAAAAGCAG R:CTCCACCCTTGGATTTACGA
10	LS-ZJU40	$(TC)_6$	F:ATATCTTGACCCGCAGCATC R:AGCTCTGCAGGACGTTTGTT
11	LS-ZJU48	$(TGC)_6$	F:CTGCAGATGGAGATGCTGAA R:CCGTGAGAATGGTGTGAATG
12	LS-ZJU56	$(GA)_9$	F:TGAATGGGTAGGAGACGGAG R:TCCCCAATCAGACAATGTGA

3. RESULTS and DISCUSSION

3.1. Initation of *L. candidum in vitro* Cultures

After surface sterilization, *in vitro* culture initiation was provided by transferring *L. candidum* bulbils to MS initation medium described above, and obtained clean material percentage was calculated as 65.5%. The regeneration percentage of clean bulbils was calculated as 89.5% after four weeks incubation on OM regeneration medium described above. We used ~ 29 stem bulbis for each experiment, the clean material obtained showed regeneration of ~ 89.5%.

3.2. Induction of Calli and Somatic Embryogenesis

For each experiment, approximately 30 leaf fragments were used as the explant source for callus induction, The callus induction was achieved by using leaf fragments of *in vitro* regenerated *L. candidum* bulbils on MS callus induction medium described above and after two weeks incubation, the smooth whitish-yellow colored calli were successfully formed from widening and hardening leaf segments. Callus development was observed in each explant. The average callus induction rate per explant was calculated as 100%. After three weeks incubation, all cells of well formed calli produced healthy somatic embryos by transferring to OM somatic embryo producing medium described above. All of somatic embryos produced well formed healthy *L. candidum* seedlings and all seedlings have proper adventitious roots to adapt greenhouse conditions.

3.3. Determination of Genetic Stability

Micropropagation and callus culture experiments were carried out during the study to determine whether there were any somaclonal variations in the plants by the effect of nutrient media and growth regulators. For this purpose in the current study SSR markers were used and scanned for whether there was a genetic variation between the materials we produced by using micropropagation. The materials obtained from callus regeneration, somatic embryogenesis and acclimated plants were checked with SSR markers and the produced band profiles of these materials were found to be the same as the mother plant. The 12 pairs of primers produced 24 scorable bands (average: 2 bands/pairs of primer). As an example, SSR-PCR amplification of primer LS-ZJU 11 in DNAs of *L. candidum* mother plant, *in vitro* regenerated bulbils, *in vitro* grown somatic embryos and acclimatized plantles were visualized on agarose gel mixture is shown (Figure 3).

The plant tissue culture technologies having wide application area such as single *in vitro* cells, tissues and organs production, calli and suspensions in big-mass scale production, has become an important tool for plant biotechnology [27]. Plant tissue culture generally defining all procedures of *in vitro* cultivation, growth and maintenance of plant materials, has been developed and used for basic studies on cell differentiation, growth, division and fusion, plant physiology and biochemistry experiments, metabolic and genetic engineering, gene transformation, conservation of plant biodiversity [28]. In the current study, indirect somatic embryogenesis were achieved by using calli induced from *in vitro* grown *L. candidum* bulbils.

There are many studies on the selection of suitable nutrient media through the tissue culture of *Lilium.* These studies include studies on plant growth regulators [29-32], photoperiod application [33-37], explant size [29,30,33,36,38] and sugar concentration [29,30,33,36,38]. Altan and Bürün [39] reported that the MS medium supplemented with 0.1 mg L^{-1} NAA+ 0.01 mg L^{-1} BA, 30 g L^{-1}sugar and 8 g L^{-1} agar used was optimal in experiments for micropropagation of *L. candidum*. [39]. In the current study, it was determined that OM medium were supplemented with 10.7 µM NAA (2 g L^{-1}), 20 g L^{-1} sucrose and 7 g L^{-1} agar is effective for the development of somatic embryogenesis from callus obtained from *in vitro* regenerated bulbils.

Although callus induction was achieved by using MS medium, the somatic embryogenesis were obtained by using OM medium, both of media were supplemented with NAA. One of the main differences between the two media is that OM contains a different nitrogen salt [The OM medium contains a different NO_3^- [$Ca(NO_3)$, 2.54 mM] as nitrogen source and it also contains lower concentrations of other nitrate salts than the MS medium (NH_4NO_3, 5.15 mM; KNO_3, 6.09 mM)]. There have been many studies on potential benefits of different nitrogen sources such as NH_4^+ and NO_3^- and main purpose of these studies was to develop nutrient components of culture medium for different plant species. For example, the different concentrations of these forms of nitrogen in the nutrient media have produced very

positive responses on shoot regeneration [40], plant recovery efficiency in ovule cultures [41] and somatic embryo development [42]. In the current study, because of previous studies [3,43] in the literature, at first the MS medium was tested for *in vitro* propagation of *L. candidum*. However, in subsequent studies, OM [22] medium containing different concentrations of NH_4^+ and NO_3^- was reported to overcome possible growth problems after transfer to *in vitro* condition, accelerate the adaptation of the plant to the nutrient medium and provide better growth and it was obtained very positive results in comparison to MS [21] medium.

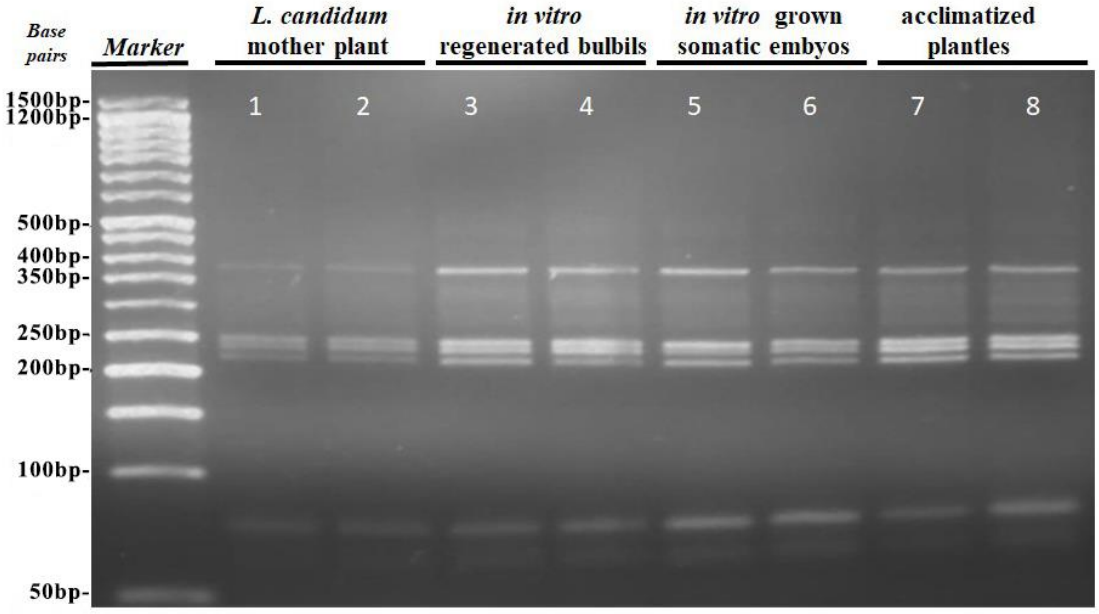

Figure 3. SSR-PCR amplification products obtained from DNAs of *L. candidum* mother plant (1, 2), *in vitro* regenerated bulbils (3, 4), *in vitro* grown somatic embryos (5, 6), and acclimatized plantles (7, 8) with SSR LS-ZJU11 [26] primers were visualized on agarose gel mixture (1.2% Biomax Basic Agarose and 2.8% Delta Micropor Agarose); M, 100 bp DNA ladder (ABM Cat: G016).

Plant cell and tissue culture have become a tool for the rapid reproduction of valuable species. Many plants can be produced with plant tissue culture by making continuous subculture under optimal conditions. However, in subculture studies performed by changing the growth environment (by accelerating or slowing down the growth), extending the changing intervals of the environment or increasing the number of subcultures have some risks. One of these risks is the emergence of somaclonal variations. Somaclonal variations are variations of genetic origin that occur between plants regenerated from somatic tissue originated callus, cell and protoplast cultures. These variations can be observed in plants as morphological, physiological and agricultural features. They are demonstrated by phenotypic, cytological and molecular level investigations [44]. Many reports have documented the assessment of genetic stability of micropropagated plants using SSR markers [12,45]. Liu and Yang [15] stated that 11 ISSR primers were used to determine the genetic stability of regenerated shoots in comparison to their mother plants. They reported that the genetic similarity between clonal samples and the mother plant was between 0.92 and 1.0. All 15 micropropagated materials and mother plants were grouped in a single master cluster with 92% similarity. They estimated the rate of somaclonal variation in plantlets to be 4.2%, emphasizing that direct shoot formation from explant regeneration indicates a safe method for the reproduction of "true–to-type" plants. Their results is acceptable for efficient mictopropagation, howewer, Asmita *et al*. [20] tested genetic stability using 10 SSR markers in twenty one *in vitro* regenerated plants. They produced a total of 273 bands from 10 SSR markers. The number of scorable bands for each primer ranged from

1 to 2. Among them, polymorphism information content was not recorded. Similar to our study, they stated that the banding profile of micropropagated plants is monomorphic and similar to the mother plant. Bi *et al*. [19] did not observe any polymorphism in embryo-like structure when analyzed with ISSR markers in five *Lilium* species and hybrids. Also, and no change in flow cytometry ploid level was observed. AFLP and ISSR markers have been used to detect genetic stability in direct shoot regenerants and ISSR markers showed no polymorphism but AFLP markers showed less than one percent [17]. Yadav *et al*. [16] used 6 RAPD markers and reported that there were no genetic variations in the regenerated micro bulbs. They stated that the results obtained through *in vitro* produced *Lilium* spp bulblets were clonally identical with mother plants. Varshney *et al*. [13] observed no change in progeny (randomly selected after four and 12 subcultures) with the RAPD marker.

4. CONCLUSION

In the current study, results of investigations based on SSR markers revealed that 12 pairs of primers produced amplified products with same monomorphic patterns of the mother plant, callus derived from *in vitro* regenerated bulbils, developing somatic embryogenesis, and acclimated material seedlings. It can be concluded that the results obtained by micropropagation protocol we employed here did not stimulate somaclonal variation in clones for these specific SSRs which were determined to be polymorphic markers in *L. candidium* genotype based on our previous studies.

Acknowledgements

This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) under Grant No: TOVAG- 217O009. Authors thank to Assoc. Prof. Dr. Ergun Kaya for support and thank to Assoc. Prof. Dr. Hasan Yıldırım and Assist. Prof. Dr. Ademi Pirhan for providing plant material.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Orcid

Hilal Busra Tokgoz https://orcid.org/0000-0002-0940-7427
Filiz Altan https://orcid.org/0000-0001-6358-2448

5. REFERENCES

[1]. Baytop, T. (1984). Türkiye'de Bitkiler ile Tedavi, İstanbul Üniversitesi, Yayın No: 3255, Eczacılık Fakültesi Yayın No: 40, İstanbul.

[2]. Khan, N., Barba-Gonzalez, R., Lim, K.B., Ramanna, M.S., Visser, R.G.F., Van Tuyl, J.P. (2010). Relevance of Unilateral and Bilateral Sexual Polyploidisation in Relation to Intergenomic Recombination and Introgression in *Lilium* Species Hybrids. *Euphytica 171*, 157–173. https://doi.org/10.1007/s10681-009-9998-0

[3]. Azadi, P., Kosh, K.I.U. (2007). Micropropagation of *Lilium ledebori* (Baker) as Effected by Plant Growth Regulators, Sucrose Concentrations, Harvesting Time and Cold Treatment. *Electronic Journal of Biotechnology, 10*(4), 587–591. https://doi.org/10.2225/vol10-issue4-fulltext-7

[4]. Özüdoğru, E.A., Kaya, E., Kirdok, E., Issever-Ozturk, S. (2011). *In vitro* Propagation From Young and Mature Explants of Thyme (*Thymus vulgaris* and *T. longicaulis*) Resulting in Genetically Stable Shoots. *In Vitro Cellular & Developmental Biology–Plant, 47(2),* 309–

320.
https://doi.org/10.1007/s11627-011-9347-6
[5]. Yeole, M.P., Gholse, Y.N., Gurunani, S.G., Dhole, S.M. (2016). Plant Tissue Culture Techniques: A Review for Future View. *Critical Review in Pharmaceutıcal Sciences 5*, 16–24.
[6]. Klimaszewska, K., Park, Y.S., Bonga, J.M. (2011). Tissue Culture Research at The CFS: Its History, Current Status and Potential Benefits. *Canadian Forest Service Publications*, Catalog ID: 32295.
[7]. Korban, S.S., Sul, I.W. (2007). Micropropagation of Coast Redwood (*Sequoia Sempervirens*). In: Jain SM, Häggman H (eds) *Protocols for Micropropagation of Woody Trees and Fruits* (pp. 23-32). Springer, Dordrecht.
[8]. Yam, T.W., Arditti, J. (2018). Orchid Micropropagation: An Overview of Approaches and Methodologies. In: Lee YI, Yeung ET (eds) Orchid Propagation: From Laboratories to Greenhouses Methods and Protocols. Springer Protocols Handbooks. Humana Press, New York.
[9]. Gaj, M.D. (2004). Factors Influencing Somatic Embryogenesis Induction and Plant Regeneration with Particular Reference to *Arabidopsis thaliana* (L.) Heynh. *Plant Growth Regulation, 43*, 27–47.
https://doi.org/10.1023/B:GROW.0000038275.29262.fb
[10]. Singh, N.D., Sahoo, L., Sarin, N.B., Jaiwal, P.K. (2003). The Effect of TDZ on Organogenesis and Somatic Emyogenesis in Pigeonpea (*Cajanus cajan* L. Millsp). *Plant Science, 164*, 341–347.
[11]. Butiuc–Keul, A., Farkas, A., Cristea, V. (2016). Genetic Stability Assessment of *in vitro* Plants by Molecular Markers. *Studia Universitatis Babeş-Bolyai Biologia*, *61*(1), 107-114.
[12]. Kalia, R.K., Rai, M.K., Kalia, S., Singh, R., Dhawan, A.K. (2011). Microsatelite Markers: An Overview of The Recent Progress in Plants. *Euphytica, 177*, 309–334. https://doi.org/10.1007/s10681-010-0286-9
[13]. Varshney, A., Lakshmikumaran, M., Srivastava, P.S., Dhawan, V. (2001). Establishment of Genetic Fidelity of *in vitro*-raised *Lilium* Bulblets Through RAPD Markers. *In Vitro Cell Developmental Biology-Plant, 37(2)*, 227–231.
https://doi.org/10.1007/s11627-001-0040-z
[14]. Khawar, K.M., Cocu, S., Parmaksiz, I., Sarihan, E.O., Özcan, S. (2005). Mass proliferation of Madonna Lily (*Lilium candidum* L) Under *in vitro* Conditions. *Pakistan Journal of Botany, 37*(2), 243–248.
[15]. Liu, X., Yang, G. (2012). Adventitious Shoot Regeneration of Oriental Lily (*Lilium orientalis*) and Genetic Stability Evaluation Based on ISSR Marker Variation. *In Vitro Cell Development-Plant*, *48*(2), 172–179.
https://doi.org/10.1007/s11627-012-9429-0
[16]. Yadav, R., Yadav, N., Pal, M., Goutam, U. (2013). Multiple Shoot Proliferation, Bulblet İnduction and Evaluation of Genetic Stability in Asiatic Hybrid Lily (*Lilium* sp.). *Indian Journal of Plant Physiology*,*18*(4), 354–359.
https://doi.org/10.1007/s40502-014-0060-4
[17]. Yin, Z.F., Zhao, B., Bi, W.L., Chen, L., Wang, Q.C. (2013). Direct Shoot Regeneration From Basal Leaf Segments of *Lilium* and Assessment Of Genetic Stability in Regenerants by ISSR and AFLP Markers. *In Vitro Cell Development-Plant, 49*(3), 333–342. https://doi.org/10.1007/s11627-013-9501-4
[18]. Bürün, B., Şahin, O. (2013). Micropropagation of *Lilium candidum* L.: A Rare and Native Bulbous Flower of Turkey. *Bangladesh Journal of Botany, 42*(2), 185–187. https://doi.org/10.3329/bjb.v42i1.15913

[19]. Bi, W.L., Yin, Z.F., Guo, L., Chen, L., Pan, C., Wang, Q.C. (2015). Plant Regeneration From Shoot Regrowth and De Novo Embryo-Like Structures from Cryopreserved Shoot Tips of *Lilium* spp. *In Vitro Cellular & Developmental Biology-Plant, 51*(4), 390–398. https://doi.org/10.1007/s11627-015-9696-7

[20]. Asmita, Sindhu, S.S., Jayanthi, M., Dhiman, M.R., Singh, M.K., Hossain, F. (2018). Genetic Fidelity Study of The *in vitro* Regenerated Plants in L.A. Hybrids of *Lilium* cv. Pavia. *International Journal of Current Microbiology and Applied Sciences, 7*(9), 1223–1231. https://doi.org/10.20546/ijcmas.2018.709.146

[21]. Murashige, T., Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum, 15*(3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[22]. Rugini, E. (1993). *In vitro* Propagation of Some Olive Cultivars, *Scientia Horticulturae, 24*, 123–134. https://doi.org/10.1016/0304-4238(84)90143-2

[23] Kaya, E., Souza, F., Gökdoğan, E. Y., Ceylan, M., & Jenderek, M. (2017). Cryopreservation of Citrus Seed via Dehydration Followed by Immersion in Liquid Nitrogen. *Turkish Journal of Biology, 41*(1), 242-248.

[24]. Marascuilo, L.A., McSweeney, M. (1977). Post-hoc Multiple Comparisons in Sample Preparations for Test of Homogenesity. In: McSweeney M, Marascuilo LA (eds) Non-parametric and Distribution Free Methods The Social Sciences. Books/Cole Publication, Belmont, pp 141–147.

[25]. Lodhi, M.A., Ye, G.Y., Weeden, N.F., Reisch, B.I. (1994). A Simple and Efficient Method for DNA Extraction from *Grapevine* Cultivars and *Vitis* Species. *Plant Molecular Biology Reporter, 12*(1), 6–13. https://doi.org/10.1007/BF02668658

[26]. Du, F., Wu, Y., Zhang, L., Li, X.W., Zhao, X.Y., Wang, W.H., Gao, Z.S., Xia, Y. (2015). De Novo Assembled Transcriptome Analysis and SSR Marker Development of a Mixture of Six Tissues from *Lilium* Oriental Hybrid 'Sorbonne'. *Plant Molecular Biology Reporter, 33*, 281–293. https://doi.org/10.1007/s11105-014-0746-9

[27]. George, E.F. (1993). Plant Propagation by Tissue Culture I: The Technology. Exegetics Ltd, Edington, UK.

[28]. Loyola-Vargas, V.M., Ochoa-Alejo, N. (2012). An Introduction to Plant Cell Culture: The Future Ahead. Methods In Molecular Biology, *Plant Cell Culture Protocols*, Springer Protocols.

[29]. Jeong, J.H. (1996). *In Vitro* Propagation of Bulb Scale Section of Several Korean Native Lilies. *Acta Horticulturae (414)*, 269-276. https://doi.org/10.17660/ActaHortic.1996.414.33

[30]. Varshney, A., Dhawan, V., Srivastava, P.S. (2000). A protocol for *In vitro* Mass Propagation of Asiatic Hybrids of Lily Through Liquid Stationary Culture. *In vitro Cellular and Development Biology-Plant, 36*(5), 383-391. https://doi.org/10.1007/s11627-000-0069-4

[31]. Kumar, S., Sharma, D.R., Sharma, Y.D., Pathani, N.S. (2007). Influence of Growth Regulators and Nitrogenous Compounds on In Vitro Bulblet Formation And Growth in Oriental Lily. *Horticultural Science, 34*(2), 77-83.

[32]. Saifullah, K., Sheeba, N., Mariam, R., Naheed, K., Asma, N., Bushra, S. (2010). Cultivation of Lilies (*Lilium regale*) for Commercialization in Pakistan. *Pakistan Journal of Botany, 42*(2), 1103-1113.

[33]. Leshem, B., Lilien-Kipnis, H., Stenitz, B. (1982). The effect of light and of explant Orientation on The Regeneration and Subsequent Growth of Bulblets on *Lilium longiflorum*

Thunb. Bulb-scale Sections Cultured *in vitro*. *Scientia Horticulturae*, *17*, 129-136. https://doi.org/10.1016/0304-4238(82)90005-X

[34]. Niimi, Y., Nakano, M., Isogai, N. (1999). Effects of Temperature and Illuminating Conditions on Regeneration and Development of Bulblets In Scale Culture Of Seven *Lilium* spp. *Journal of The Japanese Society for Horticultural Science*, *68*(1), 28-34. https://doi.org/10.2503/jjshs.68.28

[35]. Lian, M.L., Murthy, H.N., Paek, K.Y. (2002). Effects of Light Emitting Diodes (LEDs) on The *In vitro* Induction and Growth of Bulblets of *Lilium* oriental Hybrid 'Pesaro'. *Scientia Horticulturae*, *94*(3), 365-370. https://doi.org/10.1016/S0304-4238(01)00385-5

[36]. Nhut, D.T., Huong, N.T.D., Le, B., Van, D.S., Jaime, T., Fukai, S., Tanaka, M. (2002). The Changes in Shoot Regeneration Potential of Protocorm-Like Bodies Derived from *Lilium Iongiflorum* Young Stem Explants Exposed to Medium Volume, pH, Light Intensity and Sucrose Concentration Pretreatment. *The Journal of Horticultural Science and Biotechnology*, *77*(1), 79-82. https://doi.org/10.1080/14620316.2002.11511461

[37]. Kumar, S., Kashyap, M., Sharma, D.R. (2005). *In vitro* Regeneration and Bulblet Growth From Lily Bulb Scale Explants as Affected by Retardants, Sucrose and Irradiance. *Biologia Plantarum*, *49*(4), 629-632. https://doi.org/10.1007/s10535-005-0064-z

[38]. Stimart, D. P., DP, S., PD, A. (1978). Tissue Culture of Bulb Scale Sections for Asexual Propagation of *Lilium longiflorum* Thunb. *Journal of American Horticultural Science*, *103*(2), 182-184.

[39]. Altan, F., Bürün, B. (2017). The Effect of Some Antibiotic And Fungucide Applications on the Micropropagation of *Lilium candidum* L. *Mugla Journal of Science and Technology*, *3*(1), 86-91. https://doi.org/10.22531/muglajsci.307105

[40]. Vinterhalter, B., Ninković, S., Zdravković, K.S., Subotić, A., Vinterhalter, D. (2007). Effect of Nitrogen Salts on The Growth of *Ceratonia siliqua* Shoot Cultures. *Archives of Biological Sciences*, *59*(3), 217–222.

[41]. McCoy, T.J., Smith, L.Y. (1986). Interspecific Hybridization Of Perennial Medicago Species Using Ovule-Embryo Culture. *Theoretical and Applied Genetics*, *71*, 772–783. https://doi.org/10.1007/BF00276417

[42]. Leljak-Levanic, D., Bauer, N., Mihaljevic´, S., Jelaska, S. (2004). Somatic Embryogenesis in Pumpkin (*Cucurbita Pepo*): Control of Somatic Embryo Development by Nitrogen Compounds. *Journal of Plant Physiology*, *161*, 229–236. https://doi.org/10.1078/0176-1617-01055

[43]. Altan, F., Bürün, B., Şahin, N. (2010). Fungal Contaminants Observed During Micropropagation of *Lilium Candidum* L. and The Effect of Chemotherapeutic Substances Applied After Sterilization. *African Journal of Biotechnology*, *9*(7), 991–995.

[44]. Saker, M., Bekheet, S., Taha, H., Fahmy, A., and Moursy, H. (2000). Detection of Somaclonal Variations in Tissue Culture Derived Date Palm Plants Using Isoenzyme Analysis and RAPD Fingerprints. *Biologia Plantarum*, *43* (3), 347–351. https://doi.org/10.1023/A:1026755913034

[45]. Osena, G., Amugune, O.N., Nyaboga, E.N. (2017). Genetic Stability of Cassava Plants Regenerated Through Organogenesis Using Microsatellite Markers. *Journal of Plant Sciences*, *5*(1), 19–28. https://doi.org/10.11648/j.jps.20170501.13